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Tuberculosis

Current Issues in Diagnosis and Management

Edited by Bassam H. Mahboub and Mayank G. Vats

TUBERCULOSIS - CURRENT ISSUES IN DIAGNOSIS AND MANAGEMENT

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and **Mayank G. Vats**

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Notice

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Meet the editors

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Contents

Preface

One famous saying by Robert Louis Stevenson “It is not a hard thing to know what to write; the hard thing is to know what to leave out" holds very true for us while writing the preface of this book.

Tuberculosis (TB) is as old as mankind and continues to haunt the mankind despite several spectacular advances in the diagnosis and management of TB. TB is the commonest single infectious cause of death and accountable for over 25% of avoidable deaths worldwide but can still be labeled as “Captain of all these men of death”.

In the initial sections of the book chapters covering the basic pathophysiology and the important factors contributing to the same viz. epidemiology of TB, iron metabolism, unusual properties of M. Tuberculosis, lipid inclusion, role of small regulatory RNA and adaptation to survival in human host, which makes it “tough bug” to treat, has been included in details. Our understanding of the host pathogen interaction at the molecular level, especially immunopathogenesis of TB has improved enormously and has been extensively covered in the book. Chapters have been included to cover several new drug and potential vaccines for TB. New development such as the interferon-gamma release assays [IGRAs] for latent TB infection, use of liquid culture and molecular method of diagnosis are ushering in a new era in TB diagnostics. Comprehensive knowledge of latest modes of diagnosis has been also incorporated in the book. Furthermore, issues concerning quality assurance in antituberculosis drug susceptibility testing are getting established.

Data are rapidly accumulating from all over the world regarding the efficacy of standardized treatment regimens for drug-sensitive, drug-resistant TB and latent TB infection. While we are facing the menace of multi drug-resistant TB [MDR-TB], extensively drug-resistant tuberculosis [XDR¬ TB] has emerged threatening to undermine global efforts at TB control. Hence we have included chapters to cover all aspects of the diagnosis and management of MDR TB. This book will cover all these developments in great detail.

With the widespread availability of internet globally various standard web resources available on TB have also been included so that the readers may get the comprehensive and updated guidelines from these resources. The changing clinical presentation of TB, advances in laboratory, imaging diagnostic modalities, therapeutic measures and emergence of MDR TB all suggest a pressing need to have a updated book on TB. Furthermore, while all physicians encounter the TB disease in their clinical practice, there have been a lot of controversies and misconceptions over various issues for the diagnosis and management of TB.

Paucity of a well referenced, updated, standard book of TB has prompted us to undertake this venture sharing the clinical experience of global experts of TB.

Our book contains chapters on epidemiology, immune-pathology, diagnosis, treatment and latest advances for TB, highlighting the global perspective of tuberculosis. World-wide re-surgence of MDR TB indicates that the battle against this foe of mankind will continue in the coming years. TB still remains to be a research priority of paramount importance from medical, social and financial aspects and we have attempted to highlight all the aspects for the treatment of TB.

We believe that this book will serve as a practical guide for the diagnosis and management of TB for practicing physicians (especially pulmonologists and internists) and all those who are involved in the management of TB.

This book has several contributors, all of them leading authorities from various parts of the world. All the chapters have been thoroughly re-written and updated with preservation of the views of the contributors in a uniform format. This effort would not have been possible without the kind cooperation of our contributors who patiently went through revisions and updating of their chapters. We convey our heartfelt thanks to all contributors and to InTech Publisher, Croatia for their encouragement and excellent technical assistance as and when required.

Lastly we would like to thank the almighty god, our parents, wives and children, without their untiring support and encouragement this book would not have seen the light of the day.

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Section 1

Pathophysiology and Immunogenesis of Tuberculosis

Chapter 1

Mycobacterium tuberculosis Adaptation to Survival in a Human Host

Beatrice Saviola

Additional information is available at the end of the chapter

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1. Introduction

Mycobacterium tuberculosis exists exclusively as a pathogen of humans and in some cases of animals. It is not thought to exist in the environment other than for brief periods during transfer from an infected host to an uninfected contact. Thus *M. tuberculosis* must adapt to an *in vivo* environment by modifying gene expression. Differential expression can occur in immune cells such as macrophages, larger immune structures such as granulomas, and within liquefied lesions of the lung. Within the human body tubercle bacilli experience reactive oxygen intermediates as well as acidity within the phagosomes of macrophages. In addition within the centers of caseating granulomas bacilli experience low oxygen tension as well as toxic lipases and proteases released by dead immune cells. High temperature is present within the body of a person with active tuberculosis in the form of a fever. There may be other unrecognized signals and stresses that modulate gene expression within invading *M. tuberculosis* bacilli as well. Examination of gene expression during *in vivo* growth, within macrophages, or during application of specific stresses can illuminate which critical pathways in the mycobacterium are upregulated that lead to an *M. tuberculosis* bacillus exquisitely adapted to *in vivo* survival.

2. Adaptation to growth in the phagosomal compartment of macrophages

Macrophages are the preferred intracellular location for *M. tuberculosis in vivo.* Infected individuals cough and expel droplet nuclei which contain *M. tuberculosis* bacilli and remain suspended in the air. After inhalation and within the body, the bacilli are transported to the small alveoli in the lungs where they encounter alveolar macrophages which are relatively nonactivated (Dannenberg, 1993; Dannenberg, 1997). These nonactivated macrophages are not

efficient at killing or retarding growth of invading microbes. Initially bacilli are taken up into phagosomal compartments and may replicate. As the immune system becomes activated, macrophages are stimulated with INF-γ to increase their efficiency of mycobacterial killing, becoming more efficient at producing reactive oxygen intermediates and acidic stress. In response, *M. tuberculosis* pushes back against the macrophages and differentially regulates key genes. Within macrophages *M. tuberculosis* increases its lipid metabolism which may reflect an environment in the phagosome which lacks available carbohydrates (Table. 1). In addition the enzyme isocitrate lyase (*icl*) is strongly induced *in vivo,* and *icl* is upregulated in all macrophage models. Icl is a key enzyme in the glyoxylate shunt and utilizes fatty acids as an energy source. When *icl* and other genes in the glyoxylate shunt are mutated this results in attenuation *in vivo*. In addition within macrophages, genes involved in stress responses, cell wall component production, anaerobic respiration, siderophore production to scavange iron, diverse sigma factor production, and tranposases that may mutate the genome are all upregulated (Schnappinger et al, 2003, Beste et al, 2007, Ward et al, 2010).

3. Adaptation to granulomas and caseation

Once infection has progressed, tubercle bacilli replicate within incompletely activated macrophages. Additional macrophages arrive to the site of infection, and engulf newly liberated mycobacteria. The immune cells, T-cells, arrive to this location and an immune structure, the granuloma, composed of macrophages and a mantel of T-cells develops. If the host is resistant, and can robustly activate the body's macrophages, then *M. tuberculosis* infection is likely controlled. If the host immune system is weak, or is weakened, *M. tuberculosis* can replicate in the incompletely activated macrophages. Genes of *M. tuberculosis* required to resist macrophages will be important in resisting the environment of the granuloma as well. As the infection progresses in susceptible individuals, the centers of the granulomas degenerate and form a caseous, or cheesy, center. At the heart of this is an elevated lipid metabolism of the host that produces a variety of lipids including cholesterol, cholesteryl esters, triacyglycerol and others (Kim et al, 2010). Interestingly *M. tuberculosis* infection has been shown to induce elevated lipid metabolism in the host (Table 1.). The cell wall lipid of *M. tuberculosis*, trehalose dimycolate or cord factor, induces a granulomatous response in mice, and this was accompanied by foam cell formation which contains elevated lipids (Kim et al, 2010). It is intriguing to speculate that *M. tuberculosis* infection can induce elevated host lipid metabolism, and as discussed previously as part of adaptation to *in vivo* growth, *M. tuberculosis* also switches to lipid metabolism and lipids as a preferred carbon source (Eisenreich et al, 2010). Thus *M. tuberculosis* induces the host to produce what the microbe has evolved to utilize as an energy source.

4. Liquefied lesions and sputum

Later in infection caseating granulomas continue to breakdown. At a certain point these granulomas begin to liquefy, and host lipases and proteases are present which damage host

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tissues. Dead macrophages release lytic enzymes, and bacterial products may also result in host tissue damage and liquefaction ensues. As tissue is damaged, a cavity erodes into the lung airspace. In rabbit studies, *M. tuberculosis* can replicate to extremely high levels in this liquefied environment (Dannenberg 1993, Dannenberg et al 1997, Dannenberg 2006). For the first time *in vivo M. tuberculosis* is capable of replicating extracellularly. Liquid containing free *M. tuberculosis* is expelled through cavities in the lung by coughing.

M. tuberculosis within sputum contains elevated levels of lipid bodies and tends to be inhibited in its replicative process (Table 1.) (Garton et al, 2008). In addition, sputum transcriptome analysis of *M. tuberculosis* reveals that triacylglycerol synthase, t*gs1* part of the DosR regulon, is induced and lipid bodies may be composed of increased stores of triacylglycerol (Garton et al, 2008). Lipid bodies are correlated *in vitro* with nonreplicating persistence, and may help *M. tuberculosis* survive the harsh environment *ex vivo* before it encounters another human host.

5. *Mycobacterium tuberculosis* and dormancy

One third of the world's population is infected with *M. tuberculosis* in part because it causes a latent or dormant infection in a majority of those infected. If therapies are to be developed which can eradicate *M. tuberculosis*, a better understanding of dormancy is required. *M. tuberculosis* can persist for decades in a dormant state within hypoxic granulomas in the lung. Studies have suggested that in a dormant state *M. tuberculosis* is occupied mainly with maintaining cell wall integrity, membrane potential, and protecting its DNA structure. The mycobacterium must also resist the host's immune system. A number of *in vivo* and *in vitro* models have been used to investigate dormancy. These models include exposing mycobacteria to environments that are likely encountered within the host. In one model cultures are stirred slowly and sealed so that oxygen is gradually consumed. In another model nutrient starvation of the bacteria may induce dormancy. In addition, infection of mice, partial treatment with antibiotics, and exposure to immune suppression can lead to dormancy and reactivation (Murphy and Brown, 2007).

The gene encoding a transcriptional regulator, *dosR* (*devR*), part of a two component system that responds to low oxygen seems to be very important in a shift from replicating *M. tuberculosis* to a nonreplicating form (Table 1.). Carbohydrate limitation also upregulated *dosR* and there is indeed an overlap of genes upregulated in phagosomes of macrophages and low carbohydrate availability. In dormancy models aerobic respiratory metabolism was down regulated while anaerobic respiration was upregulated as were DosR controlled genes (Murphy and Brown, 2007). Amino acid and carbon starvation results in the activation of the stringent response. RelA (Rv2583c) mediates this stringent response in *M. tuberculosis* and can globally down regulate components necessary in protein translation, and thus conserve badly needed resources in the mycobacterium during times of stress. RelA may be a target to prevent *M. tuberculosis* from entering dormancy or a target to force *M. tuberculosis* out of dormancy (Murphy and Brown, 2007).

The ability of *M. tuberculosis* to survive in a dormant state relies on maintaining cell integrity, viability, and a proton motive (Rustad et al, 2008). Entry into a dormant state may be followed later by reactivation and growth of this microorganism, and may occur due to waning immunity, age, or disease. T-cells originally controlling infection may become less activated and numbers of T-cells may decrease allowing mycobacteria increased ease of replication in host macrophages. *M. tuberculosis* needs energy to exit this dormant phase, and this may be found in the form of triacylglycerol which is known to accumulate in response to acidic stress, nitric oxide exposure, and lowered oxygen tension (Table 1.) (Sirakova et al, 2006; Garton et al, 2008). In fact triacylglycerol has been shown to be important to transition from dormancy to active growth (Low et al, 2009). The highly pathogenic strain of *M. tuberculosis*, the Beijing lineage strain, over produces triacylglycerol perhaps giving the microorganism a competitive edge in resisting hypoxic stress and dormancy (Fallow et al, 2010).

6. *Mycobacterium tuberculosis* responses to acidic stress

M. tuberculosis encounters acidity in the body in a number of locations including within immune cells, macrophages. When macrophages phagocytose tubercle bacilli, phagosomes of unactivated macrophages are limited in their ability to acidify due to the presence of live *M. tuberculosis*. Bacilli can inhibit phagosomal maturation and also inhibit phagosome lysosome fusion (Armstrong and Hart, 1971; Sturgill-Koszycki et al, 1994; Huynh and Grinstein, 2007). Virulent *M. tuberculosis* can exclude a proton ATPase from the phagosome in non-activated macrophages. Exposure to the cytokine INF-γ can result in increased activation of macrophages and these macrophages that phagocytose live virulent *M. tuberculosis* can lower the intra phagosomal pH (Schaible et al,1998; Via et al, 1998; MacMicking et al, 2003; Ehrt and Schnappinger, 2009). This pH's can be toxic to bacilli either killing them, or inhibiting their growth. The robustness of the response seems to lie in the activation and efficiency of the host's immune response. Anything that interferes with the host's immune status can negatively impact acidic modulation within phagosomes, and lead to more mycobacterial replication. In addition, the tubercle bacillus' ability to respond to acidic stress will likely affect the outcome of the infection.

Mycobacteria seem to bear an intrinsic ability to resist acidic stress. They have a thick waxy cell wall as well as an outer membrane that can resist acidic stress. This physical barrier may serve to inhibit entry of toxic protons, and anything that interferes with this barrier could increase acid susceptibility. Many mutants that are acid susceptible lie in genes that affect cell wall and lipid metabolism (Table 1.). Environmental mycobacteria are found in conditions that may be acidic and can grow at pHs as low as 4.0 (Santos et al, 2007). Pathogenic mycobacteria have evolved to resist acidic stress, and potentially share similar mechanisms with their environmental cousins (Kirschner et al, 1992; Kirschner et al, 1999).

Although *Mycobacterium smegmatis* has been found to have an acid tolerance system it is not known if *M. tuberculosis* also possesses one. However, a large number of genes are upregulated due to acidic stress in *M. tuberculosis*. Interestingly when *M. tuberculosis* is engulfed by the

phagosomes of macrophages many genes are upregulated, and when cocanamycinA is added which interferes with the development of acidity, 80% of genes in *M. tuberculosis* that are normally upregulated in the phagosomes fail to do so (Rohde et al; 2007). This is an indication that acidity is one of the main environmental signals *M. tuberculosis* experiences *in vivo*.

A number of genes that are upregulated by acidic stress have been identified in previous studies. Looking at rapid response to acidity at 15 or 30 minutes it was found that genes involved in cell wall ultrastructure were induced (Fisher et al, 2002). The *mymA* operon was induced in this study, and is under the control of VirS which is an AraC/XylS family transcription factor (Singh et al, 2005). The *lipF* promoter of *M. tuberculosis* is upregulated, but requires a longer time frame (Saviola et al, 2001). It fails to be upregulated at 30 minutes, instead needing more extended exposure to acidic stress of 1.5 hours. LipF is annotated to be an esterase and may also function to alter the cell wall structure. LipF has been shown to be part of the two component system *phoP/R* regulon. In fact many genes involved in the PhoP/PhoR regulon including *pks2*, *pks3*, and *pks4* are responsive to acidic stress (Table 1.) (Gonzalo-Asensio et al, 2009; Rohde et al, 2007). Thus PhoP/R may be responding to acidic stress or conversely PhoP/R controls a downstream regulator that responds to acidity. The *ompATb* gene encodes a porin that is active specifically at low pH and functions to pump ammonia into the phagosomal environment which serves to neutralize acidity (Song et al, 2011). Longer term exposure to acidic stress seems to stimulate production of triacylglycerol. *Tgs1* is not upregulated by short term acid exposure but exposure of three weeks duration or more (Sirakova et al, 2006; Low et al, 2009; Deb et al, 2009). Triacylglycerol production may be important for mycobacteria to resist stress and survive a dormant period which is induced by stress conditions. An energy source such as triacylglycerol may be needed to reanimate from dormancy once stresses such as acidity are removed. Mutatagenesis studies also revealed genes involved cell wall/cell envelope synthesis when mutated resulted in mycobacteria which were unable to maintain neutral pH within their microbial cytoplasm in the presence of acidic stress (Vandal et al, 2008; Vandal et al, 2009, Biswass et al, 2010).

The type VII secretion system, Esx-1, may also may be involved in response to acid stress (Abdallah et al, 2007). The 6 kDa early secreted antigenic target (Esat-6) and the 10kDa culture filtrate protein (CFP-10) are secreted by Esx-1. These two proteins form a heterodimer that can dissociate at acidic pH. Esat-6 is capable of lysing membranes, and *M. tuberculosis* has been identified to reside extraphagosomally in the cytoplasm of macrophages in some cases. In addition when the *esx-1* gene was mutated it could result in an *M. tuberculosis* strain that fails to escape from the phagosomal compartment into the cytoplasm (Simeone et al, 2009). Thus Esat-6 may be involved in mycobacterial responses to acidity and adaptation to *in vivo* stressors.

7. Response to oxidative damage

Inside phagosomes of activated macrophages tubercle bacilli are exposed to reactive oxygen intermediates. *M. tuberculosis* traffics to phagosomes, and a large number of genes are upre-

gulated by oxidative stress indicating this is an important stress *in vivo* (Wu et al, 2007). In addition nutrients are limited in the phagosome which may cause *M. tuberculosis* to enter a stationary phase of growth, which has been shown to induce internal oxidative damage. The gene *whiB1* is more active during stationary phase, and the protein produced by this gene has been shown to reduce cellular disulphide bridges that may predominate during this adaptational phase (Garge et al, 2009).

Mycobacteria contain a unique substance, mycothiol, which combats oxidative stress. Other bacterial species utilize glutathione which can also neutralize oxidative stress. Mycothiol contains cysteine residues which are oxidized when that condition predominates thus forming disulfide bonds, creating mycothione, and preventing other molecules in the mycobacterial cell from becoming oxidized (Table 1.). Human cells produce glutathione to combat oxidative damage, and glutathione is toxic to mycobacterial cells perhaps due to a redox imbalance generated by this substance in the mycobacteria (Venketaraman et al, 2008; Connell et al, 2008)). Mycobacteria also contain other molecules to detoxify oxidative damage including superoxide dismutase (SOD) and catalase (KatG) which can inactivate superoxide (Table 1.) (Shi et al, 2008). SOD and KatG are upregulated early in infection indicating an increase in oxidative damage due to superoxide. Oxidative damage is capable of harming DNA, and histone like proteins (LSR2) can protect against damage by compacting DNA and acting as a physical barrier. UvrB which repairs mycobacterial DNA damage also protects against oxidative damage (Darwin and Nathan, 2005; Colangeli et al, 2009).

8. Heat shock

One of the hallmarks of tuberculosis is fever and night sweats in which body temperature increases and is suboptimal for *Mycobacterium tuberculosis* replication and survival. This allows the immune system a competitive edge over the invading microbes. Heat stress can cause damage to *M. tuberculosis* by causing proteins to unfold which may then be degraded. In response, *M. tuberculosis* can upregulate chaperonins which complex with unfolded proteins and help them refold (Table 1.). The α-crystalline protein, or Acr-2, is activated by heat shock, and has demonstrated chaperonin activity (Pang and Howard, 2007).

Many proteins that are upregulated in *M. tuberculosis in vivo* are heat shock proteins that have chaperonine activity. While these proteins may benefit the organism by complexing with and refolding heat damaged proteins, they are also recognized by the immune system. Both the 65Kd heat shock protein and the HSP70 protein can be found extracellularly to *M. tuberculosis,* and are potent stimulators of an inflammatory response (Anand et al, 2010).

9. Low iron

Normally iron taken up by intestinal epithelial cells and bound to transferrin circulates within the body. This complex binds to cell surface receptors, and is internalized where it releases its

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iron to be bound by the host cellular factor ferritin. Infection and inflammation are natural signals to the host to limit availability of iron. Proinflammatory cytokines stimulate hepcidin production, decrease iron uptake from the gut, and inhibits the iron efflux protein ferroportin (Johnson and Wessingling-Resnick, 2012). Inflammation thus inhibits iron uptake by the intestinal epithelium thus preventing iron from being loaded onto transferrin. Interfering with uptake limits iron availability in the host, and *M. tuberculosis* has been shown to be severely growth restricted in a low iron environment. It has been demonstrated in African studies that iron supplementation increases incidence of tuberculosis. Thus being anemic may be protective against infectious processes. Within human macrophages, Nramp1 (natural resistance associated macrophage protein) is produced and localizes to the phagosomal compartment where it reduces iron within this site possibly by extrusion. This function confers resistance to *M. tuberculosis* infections and mutations in the *nramp1* gene can result in increased susceptibility to active disease due to *M. tuberculosis* infection (Johnson and Wessingling-Resnick, 2012).

Mycobacteria have a variety of systems which aid in the uptake of iron and the regulation of iron responsive genes. As mycobacteria have been shown to be somewhat novel among gram positive bacteria, they possess an outer mycolic acid based membrane, as well as an inner membrane and periplasmic space. Porins in the outer membrane appear to transport iron in the presence of high iron conditions (Jones and Niederweis, 2010). *M. tuberculosis* under low iron conditions can produce the siderophore carboxymycobactin as well as mycobactin (Table 1.) (Banerjee et al, 2011). These molecules bind with a higher affinity to iron than the human host's storage proteins and steal iron from the host. Mycobactin is present within the inner membrane and thus can only bind iron imported into the periplasmic space. Interestingly lipid membranes with associated mycobactins may diffuse out, travel to lipid vesicles in the host cell, and sequester iron. These structures may recycle back to interact with the mycobacterium. Disruption of the genes responsible for production of mycobactins can cause these mutant mycobacteria to replicate less well in macrophages (Banerjee et al, 2011). Carboxymycobactins are excreted possibly by the type VII secretion or ESX system. Externally the carboxymycobactins bind available iron from transferrin (Banerjee et al, 2011). Porins and also ABC transporters may allow import of these iron loaded carboxymycobactins (Banerjee et al, 2011). The host cell, in response to infection and inflammation, produces siderocalins such as lipocalin-2 that can bind to and inactivate mycobactin from *M. tuberculosis* thus interfering with mycobacterial iron acquisition (Johnson and Wessingling-Resnick, 2012). In fact mice deleted for genes involved in production of siderocalin are much more susceptible to mortality due to *M. tuberculosis* infection (Johnson and Wessingling-Resnick, 2012). Inside the mycobacterial cell, iron is stored in bacterioferritin and a ferritin like protein. These proteins are required for replication in human macrophages and guinea pigs, act to store iron, and also to limit excess iron in the cells that can lead to iron mediated oxidative damage due to the Fenton reaction (Reddy et al, 2011).

Iron responsive genes in *M. tuberculosis* are controlled in part by the iron dependent regulator IdeR. This protein can act both as an activator and a repressor depending on where it binds within a mycobacterial promoter region (Manabe et al, 1999; Banerjee et al, 2011). Within

promoters of genes involved in mycobactin synthesis it acts as a repressor, inhibiting expression of these genes at high iron concentrations. In promoters of iron storage proteins it acts as an activator, stimulating expression of these genes at high iron concentrations and thus avoiding iron stimulated oxidative damage.

10. Hypoxic growth

In vivo M. tuberculosis experiences low oxygen tension that may be encountered in the centers of granulomas as previously described. Studies have shown that tuberculous granulomas are hypoxic in a variety of animal models including rabbits, guinea pigs, and nonhuman primates (Via et al, 2008). The response to low oxygen tension is biphasic. There is an initial response that predominates and is controlled by the two component system DosS/DosT-DosR (Table 1.). This two component system upregulates genes that are known to be part of the "dormancy regulon". DosR is the transcriptional regulator, and Dos T and DosS are the sensor kinases that respond to low oxygen tension as well as nitric oxide (Park et al, 2003; Kumar et al, 2007). *hspX* (*acr*, *Rv2031c)* is upregulated by low oxygen, is regulated by DosR, and has chaperonin activity that may aid in refolding proteins which are damaged by low oxygen tension (Vasudeva-Rao and McDonough, 2008; Florczyk et al, 2003). It is known that this protein is expressed *in vivo* as latently infected individuals possess T-cells that are reactive to the HspX protein (Geluk et al, 2007). Interestingly one half of the genes in the DosR regulon return to their baseline level after 24 hours. After this initial 24 hour period other regulators play a role in hypoxic responses such as sigE and sigC (Table 1.). An enduring hypoxic response begins after the initial response, and this may be important for *M. tuberculosis* to enter and stay in a dormant state (Rustad et al, 2008).

11. Toxin-antitoxin systems

Interestingly there are many toxin-antitoxin systems within the *M. tuberculosis* genome. These systems seem to provide a mechanism by which bacteria can alter growth rate rapidly, potentially in response to environmental stressors. The toxin is not a protein secreted and targeted against the human host, but targeted against mycobacterial cellular components. The toxin is a stable protein which may be complexed with an antitoxin forming a toxin-antitoxin pair. The antitoxin is relatively unstable and environmental stressors can inactivate it causing release of a free toxin. The toxin is then available to interact with cellular components, and may function to cleave mRNA thus inhibiting subsequent translation and rapidly halting growth of the bacterium. As static bacteria are more resistant to environmental stressors and antibiotics, this system may allow *M. tuberculosis* to survive in the face of external stressors. *M. tuberculosis* possesses 88 toxin-antitoxin systems and four of these have been shown to be activated by phagocytosis of bacilli, by macrophages, or hypoxia (Table 1.). It appears that the toxin in these systems acts by cleaving mRNA (Rapage et al, 2009).

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In Vivo Condition or Location	Mycobacterial Response
macrophages, granulomas, liquified lesions and sputum	increased lipid metabolism in bacillus, or induction of same in host
macrophages, granulomas, low iron	Siderophore production
all stress conditions, macrophages, granulomas, sputum	differential sigma factor utilization
liquified lesions, sputum, conditions leading to dormancy	lipid body production
low oxygen, macrophages, conditions leading to dormancy	DosR two component system activity
low oxygen, macrophages, possibly acidity	PhoP two component system activity
In all conditions *in vivo*	Constitutive thick waxy cell wall construction, may be upregulated
oxidative stress, macrophages	Mycothiol, SOD, & KatG production
Fever	Heat shock protein production
macrophages, phagocytosis, hypoxia	toxin-antitoxin system function

Table 1. Mycobacterial responses to in vivo stressors and conditions.

12. Two component systems

Two components systems are common in many bacteria. These systems are comprised of a sensor kinase which phosphorylates the response regulator as a result of an environmental signal, which is often a stress. The sensor kinases are trans membrane proteins which are embedded into membranes. They sense external stresses and transmit these signals internally into the bacterial cell by phosphorylating a response regulator that binds to its cognate promoter DNA, and regulates transcription. The mycobacterial genome contains 11 two component systems (Hett and Rubin, 2008). The large number of these systems in the mycobacterial coding regions is likely the result of evolution to accommodate bacterial responses to diverse stresses.

DosS/DosT-DosR was previously described, and responds to initial hypoxic stress (Table 1.) (Park et al, 2003). Some of the genes controlled by the transcriptional regulator DosR are upregulated by hypoxic stress, and are also part of the transcriptional regulator PhoP regulon, a member of the PhoP/R two component system. While it is unknown what environmental signal PhoP or the sensor kinase PhoR are responding to, genes controlled by PhoP either directly or indirectly are upregulated by such stresses as acidity and low oxygen (Table 1.) (Gonzalo-Asensio et al, 2008).

13. Sigma factors

Mycobacterial RNA polymerase catalyzes RNA synthesis from specific promoter sequences. This RNA polymerase is composed of subunits that comprise the core holoenzyme, and include two α subunits, a β, a β' and a ω subunit. The core enzyme, however, cannot target specific promoter sequences. A sigma factor is required for this function, and can bind and recognize specific -10 and -35 promoter sequences. As the mycobacterial genome possesses many different sigma factors, these RNA polymerase components can recognize diverse mycobacterial promoter sequences to activate a whole class of genes. This activity is in addition to specific transcription factors which bind to promoters, regulate transcription, and are not part of the RNA polymerase enzyme.

The mycobacterial genome possesses many different sigma factors that belong to different categories. The *M. tuberculosis* σ^A is responsible for regulating housekeeping genes, and is also an essential gene for mycobacterial growth *in vitro* and *in vivo*. While the sigma factor σ^B is highly similar to σ^A, it is nonessential and is induced by a variety of stresses including oxidative stress, heat shock, cold shock, stationary phase, and low aeration (Lee et al, 2008). There are a number of sigma factors designated to have extracellular function, and some respond to environmental stresses and are involved in the synthesis of the mycobacterial envelope. These sigma factors are SigC, SigE, SigF, SigG, SigH, SigI, SigJ, SigK, SigL, and SigM. One sigma factor that is known to respond to nutrient starvation is SigF. The sigma factor SigE is involved in response to heat shock and SDS exposure (Manganelli et al, 2004). Both SigJ and SigF are induced in response to antibiotic exposure (Manganelli et al, 2004). The sigma factor SigH also responds to heat shock and oxidative stress (Manganelli et al, 2004). Thus the use of sigma factors by the mycobacterial cell is a manner in which "master regulators" can control whole classes of genes to rapidly facilitate gene regulation in response to specific environmental stresses (Table 1.).

14. Summary

As mycobacteria invade their human hosts they must respond to a plethora of stresses many of which are generated by the host's immune system. Under this selective pressure, *M. tuberculosis* has evolved mechanisms to combat the toxic insults of the host. Although myco-

bacteria are inherently resistant to environmental stresses due to their thick waxy cell envelope, upregulation of genes further reinforce this defense. In addition there are proteins upregulated by environmental stressors which can detoxify the mycobacterial cell as is the case of acidic stress and upregulation of ammonia extruding pumps that neutralize acidic pH of the macrophage phagosome. Thus inducible systems allow *M. tuberculosis* to resist environmental stresses and persist in the human body to cause active or latent disease.

Understanding the specific steps in infection, the stresses associated with each step, and the mycobacterial response may be of clinical relevance. The knowledge that oxidative stress and acidic stress may predominate as adaptive immunity makes the host's macrophages more activated, may lead to the development of chemotherapeutic agents that target mycobacterial components produced by these stressors during this infective stage. In addition, the knowledge that mycobacteria may utilize toxin-antitoxin systems to slow their growth and to enhance their innate antibiotic resistance may spur the development of therapies that target these systems which could be used in conjunction with traditional antibiotic treatments. Chemotherapeutic agents given to decrease activity of triacylglycerol synthase may decrease infectivity of sputum positive individuals by inhibiting lipid body production in the bacilli while antibiotic treatment lags in its sterilizing activity. Ultimately treatments may be developed which target inducible systems upregulated by stresses, and may interfere with mycobacterial responses to these stressors. By thwarting these adaptive responses potentially with chemotherapeutic agents, mycobacteria may be rendered more fragile and susceptible to the host's immune system. In addition a greater understanding of how *M. tuberculosis* enters a latent state of persistence could lead to treatments that prevent this microbe from reactivating from the dormant state, or from becoming dormant to begin with. Greater understanding of *M. tuberculosis* responses to *in vivo* growth will hopefully lead to the development of technologies that lessen *M. tuberculosis'* global impact on human health.

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Chapter 2

The Immune Response to *Mycobacterium tuberculosis* Infection in Humans

Zeev Theodor Handzel

Additional information is available at the end of the chapter

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1. Introduction

The microbe *Mycobacterium tuberculosis* (MTB) is an ancient cohabiter with humans, infecting almost 3 billion people worldwide, 10% of them developing clinical disease. The 20th century dream of eradicating the global scourge of tuberculosis (TB) evaporated with the failure of the old BCG vaccine to protect the populations at greatest risk, low compliance at following the complicated and lengthy treatment in countries with limited resources, which was followed by the spread of multiple-drug resistant (MDR) strains. Actually the situation has worsened with a peak of 9.4 millions of new clinical cases in 2009 and 1.7 million deaths/year [1,2,3].

However, it is intriguing to observe that the incidence and morbidity of the disease varies greatly in different regions of the globe, being highest in Africa and Asia, as well as the response to BCG vaccination [1,4]. That, in spite of the fact that there are no structurally variable strains of MTB, therefore all have a similar virulence capacity. One important factor is the introduction of the human immunodeficiency virus (HIV) into areas and populations already having a high TB incidence [5], the resulting double infections having a disastrous effect. This is especially prominent in sub-Saharan Africa. But that factor alone can not explain the global epidemiological variability in the disease. Also, why only one in ten carriers of the microbe become clinically sick?

In order to address these questions, in the present chapter we will try to delve into the intricacies of the human immune response to MTB infection and to explore possible differences in the genetic regulation of the host immune responses in various human populations.

2. The encounter of Mtb with the innate immune system

Most human infections with MTB occur through inhaled carrier droplets into the lower airways. There the microbe encounters the alveolar macrophage (AMac) and submucosal

dendritic cell (DC). The outcome of the ensuing battle will determine whether the infection will remain locally limited within the engulfing cells of the innate immune system, or will continue to spread, causing the individual to become a clinically active TB patient [1,6,7,8]. During the first contact, the AMac recognizes the microbe through pattern recognition receptors (PRRs), which sense microbial biochemical components, such as outer coat mannosylated lipoarabinomannan (ManLam), trehalose dimycolate and N-glycolymuramyl dipeptide. These molecules act as pathogen-associated molecular patterns (PAMPs), which trigger an intracellular signaling cascade in the AMac, which leads to a phagocytic activity, which, if successful, will result into the complete engulfing of the microbe into cytosolic vesicles- the phagolysozomes and secretion of pro-inflammatory cytokines, such as tumor-necrosis factor alpha (TNFα). ManLam also binds directly to mannose receptors on macrophages and DCs.

The best studied PRRs are Toll-like receptors (TLRs) [6,9,10], of which 10 have been identified in humans. TLR- 2 and TLR-4 recognize bacterial products [9,11], TLR-2 having a major role in recognizing MTB in the lung. All contain an intracellular TIR domain, the activation of which initiates a signaling cascade via adapter proteins such as MyD88, interferon-inducing TRIF and TRIF-related adapter molecule TRAM, which results in the recruitment of interleukin-1(IL-1) receptor-associated kinase (IRAK) 4, which phosphorylates IRAK-1. The latter binds to TNFα receptor-associated factor (TRAF) 6, leading to kinase-dependent IkBα phosphorylation, the degradation of which leads to the activation of nuclear NF-kB, which is the main nuclear activator of proinflammatory cytokines. Another intracellular PRR is nucleotide-binding oligomerization domain 2 (NOD2), which binds bacterial cell-wall muramyl-dipeptide, eliciting secretion of TNFα, IL-1β, IL-6 and bacteridal LL-37 [12,13]

Neutrophils also play a defensive role, not only as first-line non-specific phagocytes, but also by secreting anti-bacterial proteins, mainly the cathelicidin LL-37 [1,14]. Neutrophils loaded by phagocytized bacteria become apoptotic, thereby eliciting macrophage activation [15].

NK cells, which are large granular circulating lymphocytes, are attracted to the sites of bacterial infections, where they specialize in recognizing and destroying infected host cells. During this process they secrete interferon gamma (IFNγ), which activates macrophages, inducing them to secrete the cytokines IL-12, IL-15 and IL-18, which activate CD8+T-cells, thus forming the link to the adaptive immune system [7,16]

The complement is the humoral arm of the innate immune system. It has been shown that M. bovis BCG may activate the three pathways of complement: the classical pathway by binding to the C1q protein, the lectin pathway by binding to the bacterial cell surface mannose-binding lectin (MBL) or L-ficolin and the alternate pathway through the deposition of C3b on the bacterial surface. Mtb can activate the classical and alternate pathways by binding C3. This enables complement to perform its major functions-microbial opsonization, microbial cell lysis through the formation of the attack complex and leukocyte recruitment by eliciting chemokine secretion [7,17].

Another recently discovered anti-microbial mechanism of phagocytic cells is the use of vital transition metals, such as iron, zinc and copper, to poison intracellular microorganisms. However, mycobacteria have developed a resistance mechanism to such intoxication [18,19].

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This contrasts with the function of the phagosomal metal transporter natural resistance-associated membrane protein (NRAMP) 1 to deprive the microorganisms from essential nutrients, such as iron and manganese [20]. Such duality existing in the same cell is of interest.

Virulent Mtbs have acquired the capability to dampen the activity of NF-Kb by some of their antigens [6,7], such as ESAT-6 and ManLam. The latter also inhibits the secretion of IL-12, an essential cytokine in the anti-MTB inflammatory response. ESAT-6 downregulates MyD88-IRAK 4 interaction, thereby also interfering with TLR signaling to NFkB. A third antigen-CFP-10 markedly reduces nitric oxide (NO) and reactive-oxygen species (ROS) production by the macrophages, thereby inhibiting their non-specific killing ability. The microbe may also regulate macrophage apoptosis to its advantage and to inhibit IFNγ- mediated macrophage activation [7]. ESX is a recently discovered protein transport system through the outer membrane of the microbe, which is essential for its survival. It has been demonstrated, in an experimental model, that ESX-5 may modulate macrophage reactivity by dampening the inflammasome activation [21]. These mechanisms enable the microbe to survive in the macrophage phagosome in a balance which is precarious to the host. In addition Mtb may escape the phagolysozome into the cytosol by damaging its membrane. Most recently it has been described that the microbe may secrete toxins, such as the newly discovered MtpA protein, through its outer membrane into the macrophage cytosol, which may cause the death of the later by cell necrosis [22].

Vitamin D seems also to play an important role in the microbe-host pull-of-arms [23]. It may modulate the inflammatory effect of some metalloproteinases (MMPs) in the lung [24] and Vitamin D supplementation has hastened bacterial eradication in pulmonary tuberculosis in a clinical trial [25].

Thus, the encounter between MTB and the various components of the innate immune system induce a complicated and sophisticated series of host responses and counter responses by the microbe. The later is one of the most ancient human infections, carried by our ancestors since they fanned-out from Africa across the globe, therefore enabling it to adapt to the human immune response (26-Cole S, Tuberculosis in time and space, Econference).

However, the next long-term phase of the encounter is played by the activation of the adaptive immune system, as described in the next section.

3. The role of adaptive immunity in the outcome of the Infection

In the previous section the importance of the host innate immune response in the encounter with MTB was described. However, it is generally accepted that the long-term outcome of the primary infection is determined by the effective mobilization of the adaptive immune response. Active TB patients, as well as latently infected carriers, do not suffer from a general innate or adaptive immune defect. On the contrary, ex-vivo studies of their immunocyte function demonstrate increased lymphocyte proliferation and the secretion of numerous cytokines [27]. Thus the disease, in people generally healthy, is a result of a very specific immune failure in face of MTB, or other mycobacteria.

It was thought that the CD4+T cell is the omnipotent determinant of the adaptive immune response in TB. However, lately it became clear that more T-cell subsets, including CD8+ and TH17 cells and even B cells participate in the process [1,7,28]. The induction phase seems to be delayed relatively to the response to more common pathogens. It is initiated by signaling and presentation of the microbial peptides by the macrophages and DCs to the CD4+ cells via MHC class II molecules, while mycobacterial membranal lipids are presented through MHC-I molecules of the CD-1 family [29]. The presentation of mycobacterial antigens occurs within the draining lung lymph-nodes to which the macrophages have migrated, followed by the activation of CD4+ and other T cells. These T cells use various receptors, such as TLRs, NOD-like receptors and C-type lectins, for this purpose. The peptides considered as potentially immunodominant are the already mentioned ESAT-6 and CFP10 and others, such as Rv2031c, Rv2654c and Rv1038c. The T cell response to these antigens is not homogenous, various T cell epitopes being engaged during the different phases of the infection [30]. Other Rv proteins are binding to T cells mainly during the latent phase [31]. T cell activation, by the recognition of these antigens in the initiating phase, results in the secretion of numerous cytokines, mostly proinflammatory, such as IL-1β, IL-6, IL-21 and IL-12p40. The later activates CD4+TH1 cells, but p40 is also a subunit of IL-23, which induces the TH17 cell lineage, which secretes IL-17, IL-21 and IL-22. These cytokines are considered to be essential for anti-microbial protection and IL-17 is thought to have a major role in granuloma formation [32], as well as TNFα, which is also secreted by CD4+ cells and promotes intra-phagosomal killing of the bacteria in macrophages. During an acute mycobacterial infection γδ T cells secrete much IL-17 [33], which also promotes the secretion of IL-12, thus a self-enhancing inflammatory loop is being formed. This is balanced by the secretion of TGFβ, the role of which is to dampen an over-reactive inflammatory response, partly so by inducing T-reg cells. The later may inhibit TH1 responses, thus potentially facilitating mycobacterial replication within macrophages [34]. A high incidence of T reg Foxp3 cells has been found in extra-pulmonary TB [35].

The activated T cells undergo clonal expansion and migrate out of the lymph nodes into the site of the infection in the lung, as effector T cells. This process is driven by chemokines, secreted by various inflammatory cells. Upon arrival to the battle ground they secrete interferon gamma (IFNγ), which is a key cytokine in the ensuing confrontation, by further activating the microbicidal machinery of the macrophage and causing it to secrete IL-18, amongst other cytokines, which seems to be part of the protective TH1 type response. IFNγ also induces the production of toxic NO via inducible NO synthase (iNOS). Casanova et al [36,37,38] have described in detail the importance of the IFNγ-IL-12 cytokines loop, including their receptors, for TB immunity. Furthermore they have described rare Mendelian genetic defects in this system, resulting in susceptibility to serious mycobacterial and sometimes salmonellar infections.

CD8+ T cells also participate in the immune reaction, as they have been found in the mediastinal lymph nodes, mixed with CD4+ cells and later at the infection site in the lungs. Most evidence about them has been collected in mouse and primate models and their role in human infections has not been fully elucidated [7].It has been demonstrated in vitro that CD8+ cells recognize bacterial peptides and lipids through the MHC-I CD-1 mol-

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ecules, which induce a cytotoxic response toward the bacteria and to the phagocytes in which they reside. They also secrete IFNγ and TNFα. Humans with latent TB develop a high level of mycobacteria-specific CD8+ T cells [39].

From all the above it is clear that the dominant protective response in TB is Th1 type. However in multiple-drug resistant (MDR) [40] and in young children [41] there is a skewing towards a Th2 type response, with greater secretion of IL-4. This may explain why children tend to develop pulmonary milliary and extrapulmonary disease. In addition it seems that the disease in children tends to have a Mendelian heritability of specific defects, while in adults there is no such background, rather some discrete polymorphisms may be found in different populations, such as in the natural resistance-associated macrophage protein 1 (NRAMP1) [42].

For a long time it was generally accepted that B-cells and specific antibodies have no protective role against TB. However monoclonal antibodies against some mycobacterial antigens have shown a clear protective effect in mice [43]. It has been postulated that the unique phenomenon of BCG protection against pediatric TB meningitis may be due in part to specific antibodies. Presently the exact role of B-cells in human TB remains to be determined.

Similarly to the innate immune system, mycobacteria have also developed evasion tactics from the adaptive immune system [44]. They may interfere with the antigen presentation process, promote the secretion of IL-10 by T cells, thereby polarizing them toward a TH2 type response, in which the essential IFNγ secretion is inhibited [7]. They may also attract more T-reg cells to the infection site, thereby further dampening the protective inflammatory response. It was demonstrated in a tuberculosis rabbit model, that mycobacteria may delay the macrophage and T-cells activation process, thereby enabling them to form a permanent infection and damaging pulmonary tissue [45]. More specifically, the bacteria possess a set of genes- rpf, which code for the regulatory Rpf proteins, which are believed to be responsible for activating bacteria from a dormant state in latency. In addition the bacteria have also a set of "anti-dormancy genes"-DosR, which induce bacterial growth, when appropriate [46].

4. The tuberculous granuloma

The formation of granuloma is the host's containment effort in response to an infection which he can not eradicate. In most cases it results in a state of latency, with dormant, but viable, bacteria residing in it [7, 45, 47]. Therefore the granuloma benefits also the bacteria, who may emerge from dormancy, proliferate again and cause an active disease, if the host's immune system is weakened due to any reason. HIV coinfection, with its damage to T cells, has become the most prominent example of this situation.

The granuloma contains a nucleus of necrotic lung tissue and intraphagosomal bacteria-containing macrophages, surrounded by fibroblasts, DCs, neutrophils, B cells and various subsets of T cells, all of those secreting cytokines, mainly IFNγ and TNFα, and chemokines which ensure a continuous mobilization of granulocytes to the granuloma. TNFα activates adhesion molecules on the immunocytes [48]. Thus the granuloma is a dynamic and continu-

ous battlefield balancing the bacteria against the immune system. Occasionally, as described before, the bacteria may damage the phagosomal membrane and escape, inducing an apoptotic or necrotic death of the macrophage. This enables the bacteria to proliferate with enhancement of tissue damaging inflammation, which may result in cavity formation.

5. Shall we ever have an effective immunotherapy or anti-TB vaccine?

Application of highly effective vaccines across the globe is the only way to control and arrest the spread of infectious diseases. So far BCG is the only available anti-TB vaccine. It is one of the oldest vaccines and has remained unchanged for a long time. It does confer reasonable protection to infants at risk and prevents pediatric TB meningitis. However it is ineffective for protection of large adult populations and has failed to prevent the rise in new infections and active disease patients and especially in MDR and extreme drug resistant (XDR) cases [49]. Therefore many efforts have been invested in trying many forms of various extracts of other mycobacteria, such as M. vaccae, which may be considered as immunostimulants of TH1 responses or a kind of vaccines. Most have resulted in a transient enhancement of the anti-tuberculous inflammatory response, sometimes with severe side-effects, but without long-term clinical benefit [50]. How can this be explained?

The main reason is that decades of research have not, as yet, demonstrated a universal clearly immunodominant and protective T cell epitope to one of the bacterial antigens-mainly to cell-wall peptides, lipids or glycolipids. An exception may be the 85A and 85B antigens, which may be suitable candidates for a widely used anti-tuberculous vaccine under various constructs [51]. They show enhancement of TH1-type responses, but long-term clinical results are still unknown. Additional vaccines are under trials, such as MTB subunit and DNA preparations [52].

In addition there is the problem in the variability of the host immunogenetic response, both to BCG and to MTB [53]. Therefore various research projects are trying to identify, already mentioned, polymorphisms in immune-associated and other genes, which may increase or decrease the susceptibility to TB, such as the one which has been recently identified in a Moroccan population [54] and another one in a Chinese ethnic group [55]. This subject lies outside of the scope of this chapter, but it may lead to a better understanding of the processes determining the fate of a MTB infection and assist in designing better vaccines, although they may need to be population-targeted.

6. Summary

It has been attempted, in the present chapter, to describe in some detail the arms race between MTB and its ancient human host, who uses the full scope of his sophisticated innate and adaptive immune mechanisms to placate the enemy. The bacteria, which succeed to break the physical barriers in the respiratory tract and reach the lung, are immediately surrounded by residing

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DCs and AMa, which recognize the bacterial PAMPs with their PRRs, such as surface TLRs. This recognition triggers DC and macrophage activation, which results in the phagocytosis and internalization of the bacteria in the phagolysosome, where they are submitted to toxic lysis. Meanwhile the macrophages emigrate to the mediastinal lymph nodes, where the bacterial lipid and peptide molecules are presented to CD4+ and CD8+ T cells via MHC-I and MHC-II, causing T cell activation and clonal proliferation. The later return to the battlefield at the site of the lung infection and try to complete bacterial elimination, by intensifying local inflammation. To achieve that, the T cells and the macrophages secrete a series of cytokines, such as IFNγ, IL-12 and TNFα. Secreted chemokines attract more inflammatory cells, such as neutrophils.

Nevertheless, 90% of infected persons, who remain clinically asymptomatic, enter the stage of latency, in which they continue to harbor dormant, albeit viable, bacteria in their macrophages and 10% develop active clinical disease. This is due to numerous evasion tactics from the immune system, that MTB has developed during its long cohabitation with the human host. The bacterium may damage the phagosomal membrane and escape into the macrophage cytosol, inducing necrotic cell death. It may interfere with the signaling to T cells via MHC molecules, downregulate the secretion of IFNγ, promote the secretion of IL-10 and the activity of CD4+Foxp3 T reg cells, thus dampening the protective inflammatory response. A hallmark of the latency stage is granuloma formation, which is a complex structure, containing a core of dormant bacteria in necrotic tissue, surrounded by neutrophils, macrophages, DCs and T cells. This precarious balance may be easily disrupted, if, for whatever reason, immune surveillance is weakened, causing bacterial breakthrough and clinical relapse.

So far, BCG is the only antituberculous vaccine widely available, which does confer a measure of protection in children, but failed to arrest the spread of the infection in adult populations. Many centers around the world are trying to identify immunodominant bacterial epitopes, which could form the basis of a universal efficacious vaccine. So far, the 85A and 85B antigens, in various constructs, seem to be presently the most promising, at least in animal models and limited clinical trials. In addition, since the beginning of the 20th century, many mycobacterial formulations and lately also cytokines, have been tried as specific immune stimulants. In most cases they did induce generalized inflammation with significant side-effects, but with little clinical benefit. However, recent technological developments, such as recombinant preparations and DNA extracts, may obtain better results. To those have to be added numerous projects trying to unravel the immunogenetic susceptibility or resistance factors.

One may estimate that within a decade, or so, better anti-tuberculous vaccines and treatments will be developed, possibly targeted to specific populations.

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Chapter 3

Lipid Inclusions in Mycobacterial Infections

Matthias Stehr, Ayssar A. Elamin and Mahavir Singh

Additional information is available at the end of the chapter

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1. Introduction

M. tuberculosis and *M. leprae* are intracellular pathogens. *M. tuberculosis* can survive up to decades in a phenotypically non-replicating dormant state, primarily in hypoxic granulomas in the lung [1]. The otherwise drug-susceptible dormant mycobacteria show the remarkable property to develop drug resistance within the granulomas of the host. These nonreplicative drug-resistant bacteria within the host´s tissues are called persisters [2].

Mycobacteria have outstanding mechanisms to escape from elimination and have a high degree of intrinsic resistance to most antibiotics, chemotherapeutic agents and immune eradication [3,4]. One major obstacle for host defence mechanisms and therapeutic intervention is the robust, mycolic acid-rich cell wall, which is unique among prokaryotes [3,5]. In the last years it has become apparent that mycobacteria induce the accumulation of lipids in the host cells and use them as energy and carbon source. This strategy is regarded as another crucial factor for the long term-survival of *M. tuberculosis* and *M. leprae* in the host. Most mycobacteria have the ability to synthesize lipid bodies as reservoirs for fatty acids. The lipid droplet- containing macrophages are called "foamy macrophages" and are the hallmark of *M. tuberculosis* and *M. leprae* infection.

M. leprae is the causative agent of leprosy. Leprosy is a chronic infectious disease caused by the obligate intracellular bacterium *Mycobacterium leprae* and is a major source of morbidity in developing countries [6,7]. Leprosy patients show two major manifestations of the disease, known as as lepromatous leprosy (LL), and tuberculoid leprosy (TT) [6]. TT is observed in patients with good T-cell mediated (Th1) immunity and is characterized by granuloma formation and death of Schwann cells (Scs) leading to myelin degradation and nerve destruction [8,9]. Patients with poor T-cell mediated immunity show the lepromatous type leprosy (LL), which leads to a high bacterial load inside host cells specially in Schwann cells and macrophages [8,10-12]. For both forms of leprosy damage of the nerves is observed [12].

Lepromatous leprosy lesions of the skin, eyes, nerves, and lymph nodes are characterized by tumor-like accumulations of foamy macrophages. The foamy macrophages are fully packed with lipid droplets (LDs) and contain high numbers of leprosy bacilli. These aggregations of foamy macrophages expand slowly and disfigure the body of the host [13].

The finding that *M. leprae* has insufficient fatty acid synthetase activity to support growth lead to the hypothesis that *M. leprae* scavenges lipids from the host cell [14]. Over the last years it has become evident that survival and persistence of *M. tuberculosis* is critically dependent on lipid body formation. Furthermore lipid body formation seems to be the prerequisite for transition of *M. tuberculosis* to the dormant state. The formation of foamy macrophages is a process which appears to be a key event in both sustaining persistent bacteria and release of infectious bacilli [15]. This goes along with the important observation that sputum from tuberculosis patients contains lipid body-laden bacilli [16,17].

In the dormant state lipids from lipid bodies appear to be the primary carbon source for *M. tuberculosis* in vivo. For *M. tuberculosis* several bacterial genes are upregulated during the dormant state and have been reported to be involved in lipid metabolism such as diacylglycerol acyltransferase (tgs1), lipase (lipY), and isocitrate lyase (icl) [18,19].

M. leprae has a small genome (3.2 Mb). The obligate intracellular organism shows a moderate genome degradation and several genes are absent when compared with other mycobacterial species. Due to the gene loss *M. leprae* is strongly dependent on the host for basic metabolic functions [8,20]. Macrophages infected with *M. leprae* contain oxidized host lipids and it has been observed that *M. leprae* upregulates 13 host lipid metabolism genes in T-lep lesions and 26 in L-lep lesions. The oxidized lipids inhibit innate immune responses and thus seem to be an important virulence factor for the organism [21].

This review highlights the importance of the LDs as one of the most unique determinant for persistence and virulence of *M. tuberculosis* and *M. leprae*. The formation of LDs in *M. tuberculosis and M. leprae* in infected host cells shall be compared and the lipid metabolism of both organisms will be discussed.

In this review we will use the term “lipid droplets” for lipid-rich inclusions in the host and “lipid bodies” for lipid-rich inclusions in the pathogen.

2. Biogenesis of lipid inclusions in bacteria and eukaryotes

The current models of lipid droplet biogenesis are still hypothetical and have been reviewed extensively by Murphy in 1999 and Ohsaki in 2009 [22,23]. The most common model supposes that the membrane protein diacyltransferase DGAT1 synthesizes triacylglycerols (TAG), which accumulate between the two membrane leaflets of the endoplasmic reticulum (ER) to be finally released by budding. The lipids are covered by a phospholipid monolayer from the ER membrane.

The formation of lipid bodies in bacteria has been even less characterized. Wältermann et al. suggested in 2005 that a bifunctional wax ester synthase/acyl-CoA:diacylglycerol acyltrans-

ferase, (WS/DGAT) synthesizes TAG for lipid body formation. WS/DGAT is an integral membrane protein and synthesizes a growing globule around the cytoplasmic portion of the enzyme. Finally the lipid body is released to the cytoplasm. The origin of the surface phospholipid monolayer is not known [22,24].

2.1. Lipid droplets in the host

The accumulation of lipid droplets occurs also in several infectious, and inflammatory conditions, including in atherosclerosis [25], bacterial sepsis [26], viral infections [27], and in mycobacterial infections [15,28,29]. *M. tuberculosis* infected macrophages store mostly neutral lipids, while cells infected with *M. leprae* seem to accumulate next to TAG a high degree of cholesterol and cholesterol esters [10,30].

LDs are observed in various cells of the immune system including macrophages, neutrophils, and eosinophils. The structure and composition of LDs is highly conserved. They contain a core of neutral lipid esters typically TAG, but also sterols and sterol esters [31-36]. The surface is covered by a phospholipid monolayer, which is composed at least in some cells by unique fatty acids [37].

M. leprae infects preferentially macrophages and Schwann cells [11]. A typical feature of lepromatous leprosy is the survival and replication of. *M. leprae* within the lipid droplets stored in the enlarged phagosome of histiocytes. Lipid droplets are thought to be an important nutrient source for the bacillus. A major concern in leprosy is peripheral neuropathy. The damage to nerves of the peripheral nervous system is caused by the the infection of Schwann cells (SCs) by *M. leprae*. In LL nerve biopsies, highly infected SCs also contain lipid droplets and show a foamy appearance, such as Virchow cells found in dermal lesions [38]. The biology of the these foamy cells has been characterized poorly until now. Neither the origin or nature of the lipids has been elucidated yet. Only recently it *in vitro* studies by Mattos could show that ML induces the formation of lipid droplets in human SCs [10]. Moreover, the group found that LDs are promptly recruited to bacterial phagosomes. In SCs LD recruiting by bacterial phagosomes depends on cytoskeletal reorganization and PI3K signaling, but is independent of TLR2 bacterial sensing [10].

Important markers for the lipid accumulation in adipocytes or macrophages are lipid-droplet-associated proteins such as adipose differentiation-related protein ADRP and perilipin, which play essential roles in lipid-droplet formation [39]. After phagocytosis of live *M. leprae* ADRP expression is constantly upregulated in human monocytes. ADRP and perilipin are localized at the phagosomal membrane (Figure 4) [39].

2.2. Lipid bodies in the pathogen

Prokaryotes do not generally produce lipid bodies containing TAG. Accumulation of TAG in intracellular lipid-bodies is mostly restricted to bacteria belonging to the actinomycetes group [40].

Most mycobacterial species accumulate considerable amounts of TAG during infection [24,41-44]. The intracellular pathogen *M. tuberculosis* can survive up to decades in a pheno-

typically non-replicating dormant state, primarily in hypoxic granulomas in the lung [1]. The otherwise drug-susceptible dormant bacteria develop drug resistance within the granulomas of the host. These nonreplicative drug-resistant bacteria within the host´s tissues are called persisters [2].

It has been observed that persisters store large amounts of intracellular triacylglycerol lipid bodies (LBs) [15,17,28,45,46]. *M. tuberculosis* uses TAG from the lipid bodies as energy and carbon source under conditions such as starvation [47], oxygen depletion [48], and pathogen reactivation [49]. The observation that sputum from tuberculosis patients contains lipid body-laden bacilli, proves the importance of lipids for the survival of the bacterium in the host [17].

3. *M. tuberculosis* induces foamy macrophages in the host

M. tuberculosis infects primarily alveolar macrophages, which reside within alveoli. The infected macrophage leaves the alveoli and migrates then towards the next lung draining lymph node. *M. tuberculosis* inhibits the generation of the phagolysosome and the bacteria begin to multiply within the macrophage [50]. The host's immune response seems to be unable to clear the bacillus from the infected macrophages. Infected macrophages secrete TNF-α and chemokines, which recruit systemic monocytes. The macrophages start to enlarge and accumulate TAG in lipid droplets. These lipid-filled foamy macrophages (FM) are surrounded by an outer layer of lymphocytes. Within the foamy macrophages the bacteria resist in phagosomes, packed with lipid droplets.

Over the last years it has become evident that survival and persistence of *M. tuberculosis* is critically dependent on lipid body formation, and induction of foamy macrophages appears to be a key event in both sustaining persistent bacteria and and release of infectious bacilli [15].

M. tuberculosis-infected phagosomes engulf cellular lipid droplets and finally the bacteria are completely enclosed by cellular lipid droplets. Only enclosed by lipid droplets the bacteria form lipid bodies and cell replication comes to a halt and finally the bacteria enter the state of dormancy and induced drug resistance [19,28]. In the nonreplicative state *M. tuberculosis* induces several bacterial genes involved in lipid metabolism such as diacylglycerol acyltransferase (tgs1), such as diacylglycerol acyltransferase (*tgs1*), lipase (*lipY*), and isocitrate lyase (*icl*) are upregulated [19,46]. In conclusion lipid body formation seems to be absolutely necessary for transition of *M. tuberculosis* to the dormant state. This goes along with the important observation that sputum from tuberculosis patients contains lipid body-laden bacilli [17].

The final granuloma consists of a core of infected, lipid-laden macrophages, which are surrounded by an outer layer of additional differentiated macrophages. The outer shell consists of T lymphocytes, B lymphocytes, dendritic cells, neutrophils, fibroblasts and an extracellular matrix [29,51-53].

The development and composition of a human tuberculosis granuloma is depicted in Figure 1.

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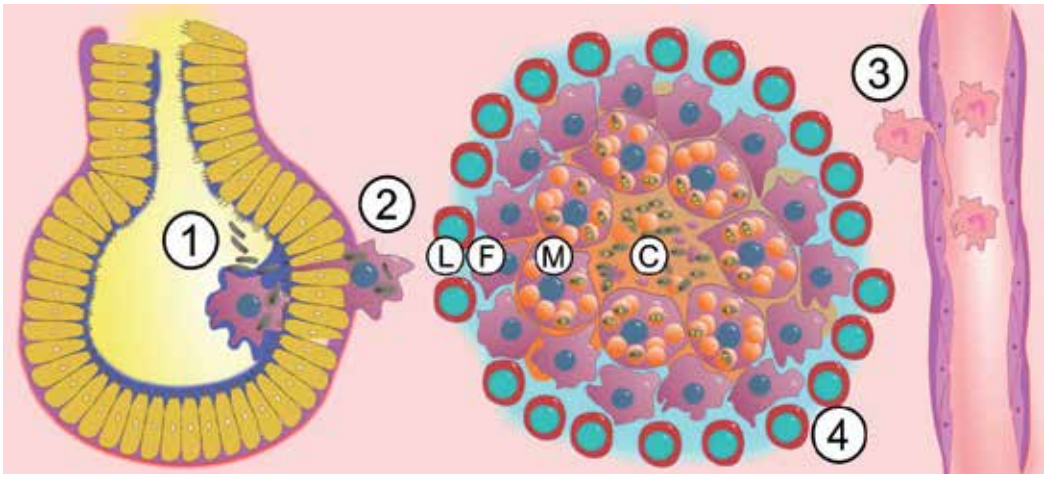

Figure 1. Development and structure of the human tuberculosis granuloma. 1, Uptake of *M. tuberculosis* by alveolar macrophages. 2, Migration of the infected macrophage towards the next lung draining lymph node. 3, Recruitment of systemic monocytes. 4, Granuloma formation. L, Lymphocytes at the periphery of the granuloma outside the fibrous outer layer. F, Fibrous capsule. Contains fibroblasts, collagen and other extracellular matrix proteins. M, Macrophage region with foamy macrophages. C, Caseum. Contains debris and lipids from necrotic macrophages. Orange, Lipid droplets of the macrophage. Yellow dots, Lipid bodies.

3.1. Lipid body formation in *M. tuberculosis* is critically dependent on lipid droplets from the host

Host lipids from lipid droplets are used by the pathogen as substantial nutrient source. Middlebrook already demonstrated in the late 1940s that mycobacterial growth *in vitro* was enhanced by supplementation with oleic acid [54]. Over the last years several groups have reported that *M. tuberculosis* within foamy macrophages produces lipid bodies, suggesting that they are able to accumulate host cell lipids [19,55]. Mycobacterial growth inside adipocytes is strictly dependent upon TAG provided by lipid droplets in host cells [55], and it has been shown that *M. tuberculosis* incorporates intact host TAG into bacterial TAG [46].

The utilization of host lipids in vivo does not only promote survival but may also increases virulence and modulate the immune response to infection. Growth of *M. tuberculosis* on fatty acids such as such propionate or valerate during infection leads to increased production of the surface-exposed lipid virulence factors, phthiocerol dimycocerosate (PDIM) and sulfolipid-1 (SL-1) [56].

Cholesterol utilization was also identified to be required for mycobacterial persistence [57]. In 2008 Pandey and Sassetti found that *M. tuberculosis* can grow using cholesterol as a primary carbon source and that the mce4 transporter is required for cholesterol uptake. *M. tuberculosis* contains four homologous mce operons, mce1–mce4, which are thought to encode lipid transporters [57,58].

Especially *M. leprae* infected macrophages show an increased accumulation of cholesterol and cholesterol [10,30]. But in contrast to *M. tuberculosis* the *M. leprae* genome encodes only one

operon for cholesterol uptake (mce1). All *M. leprae* five *mce* genes were overexpressed during intracellular growth in mouse and human biopsies [59,60]. This observation suggests, that the intracellular bacilli population induces cholersterol uptake of the infected cell and subsequently uses the stored cholesterol as carbon and energy source.

Cholesterol is also essential for uptake of *M. tuberculosis* and *M. leprae* in macrophages. Cholesterol accumulates at the site of mycobacterial entry in macrophages and promotes mycobacterial uptake. Cholesterol mediates the recruitment of TACO from the plasma membrane to the phagosome [61]. TACO, also termed as CORO1A, is a coat protein that prevents phagosome-lysosome fusion and thus degradation of mycobacteria in phagolysosomes (Figure 4) [61,62]. This mechanism for the formation of TACO-coated phagosomes promotes intracellular survival [62,63].

3.2. Lipid body formation in *M. tuberculosis* is critically dependent on lipid droplets

Host lipids from lipid droplets are used by the pathogen as substantial nutrient source. Middlebrook already demonstrated in the late 1940s that mycobacterial growth *in vitro* was enhanced by supplementation with oleic acid [54]. Host lipids play an important role during infection. They appear to be the primary carbon source for *M. tuberculosis* in vivo. Over the last years several groups have reported that *M. tuberculosis* within foamy macrophages produces lipid bodies, suggesting that they are able to accumulate host cell lipids [19,55]. Neyrolles et al. showed that mycobacterial growth inside adipocytes occurs only after the formation of lipid droplets in the host cell. This result emphasizes that *M. tuberculosis* is dependent upon TAG provided by lipid droplets in host cells [55]. In 2011 Daniel et al. finally demonstrated that *M. tuberculosis* inside foamy macrophages imports fatty acids derived from host TAG and incorporates them intact into bacterial TAG. Moreover the group proved the accumulation of TAG in lipid bodies [46].

The utilization of host lipids in vivo does not only promote survival but may also increases virulence and modulate the immune response to infection. Growth of *M. tuberculosis* on fatty acids such as such propionate or valerate during infection leads to increased production of the surface-exposed lipid virulence factors, phthiocerol dimycocerosate (PDIM) and sulfolipid-1 (SL-1) [56].

Cholesterol utilization was also identified to be required for mycobacterial persistence [57]. In 2008 Pandey and Sassetti found that *M. tuberculosis* can grow using cholesterol as a primary carbon source and that the mce4 transporter is required for cholesterol uptake. *M. tuberculosis* contains four homologous mce operons, mce1–mce4, which are thought to encode lipid transporters [57,58].

3.3. Biosynthesis of TAG and formation of lipid bodies in *M. tuberculosis*

Biosynthesis of TAG consists of the sequential esterification of the glycerol moiety with fatty acyl-residues by various acyltransferases. Fatty acid biosynthesis consists of the stepwise addition of acetyl groups, which are provided by acetyl-CoA. The initial step is the transfer of an acetyl group from acetyl-CoA to a small protein, called acyl carrier protein (ACP). In the

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following two-carbon fragments are added sequentially to yield fatty acids of the desired length. *M. tuberculosis* uses both type I and type II FAS systems for fatty acid elongation. The multifunctional FAS I enzyme (*Rv2524c*) catalyzes the de novo synthesis of C_{16}- and C_{18}-S-ACP. These fatty acids are converted to the CoA derivative and used primarily for the synthesis of membrane phospholipids. By continuous elongation of these fatty acids FAS I produces specifically the C20- and C26-S-ACP products, and these fatty acids are released as the CoA derivatives. The C20 fatty acid is transferred to the FAS II system for the synthesis of the very-long-chain mero segment of α-, methoxy-, and ketomycolic acids [64]. The transfer from the FAS I to the FAS II system occurs by a key condensing enzyme, the ketoacyl ACP synthase III (FabH). FabH catalyzes the decarboxylative condensation of malonyl-ACP with the acyl-CoA products of the FAS I system (Figure 2). Two distinct cyclopropane synthases, MmaA2 and PcaA introduce cyclopropane rings into the the growing acyl chain [64-66].

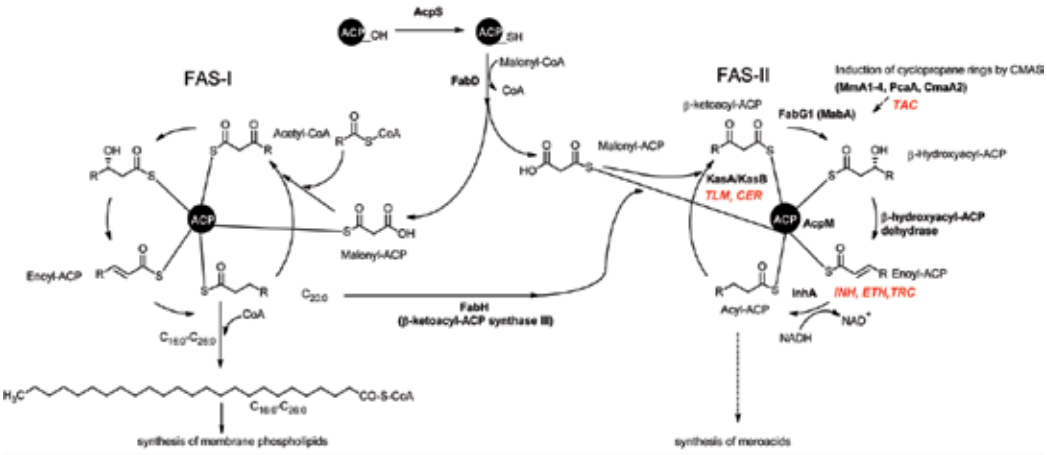

Figure 2. Fatty acid biosynthesis in *Mycobacterium tuberculosis*. The FAS-II elongation module uses the substrates R-CO-S-ACP and malonyl-S-ACP derived from malonyl-S-CoA, generated by FabD. FabH condenses both substrates R, long-chain alkyl group. Enzymes involved in these reactions are as follows: FabG1, a β-ketoacyl-ACP reductase catalyzes the reduction of beta-ketoacyl-ACP substrates to beta-hydroxyacyl-ACP. β-hydroxyacyl-ACP dehydrase. 2-trans-enoyl-ACP reductase (InhA). The β-ketoacyl-ACP synthase (KasA/KasB) catalyzes the addition of of two carbons from malonyl-ACP to R-CO-S-ACP (See text for details). R, long-chain alkyl group. ACP, acyl carrier protein. Enzymes are in bold letters. Selected inhibitors are depicted in red bold letters. TLM, thiolactomycin. CER, cerulenin. ETH, ethionamide. INH, isoniazid. TRC, triclosan. TAC, thiacetazone.

Esterification of fatty acids with glycerol-3-phosphate occurs via sequential acylation of the sn-1,2 and 3 positions of glycerol-3-phosphate, and removal of the phosphate group before the last acylation step. The terminal reaction is the esterification of diacylglycerol (DAG) with acyl-CoA by an diacylglycerol acyltransferase [40]. Animals and plants use diacylglycerol acyl-transferases (DGAT) for the terminal esterification. DGATs catalyze exclusively the esterification of acyl-CoA with diacylglycerol. Bacteria do not contain

DGATs but only bifunctional wax ester synthase/acyl-CoA:diacylglycerol acyltransferases (WS/DGAT). WS/DGATs, mediate next to TAG formation the synthesis of waxes by esterification of acyl-CoA with alcohol [67]. The genome of *M. tuberculosis* codes for 15 genes which contain the highly conserved putative active site motif of WS/DGATs (HHxxxDG). These genes were designated as "tgs", triacylglycerol synthases, but have only a weak sequence similarity to other WS/DGAT sequences. All 15 expressed mycobacterial Tgs proteins show diacylglycerol acyltransferase activity and Tgs1 has the highest activity of all enzymes [48]. Gene disruption of *tgs1* results in a drastic reduction of major C26 long-chain fatty acid in *M. tuberculosis* grown under hypoxic conditions. Thus Tgs1 appears to be a major contributor to TAG synthesis in *M. tuberculosis* so far [48,68]. And moreover two homologous proteins to Tgs1 and Tgs2 (BCG3153c and BCG3794c) and another poorly characterized acyltransferase (BCG1489c) were found to be exclusively associated to lipid bodies. The disruption of *BCG3153c, BCG3794c*, and *BCG1489c* reduces TAG accumulation during the hypoxia-induced nonreplicating state, revealing that the enzymes are involved in TAG synthesis during latency and pathogenicity [69].

Ten of the 15 tgs genes in *M. tuberculosis* are located adjacent or proximal to 11 lip genes that are annotated as probable phospholipases or lipases-esterases-carboxylesterases. Some tgs genes may be cotranscribed with neighboring lip genes and may synthesize triacylglycerols from the released fatty acids from the host [18]. Lip gene products may be important for utilization of TAGs during dormancy and upon reactivation after dormancy. The tgs gene Rv0221 is located near lipC (*Rv0220*), lipW (*Rv0217c*), acyl-CoA synthetase (*Rv0214*), acyl-CoA dehydrogenase (*Rv0215c*), and an integral membrane acyltransferase (*Rv0228*). This clustering of genes of the fatty acid metabolism suggests that these genes may be cotranscribed and may release fatty acid from host TAG, carry out the transport of fatty acids and finally catalyze the re-synthesis of TAGs in the pathogen. Rv0221 and LipC have to be shown to be catalytical active. [18,70].

In summary Tgs enzymes play a major role in TAG synthesis, lipid body formation and maintenance.

Ag85A, a mycoltransferase, that is known to catalyze the formation of the cord factor was recently found to have additional DGAT activity [71]. The kinetic parameters are quite similar to those reported for the *M. tuberculosis* Tgs1-4, but the primary sequence of Ag85A does not contain the active site motif of WS/DGATs or TGS enzymes (HHxxxDG) [48,68,71]. Ag85A belongs to the α/β hydrolase fold family and contains the consensus GXSXG sequence. The enzyme is a carboxylesterase with an additional acyltransferase activity. Overexpression of Ag85A induces lipid body formation in *M. smegmatis*. The enzyme is located in the mycobacterial cell wall, suggesting that it may be involved in the maintenance of lipid droplets in the host cell [71].

The genome of *M.leprae* contains also mycolytransferase 85 complex genes (A, B and C). Transcripts of these genes are upregulated either in infected nude mouse or human skin lesions [59].

The *M. leprae* genome shows only one predicted gene product which has has a significant degree of identity to any the Tgs enzymes from *M. tuberculosis* [18]. The tgs gene product ML1244 shows 72% identity to Rv2484c from *M. tuberculosis*. *Rv2448* is located next to a carboxylesterase lipQ, (*Rv2485c*), a probable glycerol-3-phosphate acyltransferase, (*Rv2482c*), a lysophosphatidic acid acyltransferase-like protein (*Rv2483c*), and a probable enoyl-CoA hydratase (*Rv2486*). The gene cluster of lipid metabolism genes suggests a possible involvement of the gene products in the synthesis of TAG [18]. A few tgs genes (*Rv3234c*, *Rv3233c*, *Rv2285*, and *Rv1425*) are located proximal to lipoproteins, which may serve as donors or acceptors of fatty acids [48]

3.4. Activation of TAG – Lipases and esterases of *M. tuberculosis*

Neutral lipids in the core of the lipid body are hydrolyzed by lipases or esterases, yielding fatty acids for energy generation and anabolism of membrane phospholipids.

In the genome of *M. tuberculosis* H37Rv twenty-one genes are termed as putative lipases (*lip* A to W, except K and S) [72]. The annotation was only based on the presence of the consensus sequence GXSXG, which is characteristic for the large group of the α/β hydrolase fold protein family, which includes lipases as well as esterases, proteases, peroxidases, epoxide hydrolases and dehalogenases [72]. Thus the members of the lip group have only a very low level of sequence identity of ~20% and might have another function apart from lipid hydrolysis. Only the gene product of *Rv3097c* (LipY)) shows reasonable hydrolase activity for long-chain TAG with chain lengths ranging from C4 to C18. Overexpression of LipY induces extensive TAG hydrolysis. Disruption of *lipY* markedly reduces but does not completely inactivate TAG hydrolase activity, which suggests the presence of other lipases in *M. tuberculosis* [47,49].

Overexpression of *LipY* in *M. bovis* Bacillus Calmette-Guérin reduces protection against infection in mice, indicating that lipY plays a central role in TAG hydrolysis and virulence [47,73,74]. LipY contains a PE (Pro-Glu) domain, that is involved in modulation of LipY activity [73]. The PE domain contains a signal sequence for secretion of LipY by the ESX-5 system. It has been implicated that the secreted LipY is loosely associated with the bacterial surface where it may hydrolyze host's TAG [75].

Several other esterases, next to the members of the Lip group have been identified and biochemically characterized. They all belong also to the α/β hydrolase fold family and showing the minimal GXSXG motif. In 2007 Côtes et al. characterized a novel lipase Rv0183. The enzyme is only found in the cell wall and culture medium. This observation suggests that Rv0183 is involved in the degradation of the host cell lipids e.g. when M. tuberculosis infects adipocytes [55,76]. Another probably cell wall-associated carboxylesterase is encoded by Rv2224c. The esterase Rv2224c was found to be required for bacterial survival in mice [77]. The substrate spectrum of Rv2224c is poorly characterized and until now it is unknown whether the enzyme uses TAG as substrate [77]. Furthermore the three-dimensional structures of the esterases Rv0045c (PDB 3P2M) [78], Rv1847 (PDB 3S4K), and LipW (3QH4) from *M. tuberculosis* have been determined, but unfortunately it is not known whether these enzymes are involved in TAG hydrolysis.

3.5. Lipase genes of *M. leprae*

In the *M. leprae* genome only 2 lipase genes (*lipG*, *lipU*) were found. But *M. tuberculosis* has also only six expressed Lip enzymes, showing reasonable hydrolase activity for long-chain triacylglycerols. (LipY, LipC, LipL, LipX, LipK, LipG). LipG and LipU from *M. leprae* are homologous with LipG and LipU from *M. tuberculosis* and show sequence identities of 72 and 79%, respectively. The lipases LipG and LipU from *M. tuberculosis* show very low and no activity with long chain triacylglycerols as substrates [47]. *M. tuberculosis* LipY is suspected to be a major functional lipase, which utilizes stored triacylglycerols (TAG) during dormancy and reactivation of the pathogen [47,49]. LipY shows only a weak similarity with *M. leprae* LipU (23 % identity). In summary it appears that *M. leprae* uses different lipases for the hydrolysis of fatty acids than *M. tuberculosis*.

3.6. Enzymes of the β-oxidation and glyoxylate cycle

M. tuberculosis can grow on fatty acids as sole carbon source and it has been demonstrated that fatty acid oxidation is important for survival of the pathogen in the lungs of mice [79,80]. Fatty acids are oxidized via the β-oxidation cycle and the glyoxylate shunt, to replenish TCA cycle intermediates during growth [81]. The β-oxidation cycle consists of five biochemical reactions, where one molecule acetyl-CoA of the fatty acid is split off per cycle. The genome of *M. tuberculosis* encodes around 100 genes, designated as fad genes (fatty acid degradation) with putative roles in the β-oxidation of fatty acids. While *E. coli* has only one enzyme for each step of the β-oxidation cycle, *M. tuberculosis* seems to have several backup enzymes for each reaction [82]. The initial step of β-oxidation is the formation of acyl-CoA from free fatty acids and Coenzyme A and is catalyzed by acyl-CoA synthase. In *M. bovis* BCG one Acyl-CoA synthase (BCG1721) (Rv1683) has been identified to be exclusively bound to lipid bodies. Nonreplicating mycobacteria, which overexpress a BCG1721 construct with an inactive lipase domain displayed a phenotype of attenuated TAG breakdown and regrowth upon resuscitation. These results indicate that the gene might be essential for TAG hydrolysis and growth [69].

Together with malate synthase, isocitrate lyase (ICL) is the key enzyme of the glyoxylate cycle that catalyzes the cleavage of isocitrate to glyoxylate and succinate [81,83]. The *M. tuberculosis* genome codes for two isocitrate lyases, icl and icl2, which are essential for the fatty acid metabolism and jointly required for in vivo growth and virulence. Disruption of icl has only little effect on survival in macrophages and bacterial loads in lungs of infected mice. Only disruption of both lyase genes results in a fast elimination of bacteria from lungs of infected mice and infected macrophages [79,80]. These results strongly suggest that both icl genes are required for mycobacterial persistence.

All enzymes involved in lipid metabolism in lipid bodies are summarized in Table 1.

M. leprae has approximately one-third as many potential fad enzymes with probable roles in the β-oxidation. Even though *M. leprae* genome contains less necessary β-oxidation cycle genes than *M. tuberculosis*, transcript analysis revealed expression of acyl-CoA metabolic enzymes including *echA1* (ML0120, putative enoyl-CoA hydratase), *echA12* (ML1241,

possible enoyl-CoA hydratase), *fadA2* (ML2564, acetyl-CoA-acetyltransferase), *fadB2* (ML2461, 3-hydroxyacyl-CoA dehydrogenase), *fadD19* (ML0352, acyl-CoA synthase), *fadD26* (ML2358, fatty acid-CoA-ligase), *fadD29* (ML0132, probable fatty-acid-CoA synthetase), *fadD28* (ML0138, possible fatty-acid-CoA synthetase), *fadE25* (ML0737, probable acyl-CoA dehydrogenase) and *fadE5* (ML2563, acyl-CoA dehydrogenase) [59,60]. This gives strong evidence that host lipids provide the main carbon and energy sources for *M. leprae* during infection.

The *M. leprae* genome contains a gene, coding for an isocitrate lyase, *aceA*. The amino acid-sequence of AceA (ML1985c) shows 80 % identity with its homologue from *M. tuberculosis* ICL2 (Rv1915/1916). *AceA* is upregulated in both *M. leprae*-infected nude mouse and human lesions. [59]. A second icl gene, as observed in *M. tuberculosis*, is not present in the genome of *M. leprae*. This finding is of particular interest, because both lyases, *icl* and *icl2*, are jointly required for in vivo growth and virulence [79,80]. Deletion of *icl1* or *icl2* has little effect on bacterial growth in macrophages [80]. So far the *M. leprae* AceA might play a slightly different role in as the both isocitrate lyases in *M. tuberculosis.*

4. Lipid composition in *M. leprae* infected cells

In 1863, Virchow described foamy cells, which form droplets and surround *M. leprae* within the phagolysomes. [84,85]. This lipid capsule forms a characteristic electron-transparent zone. In contrast to *M. tuberculosis*, the presence of lipid bodies seem to be rather exceptional in *M. leprae* [85]. The lipid capsule contains mycoserosoic acids of phthiocerol dimycocerosates as well as phenolic glycolipids [86,87]. Brennan reported the full characterization of three phenol-phthiocerol triglycosides by *M. leprae* [84]. It has been postulated that many of these molecules together with phosphatidylinositol mannosides and phospholipids are released from the cell wall after synthesis, forming the capsule-like region [11]. The dominant lipid in the cell wall which gives *M. leprae* immunological specificity is phenolic glycolipid-1 (PGL-1). Phenolic glycolipid 1 has been isolated in relatively high concentrations from purified bacteria and from *M. leprae* infected tissues [88]. PGL-1 is thought to be a major component of the capsule in *M. leprae* and constitutes an important interface between bacteria and host [89]. It has been suggested that PGL-1 is involved in the interaction of *M. leprae* with the laminin of Schwann cells, thus PGL-1 hight play a role in peripheral nerve-bacillus interactions [90]. Moreover, phenolic glycolipids seem to be involved in the in the stimulation of suppressor T-cells in lepromatous leprosy [91]. Recently it was reported that also LDs from *M. leprae* infected SCs and macrophages accumulate mainly host derived lipids, such as oxidized phospholipids [92]. BODIPY stains infected SCs, indicating that LDs contain neutral lipids, such as triacylglycerols (TAG), but it seems as *M. leprae*-infected cells accumulate large amounts of cholesterol and choesterol esters [10].

Gene	Protein	DGAT activity *(in vitro)*	Lipid body associated	Influence on lipid bodies / TGA accumulation	Modulation of virulence	*M. leprae homologue*	Reference
Rv3130c (*tgs1*)	DGAT	+	+	Δ*tgs1* decreases TAG accumulation	NA	ML1244	[46,48,68]
Rv3734c (*tgs2*)	DGAT	+	+	NA	NA	ML1244	[48,68]
Rv3234c (*tgs3*)	DGAT	+	NA	NA	NA	ML1244	[48,68]
Rv3088 (*tgs4*)	DGAT	+	NA	NA	NA	ML1244	[48,68]
Rv1760	DGAT	+	NA	NA	NA	ML1244	[48,68]
Rv2285	DGAT	+	NA	NA	NA	ML1244	[48,68]
Rv3804c (*85A*)	DGAT	+	NA	Overexpression increases production of lipid bodies	NA	ML0097 (85A)	[71]
BCG1489c [*Rv1428c*]	DGAT	NA	+	Δ*BCG1489c* reduces TAG accumulation	NA	ML2427c	[69]
BCG3153c (*tgs1*) [*Rv3130c*]	DGAT	NA	+	Δ*BCG3153c* reduces TAG accumulation	NA	ML1244	[69]
BCG3794c (*tgs2*) [*Rv3734c*]	DGAT	NA	+	Δ*BCG3794c* reduces TAG accumulation	NA	ML1244	[69]
BCG1489c [*Rv1428c*]	acyltransferase	NA	+	Δ*Rv1428c* reduces TAG accumulation	NA		[69]

Gene	Protein	TGA-hydrolyzing activity (*in vitro*)	Lipid body associated	Influence on lipid bodies / TGA accumulation	Modulation of virulence	*M. leprae* homologue	Reference
Rv3097c (*lipY*)	Lipase/esterase	+	NA	Δ*lipY* reduces TAG hydrolysis. Overexpression increases TAG hydrolysis	Overexpression increases virulence in mice	ML0314c (*lipU*) ML1053 ML1183c	[47]
Rv1399c (*lipH*)	Lipase/esterase	+	NA	NA	NA	ML0314c (*lipU)*	[72]
BCG1721 [*Rv1683*]	Lipase/esterase	+ (in vivo*)	+	+ (*)	NA	ML1346	[69]
Rv0183	Lipase/esterase	Hydrolyzes only monoacylglycerides	NA	NA	NA	ML2603	[76]
Rv2224c	Lipase/esterase	NA	NA	NA	Gene disruption decreases virulence in mice	ML1633c	[77]

Gene	Protein	Isocitrate cleavage (*in vitro*)	Lipid body associated	Influence on lipid bodies / TGA accumulation	Modulation of virulence	*M. leprae* homologue	Reference
Rv0467 (*icl*), *Rv1915-1916* (*icl2*)	isocitrate lyase	+	NA	NA	The Δ*icl* , Δ*icl2* strain shows no intracellular replication	ML1985c (aceA)	[79,80,93-95]

Table 1. Enzymes involved in lipid body metabolism in *M. tuberculosis* and *M. bovis* BCG. Homologous genes in *M. tuberculosis* H37Rv are written in square brackets. NA, not applicable. *, expressed in yeast as recombinant protein. DGAT, diacylglycerol acyltransferase

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5. Induction of lipid droplet biogenesis

Since the biogenesis of lipid droplets in macrophages seems an absolute requirement for intracellular bacteria to establish infections, we will discuss mechanisms involved in foam cell formation and development of lipid droplets.

5.1. Scavenger receptor mediated lipid droplet biogenesis in *M. tuberculosis*

Upon infection with pathogenic bacteria macrophages generate reactive oxygen species (ROS). The release of ROS generates oxidative stress, and results not only in damage to cellular structures but also to oxidation of fatty acids, such as low density lipoproteins (OxLDL) in granulomas. The binding of OxLDL to type 1 scavenger receptors CD36 and LOX1 induces increased surface expression of both receptors, leading to uptake of OxLDL [96-98]. In addition, CD36 increases the uptake of *M. tuberculosis* by macrophages [99]. The increased rate of OxLDL uptake results in the accumulation of oxidized lipids, which finally leads to the formation of foamy macrophages [98]. *M. tuberculosis* and *M. leprae* benefit from the accumulated OxLDL in the infected macrophage. OxLDL-laden lung macrophages show enhanced replication of intracellular *M. tuberculosis* compared to macrophages loaded with non-oxidized LDL [98]. The presence of oxidized phospholipids in *M. leprae* infected macrophages down-regulates the innate immune response and contributes to pathogenesis [92]. Moreover, scavenger receptor-deficient phagocytes are characterized by a reduced intracellular bacterial survival and a lower cytokine response [100].

5.2. TLR mediated LD formation in *M. bovis* and *M. leprae*

Mycobacterium bovis Bacillus Calmette-Guérin (BCG) and *M. leprae* are recognised by the Toll-like receptors (TLR) TLR6 and TLR2 [101,102]. Mycobacterium bovis Bacillus Calmette-Guérin induced lipid body formation is TLR2 mediated [103]. The mycobacterial surface molecule lipoarabinomannan (LAM) induces the formation of foamy macrophages by binding to TLR2 [104] (Figure 3).

M. leprae association to macrophages is mediated by binding of the bacteria to TLR2 and TLR6. Heterodimerization of TLR2 and TLR6 leads to downstream signalling and subsequent LD formation [102,105]. Macrophage association is not dependent on binding to TLR2 or TLR6. Neither a $TLR2^{-/-}$ or $TLR6^{-/-}$ knockout macrophage shows reduced binding to *M. leprae*. This suggests that both TLR2 and TLR6 can bind *M. leprae* alone, or/and the presence of other receptors, binding to *M. leprae*. The $TLR2^{-/-}$ or $TLR6^{-/-}$ knockout macrophages do also not completely abolish LD formation, but show only reduced LD formation [102]. This suggests the presence of additional signalling pathways for LD formation. In SCs TLR6, but not TLR2, is essential for *M. leprae*-induced LD biogenesis in [101]. In LL lesions, accumulated with LD enriched macropages the genes for ADRP and CD36 are up-regulated [30,92,102]. This suggests also an involvement of CD36 in LD formation of *M. leprae* (Figure 4) [99].

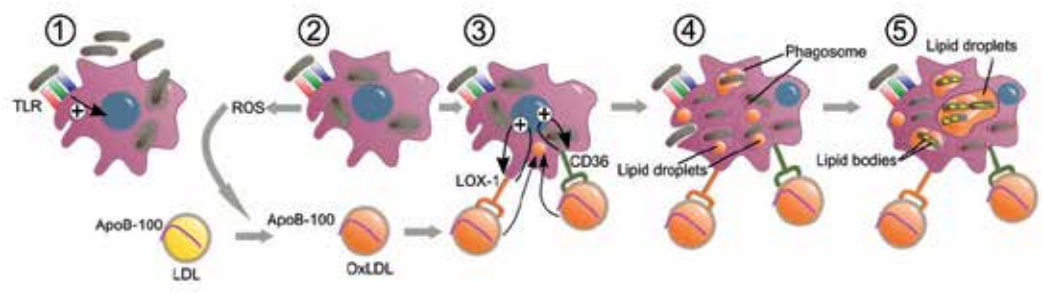

Figure 3. Induction of lipid droplet biogenesis in macrophages by *Mycobacterium tuberculosis*. 1) Recognition of bacteria by Toll-like receptors (TLR) trigger phagocytosis and subsequent formation of lipid droplets. 2) The infected macrophage produces reactive oxygen species (ROS), which oxidize LDL. 3) The binding of OxLDL to type 1 scavenger receptors CD36 and LOX1 induces increased surface expression of both receptors and increases uptake of host´s oxidized fatty acids. 4) Mycobacterium-laden phagosomes internalize lipid droplets. 5) Within the lipid droplets the bacteria form lipid bodies and finally enter the dormant state. ApoB-100, apolipoprotein B-100.

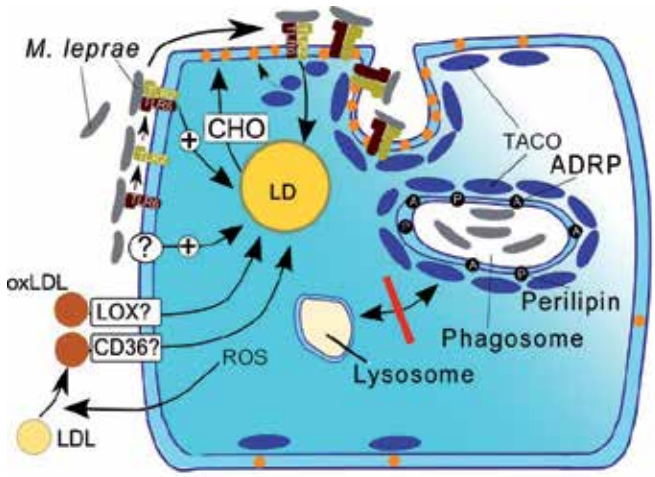

Figure 4. Basic mechanisms of lipid droplet induction in *M. leprae* infected macrophages. *M. leprae* attaches to TLR2 and TLR6. Heterodimerization of TLR2 and TLR6 induces downstream signalling and subsequent accumulation by LD formation. [102,105]. In SCs TLR6, but not TLR2, is essential for *M. leprae*-induced LD biogenesis [101]. Cholesterol from the LDs accumulates at the site of mycobacterial entry and promotes mycobacterial uptake. Cholesterol also recruits TACO from the plasma membrane to the phagosome [61]. TACO prevents phagosome-lysosome fusion and promotes intracellular survival [62,63]. Hypothetical uptake of oxidized lipids by scavenger receptors in *M. leprae*: Reactive oxygen species might oxidize low-density lipoprotein (LDL) to oxLDL, which is thought to be subsequently bound and taken up by scavenger receptors CD36 and LOX1. CHO, cholesterol. Unknown mechanisms for LD induction are indicated with a question mark.

5.3. Mycolic acids induce the formation of foamy macrophages

Mycolic acids and oxygenated mycolic acids are strong inducers of monocyte-derived macrophages differentiation into foamy macrophages [19,106]. Peyron et al. demonstrated

that that a set of oxygenated mycolic acids specifically produced by highly virulent mycobacteria species (*M. tuberculosis*, *M. avium*) were responsible for the formation of foamy macrophages [19].

6. Clinical implications

Several enzymes of the mycobacterial lipid-biosynthesis are regarded as targets for new antitubercular compounds. The research focused on enzymes, involved in the biosynthesis of lipid compounds of the mycobacterial cell wall [107]. Especially the biosynthesis of the highly toxic cord factor is an attractive target. The cord factor is synthesized by the antigen 85 complex [108,109]. It was recently shown that one member of the complex, antigen 85A is involved in the formation of intracellular lipid bodies [71]. Antigen 85 is an important virulence factor. It has been shown that *M. tuberculosis* requires the expression of Ag85A for growth in macrophages [110]. *M. tuberculosis* strain lacking Ag85C shows an decrease of 40% in the amount of cell wall linked mycolic acids [111,112]. The treatment by a trehalose analogue, 6-azido-6-deoxy-α,α'-trehalose (ADT) inhibits the activity of all members of Ag85 complex *in vitro* [108, 113]. Also ethambutol targets the synthesis of arabinogalactan, isoniazid and ethionamide inhibit biosynthesis of mycolic acids [107].

The most potent inhibitor for mycolic acid biosynthesis is isoniazid (INH). INH is a prodrug which is converted to the isonicotinoyl radical by KatG. INH forms a covalent adduct with NAD. This INH-NAD adduct inhibits FAS-II enoyl-ACP reductase InhA, which in consequence leads to inhibition of mycolic acid biosynthesis, and ultimately to cell death [114-117]. The inhibitors of fatty acid biosynthesis are summarized in Figure 2 and Table 2.

Synthesis step	Enzyme	Compound / class	References
FAS-I and FAS-II	KasA/KasB	Cerulenin (2R,3S-epoxy-4-oxo-7,10-trans,trans-dodecanoic acid amide	[118] [119]
FAS-II	KasA/KasB	TLM (Thiolactomycin)	[120-122]
FAS-II	KasA/KasB	Platensimycin	[123]
	InhA	INH (Isoniazid)	[124]
	InhA	ETH (Ethionamide)	[125]
	InhA	TRC (Triclosan)	[126]
	InhA	alkyl diphenyl ethers (Triclosan derivatives)	[127]
	InhA	2-(o-Tolyloxy)-5-hexylphenol (PT70)	[120]
Cyclopro-panation	CMASs (cmaA2, mmaA2 or pcaA)	TAC (Thiacetazone)	[128]
	MmaA4	TAC (Thiacetazone)	[128]

Table 2. Inhibitors of fatty acid biosynthesis

7. Conclusion

The formation of lipid inclusions during infection in the host as well as in the pathogen during intracellular infection with *M. tuberculosis* and *M. leprae* plays an important role in pathogenesis. A hallmark of intracellular infection is the formation of foamy macrophages. *M. tuberculosis* and *M. leprae* induce the formation of lipid droplets in the host cell. The accumulated lipids are used as energy and carbon source. In fact *M. tuberculosis* seems to switch completely to fatty acid catabolism at the transition from the acute to the chronic phase of infection. The central role of fatty acid metabolism during the dormant state of *M. tuberculosis* is underlined by the finding that both isocitrate lyase, icl and icl2, are essential for intracellular replication in the lung [79,80]. The TAG metabolism and the resulting formation of lipid inclusions of host and pathogen play a fundamental role in infection. Indeed TAG-derived fatty acids from the host cell are imported into *M. tuberculosis* and incorporated into bacterial TAG [46]. In conclusion the enzymes involved in lipid droplet metabolism are essential for survival of the pathogen in the lung and thus attractive targets for novel drugs. Especially enzymes with DGAT activity such as Tgs and Ag85A seem to be promising drug target candidates. Another promising targets seem to be the recently discovered cell wall associated and secreted esterases, which are involved in the utilization of host cell lipids such as Rv0183 and LipY [55, 75,76]. Future studies should also focus on the lipid metabolism of *M. leprae*, an organism which upregulates several genes of the host´s lipid metabolism during infection [92]. The regulation of lipid droplet formation in the host cell is another important topic. Recent sudies revealed that intracellular pathogens induce the expression of LDL receptor and scavenger receptors CD36 and LOX1 for the internalization of native and oxidized fatty acids. Especially the generation of oxidized lipids by macrophage-derived reactive oxygen species seems to be an important mechanism for the induction of scavenger receptors.

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Chapter 4

The Role of Antibodies in the Defense Against Tuberculosis

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1. Introduction

Throughout history tuberculosis (TB) has been a health problem for humanity. In the beginning of civilization, when human population densities were sparse, this disease may have been fairly harmless. However, with the increase in population densities, probably from the 17th to 19th centuries, TB took epidemic proportions [1].

Bacille Calmette Guérin (BCG) is effective to prevent miliary and meningeal TB in infants [1]. The reports about the efficacy of this vaccine for the prevention of adults pulmonary TB are contradictory and the consensus is that the protection conferred by BCG against this form of TB is questionable [1]. The wide use of BCG vaccination has been unable to prevent nearly two million deaths associated with TB that are produced every year. Currently the World Health Organization no longer recommends BCG vaccination in children born from HIVpositive mothers which complicate the implementation of BCG vaccination programs [2]. The implementation of standard drug treatment for TB is difficult in the areas of the highest incidence of the disease. Treatment is further complicated by the limited effectiveness of the current therapeutic schemes against drug resistant strains of TB [3-5].

Nowadays there is an increasing realization of the need for new animal models to test vaccine efficacy in more realistic scenarios, overcoming the limitations of current models in use. In addition, the elucidation of the significance of antibody-mediated defense against intracellular pathogens, in particular against *Mycobacterium tuberculosis*, constitutes an exciting new approach to improve the rational design of new vaccines, therapies and diagnostics.

2. Specific antibodies: Players in the defense against TB

In order to develop improved vaccines and new methods for controlling TB, an important element is the discovery of markers to measure the effectors' mechanisms of the protective immune response against *M. tuberculosis*.

For many years Cell-Mediated Immunity (CMI) was viewed as the exclusive defense mechanism against intracellular pathogens. The Th1/Th2 classical paradigm prevailed for a long time and the development of vaccines followed this theory [6]. Based on this theory, only intracellular pathogens could be effectively controlled by granulomatous inflammation induced by a Th1 response, whereas a Th2 response induces antibody production that controls extracellular pathogens and parasites. However, the question of what constitutes a true demarcation between "extracellular and intracellular" pathogens is important in this regard. During their infectious cycle, intracellular pathogens could be found in the extracellular space and *vice versa*. In the specific case of *M. tuberculosis*, it can be localized extracellularly at the beginning of the infection in the upper respiratory tract as well as during advanced stages of the disease, after rupture of granulomatous lesions occur [7]. This facultative intracellular pathogen was shown to have an extracellular phase [7] [8] that may include replication [7] which in turn could potentially be targeted by specific antibodies.

There are several prokaryotic and eukaryotic intracellular pathogens for which antibody have been shown to modify the course of infection by different mechanisms, as reviewed extensively by Casadevall and colleagues [9, 10, 11]. In the case of *Erhlichia* spp., specific antibodies were shown to mediate protection [12], possibly by blocking cellular entry or promoting the expression of proinflammatory cytokines. [13,14]. A combination of both humoral and cellular immune mechanisms could be the optimal choice controlling certain intracellular pathogens,.In this regard, de Valliere and colleagues reported that human antimycobacterial antibodies enhanced Cell-Mediated Immune responses to mycobacteria that are beneficial to the host [15].

3. Epidemiological evidence of antibody mediated protection

There is accumulating evidence, in the last few decades, regarding the effect of antibodies in the context of development of pulmonary or disseminated TB. Children with low serum IgG against sonicated mycobacterial antigens and LAM, or those who could not mount antibody responses to these antigens were predisposed to dissemination of *M. tuberculosis* [16].

M. leprae reactive salivary IgA antibodies were suggested to be important in a mucosal protective immunity [17]. In study carried out among the Mexican Totonaca Indian population, the presence of high antibody titers to Ag87 complex antigens were observed in patients with non-cavitary TB and in patients who were cured with anti-TB chemotherapy. In contrast, patients without such antibodies had a poor outcome of the disease [18].

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4. Experimental studies

4.1. Animal models

An important criterion for the evaluation of the role of specific antibodies in the protection against TB is the use of animal models. Currently, there is no optimal model to re-produce the infection as it occurs in humans [19].

The geographical location, genetic factors of the host, the presence of environmental mycobacteria and other concomitant infections like helminthiasis, are factors that have to be considered when designing animal experiments [20]. Several animal models have been used to evaluate different aspects of mycobacterial infection and disease. A crucial aspect is the delivery of mycobacterial inoculum. In this regard, several routes of inoculation have been employed experimentally, including intravenous, intraperitoneal, intranasal, intratracheal and aerosol [21, 22].

The study of the distribution of monoclonal and polyclonal antibody formulations in different organs and tissues of mice after administration by different routes, including the use of backpack models have been reported [23-27]. Each model has its advantages and drawbacks.

For example, the backpack model is very useful for the evaluation of the protective role of IgA, but poses ethical problems in long term experiments due to the increase in tumour size over time produced by the inoculated hybridoma [28]. In prophylactic and therapeutic models, antibody formulations have been administered via the intranasal [29], intravenous [30] and intraperitoneal [26] routes and combined with cytokines and antibiotics [31, 32] before and/or after the infectious challenge. The administration of *M. tuberculosis* pre-coated with antibodies [27, 33] in different models of infection has also contributed to understanding the interactions between host and microbe.

Another approach has been the use of knockout mice models for IgA [34] polymeric immunoglobulin receptor (pIgR) [34] and B cells [35,36,37,38], as will be discussed later.

4.2. Experimental studies with antibodies

A substantial number of studies utilizing anti mycobacterial antibodies have been conducted as far back as the end of the 19th century. These experiments can be grouped into several categories: serum therapies, mouse polyclonal antibodies, human polyclonal antibodies (including commercial human gamma globulins), secretory human IgA (hsIgA) and studies with monoclonal antibodies.

4.2.1. Serum therapies

Serum therapy experiments were conducted from the second half of the 19th century (reviewed in [39,40]). Immune sera was generated by immunizing animals with different mycobacterial fractions and administered either to animals or humans [39,40] The results obtained were either beneficiary, variable, inconclusive or contradictory, [39,40]. These variable results led to the perceived minor role of antibodies in the defense against *M. tuberculosis*.

What factors could have led to the heterogeneity in study results? Recognizable differences in the methods used for serum preparations and their administration, as well as the lack of appropriate experimental controls probably accounted in part for the studies outcomes. Furthermore, it is important to recognize that immune serum is a polyclonal preparation that includes antibodies with multiple specificities and isotypes. Consequently, polyclonal sera may contain antibodies of different subclasses and functional categories that can affect theoutcome of infection. For example, IgG3 murine monoclonal antibodies protected against *M.tuberculosis* [27] but failed to protect against *Cryptococcus neoformans* [41]. An IgG3 non-protectivemonoclonal antibody to *C. neoformans*, became protective upon subclass switching to IgG1 [41]. In addition to intrinsic factors associated with the antibody structure, other parameters such as the genetic background of the microbe and the immunocompetence of the host could alter the outcome of antibody protection experiments.

For some microorganisms, such as *Samonella typhimurium* and *C. neoformans,* passive antibody therapy efficacy depends on the mouse strain used [42, 43]. In the same way, some microbial strains are more susceptible to the effects of antibodies [44].

The animal model used is another important parameter that varies between different experiments cited in the literature [45]. Timing, the route of infection, the magnitude of the infecting inoculum are some additional variables that could affect antibody protection studies [46].

Despite their variability, the results obtained with serum therapy were valuable, demonstrating some beneficial effect of serum on the course of TB in humans, mainly in cases of early or localized TB [45]. Moreover, it was demonstrated that long periods of treatment were necessary to achieve a sustained effect [45].

4.2.2. Polyclonal mouse antibodies

A recent study re-examined the usefulness of immune serum in the context of a therapeutic vaccine against TB [32]. This vaccine, named RUTI, is generated from detoxified *M. tuberculosis* cell fragments that facilitate a balanced T helper response to a wide range of antigens along with intense antibody production [47]. Local accumulation of specific CD8+ T cells and a strong humoral response after immunization are characteristic features of RUTI that contribute to its protective properties. In that study, immune serum was generated by immunizing mice with RUTI [32]. Severe Combined Immunodeficiency (SCID) mice were inoculated with *M. tuberculosis* and treated with chemotherapy for 3–8 weeks. After chemotherapy they were treated for up to 10 weeks with intraperitoneal injections of the generated immune serum. Mice treated with immune serum from RUTI vaccinated animals showed significant decreases in lung CFU in addition to reduced extent of granulomatous response and abscess formation [47]. These results indicate that protective serum antibodies can be elicited by vaccination, and that antibodies may be usefully combined with chemotherapy [32, 47, 48].

4.2.3. Human gammaglobulins

4.2.3.1. Specific human polyclonal antibodies

Evidence for the stimulatory role of specific polyclonal antibodies on cellular immunity in experimental mycobacterial infections was reported by de Valliere and colleagues in 2005 [15].

In this study, serum samples containing specific antimycobacterial antibodies were obtained from volunteers vaccinated twice with BCG by the intradermal route. Significant titres of IgG antibodies against lipoarabinomannan (LAM) were detected in the sera. BCG internalization into phagocytic cells was significantly increased in the presence of these BCG induced antibodies as were the growth inhibitory effects of neutrophils and macrophages on mycobacteria. Furthermore, these antibodies induced significant production of IFN-γ by CD4+ and CD8+ T cells [15].

4.2.3.2. Commercial immunoglobulin formulations

Human Intravenous Immunoglobulin (IVIG) has been used to treat individuals with immune deficiencies and patients with inflammatory, autoimmune and infectious conditions [49, 50, 51]. Several groups tested the effect of human immunoglobulin preparation on mycobacterial infection. Roy and colleagues showed that treatment of *M. tuberculosis* infected mice with one cycle of IVIG led to the substantially lower bacterial loads in the spleen and lungs following its administration either at early or at late stage of infection 52]. The effect of the administration of a commercial preparation of human immunoglobulin (hIg) in a mouse model of intranasal infection with BCG was evaluated by Olivares and colleagues [33]. This group demonstrated the passage of specific antibodies to saliva and lung lavage following the intranasal or intraperitoneal administration of human hIg to mice. This treatment inhibited BCG colonization of the lungs of treated mice. A similar inhibitory effect was observed after infection of mice with hIg -opsonized BCG [33].

The same formulation was evaluated also in a mouse model of intratracheal infection with *M. tuberculosis*. Animals receiving human hIg intranasally 2h prior to intratracheal challenge demonstrated a significant decrease in lung bacillary load as compared with non-treated animals [29]. When *M. tuberculosis* was pre-incubated with hIg prior to challenge the same effect was observed [29].

The protective effect of the hIg formulation was abolished following pre-incubation with *M. tuberculosis* [29]. These results are suggestive of a potential role for specific human antibodies in the defense against mycobacterial infections.

Taken together, these studies provide support for the potential use of immunoglobulins against *M. tuberculosis*.

4.2.3.3. Human secretory IgA

Human secretory IgA (hsIgA) is the major class of antibody associated with immune protection of the mucosal surfaces [53]. Colostrum volume is above 102 mL in humans during the first three days after delivery [54]. The high percentage of (hsIgA) in human colostrum [55] strongly suggests its important role in passive immune protection against gastrointestinal and respiratory infections [56]. In one study performed by Alvarez and colleagues, hsIgA from human colostrum was obtained by anion exchange and gel filtration chromatographic methods, using DEAE Sepharose FF and Superose 6 preparative grade, respectively [57].

hsIgA was administered intranasally to BALB/c mice, and the level of this immunoglobulin in several biological fluids was determined by ELISA. The results showed the presence of this antibody in the saliva of animals that received the hsIgA, at all time intervals studied. In tracheobronchial lavage, hsIgA was detected at 2 and 3 hours after inoculation in animals that received the hsIgA [58]. Similar studies were performed by Falero and colleagues with monoclonal antibodies of IgA and IgG class [59]. Following demostration that hsIgA could be detected in several biological secretions after intranasal administration, the protective effect of this formulation against *M. tuberculosis* challenge was evaluated. Mice challenged with *M. tuberculosis* preincubated with hsIgA showed a statistically significant decrease in the mean number of viable bacteria recovered from the lungs compared to control mice and to the group that received the hsIgA before challenge with *M. tuberculosis*. Moreover, an increased level of iNOS production was also reported (Alvarez et al., mannuscript in preparation). Consistently with this result, a better organization of granulomatous areas with foci of lymphocytes and abundant activated macrophages were observed in the lungs of mice that received *M. tuberculosis* pre-incubated with hsIgA and sacrificed at 2 months postchallenge. Untreated animals, however, showed an increased area of bronchiectasis and atelectasis as well as fibrin deposits, accumulation of activated macrophages and lymphocytes.

The pneumonic areas were more prominent in the untreated animals than in the groups treated with hsIgA and *M. tuberculosis* pre-incubated with hsIgA (Alvarez et al., manuscript in preparation)

4.2.4. Monoclonal antibodies

Since the first report on the use of the monoclonal antibody Mab 9d8 against *M. tuberculosis* [27], many similar studies have been reported [40]. This IgG3 monoclonal antibody (Mab) generated against arabinomannan (AM) capsular polysaccharide, increased the survival of intratracheally infected mice when the *M. tuberculosis* Erdman strain was pre-coated with it [27]. In this study, a longer survival associated with an enhanced granulomatous response in the lungs was found as compared to controls receiving an isotype-specific non-related Mab [27]. Another Mab, SMITB14, directed against the AM portion of LAM prolonged the survival of intravenously infected mice associated with reduced lung CFU and prevention of weight loss [60]. In this study, the authors demonstrated that protection was independent of the antibody Fc portion, because the F(ab')2 fragment also conferred a similar protective effect [60]. In another study, mice receiving the Mab 5c11 (an IgM antibody that recognizes other mycobacterial arabinose-containing carbohydrates in addition to AM) intravenously prior to Mannosylated lipoarabinomannan (ManLAM) administration, showed a significant clearance of Man-LAM and redirection of this product to the hepatobiliary system [26]. This study strongly supports an indirect effect of certain antibodies on the course of mycobacterial infection, altering problably the pharmacokinetics of mycobacterial components and contributing to protection against TB [26].

Heparin Binding Hemagglutinin Adhesin (HBHA) is a surface-exposed glycoprotein involved in the mycobacterial binding to epithelial cells and in mycobacterial dissemination [62]. Monoclonal antibodies 3941E4 (IgG2a) and 4058D2 (IgG3) directed against HBHA were used

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to coat mycobacteria before administration to mice. In this study, spleen CFUs was reduced while lung CFUs did not [63]. These results suggest that binding of these antibodies to HBHA can impede mycobacterial dissemination.

The protective efficacy of a monoclonal antibody, TBA63 and IgA anti-Acr administered intranasally before and after the intranasal or aerosol challenge with *M. tuberculosis* was demonstrated in a study by Williams and colleagues [64]. In another series of experiments carried out by López and colleagues, the protective effect of this Mab administered intratracheally before an intratracheal challenge with virulent mycobacteria was evaluated. At 21 days post-infection, pre-treatment of mice with TBA63 caused a significant decrease in viable bacteria in the lungs compared to control mice or those treated with the Mab against the 38-kDa protein (TBA86) [65]. Consistent with the reduction of viable bacteria following treatment with TBA63, the area of peribronchial inflammation was also statistically smaller in this group compared to the control group [65].

When the lungs of mice were histologically examined, granulomas were better organized in the infected animals that had received TBA63 than in controls or mice treated with TBA86. The reduction of CFU in lungs of the treated group was associated with milder histopathological changes, as indicated by the organization of the granulomas and less pneumonic area. The fact that this Mab promotes granuloma formation in mice infected intratracheally with *M. tuberculosis* strongly suggests the close interaction between antibody mediated immunity and cell-mediated immunity to induce protection against intracellular pathogens (66).

The 16 kDa protein (Acr antigen) has been defined as a major membrane protein peripherally associated with the membrane [67] carrying epitopes restricted to tubercle bacilli on the basis of B-cell recognition [68, 69]. The Acr antigen is present on the surface of tubercle bacilli and is highly expressed in organisms growing within infected macrophages, allowing it to be potentially targeted by specific antibodies either inside infected cells as well as extracellulary. A novel immunotherapy, combining treatment with anti-IL-4 antibodies, IgA antibody against 16 kDa protein and IFN-γ, showed the potential for passive immunoprophylaxis against TB. In genetically deficient IL-4-/- BALB/c mice, infection in both lungs and spleen was substantially reduced for up to 8 weeks. Administration of rIL-4 to IL-4-/- mice with increased bacterial counts to wild-type levels and make mice refractory to protection by IgA/IFN-γ [70].

More recently, Balu and colleagues reported that intranasal administration of a human IgA1 Mab, obtained using a single-chain variable fragment derived from an Ab phage library with high affinity for hspX and the human FcαRI (CD91) IgA receptor together with recombinant mouse IFN-γ significantly inhibited pulmonary infection with *M. tuberculosis* H37Rv in mice transgenic for human CD91 but not in the CD91-negative controls. These results suggested that binding to CD91 was necessary for the IgA-imparted passive protection [71]. When the Mab was incubated with human whole-blood or monocyte cultures it inhibited H37Rv infection.

Inhibition of the infection by the antibody was synergistic with human rIFN-γ in purified human monocytes cultures but not in whole blood cultures [71]. The demonstration of the role of FcαRI (CD91) in human IgA mediated protection contributes to understanding the mecha-

nisms involved as well as for using this knowledge for the future development of new immunotherapies for TB [71].

4.2.5. Transgenic mice

Mouse models with deficiency in antibody production can provide useful information for understanding certain roles of antibodies in protection against mycobacterial infections. Rodríguez and colleagues reported that after immunization of IgA deficient (IgA-/-) and wild type mice by the intranasal route with the mycobacterial surface antigen PstS-1, IgA-/- mice were more susceptible to BCG infection than IgA+/+ mice [34]. Cytokine response analysis demonstrated reduction in the IFN-γ and TNF-α production in the lungs of IgA-/- as compared with IgA+/+ mice, suggesting that IgA may play a role in protection against mycobacterial infections in the respiratory tract. Furthermore, these authors demonstrated that immunized pIgR-/- mice were more susceptible to BCG infection than immunized wild-type mice [34].

In an attempt to elucidate whether antibody-mediated immunity has a special role in the defense against TB, different experiments with B cell knockout mice were performed by several authors. In 11016, Vordermeier and colleagues developed an infection model of TB in μ chain knockout Ig- mice. Organs from *M. tuberculosis* infected IgG- mice had three to eight fold elevated counts of viable bacilli compared with those from normal mice. This result suggested that B cells play a role in the containment of murine tuberculous infection [35]. In another study B cell KO mice and controls were infected by aerosol with *M. tuberculosis*. They were subsequently given chemotherapy to destroy remaining bacilli and then re-challenged by aerosol exposure. There were not differences in the ability of animals to control this second infection, indicating that, in this low dose pulmonary infection model, any local production of antibodies neither impeded nor enhanced the expression of specific acquired resistance [36].

In another series of experiments the role of B cells was evaluated during early immune responses to infection with a clinical strain of *M. tuberculosis* (CDC 1561). In this study, despite comparable bacillary loads in the lungs, B cell KO mice had a less severe pulmonary granuloma formation and delayed dissemination of bacteria from lungs to peripheral organs. Additional analysis of lung cells demonstrated higher numbers of lymphocytes, particularly CD8+ T cells, macrophages, and neutrophils in wild-type and reconstituted mice as compared with B cell KO mice. These results demonstrate that less severe granuloma formation and delayed dissemination of mycobacteria found in B cell KO mice were dependent on B cells, (not antibodies, at least in this study) and were associated with modification of cellular infiltrate in the lungs [37]. This latter result differs from a study carried out by Maglione and colleagues in which B cell-/- mice demonstrated exacerbated immunopathology corresponding with enhanced pulmonary recruitment of neutrophils following aerosol challenge with *M. tuberculosis* Erdman strain [38]. Infected B cell-/- mice demonstrated increased production of IL-10 in the lungs, while IFN- γ, TNF-α, and IL-10R were not significantly different from those of wild type mice [38].. B cell-/- mice demonstrated enhanced susceptibility to aerosol infection of 300 CFU of *M. tuberculosis* with elevated bacterial burden in the lungs but not in the spleen or liver [38].

Together these studies suggest that B cells may have an important role in host defense against *M. tuberculosis*.

5. Mechanisms of action

The various effects of antibodies demonstrated in the studies analyzed, suggest that different mechanisms of action are involved in the effect of monoclonal and polyclonal antibodies on *M. tuberculosis*. Secretions found on mucosal surfaces contain significant levels of Igs, particularly, IgA. IgA has both direct and indirect functional roles for combating infectious agents such as viruses and bacteria that cross the mucosal barrier [72]. Moreover, experimental evidence suggests that IgA associated with the pIgR may neutralize pathogens and antigens intracellularly during their transport from the basolateral to the apical zone of epithelial cells [73,74]. In addition, IgA may interact with Gal-3 (an intracellular binding β-galactosidase lectin), and interfere with the interaction of mycobacteria with the phagosomal membrane, resulting in the decrease of bacterial survival and replication in the phagosome [75].

Antibodies may be critical, during the extracellular phases of intracellular facultative pathogens. They may act by interfering with adhesion, by neutralizing toxins and by activating complement. Moreover, antibodies may be able to penetrate recently infected cells, bind internalised pathogens, and enhance antigen processing (76). Antibodies may also play a crucial role in modulating the immune response by activating faster secretion of selected cytokines that in turn, contribute to more efficient and rapid Th1 response [76,77], increasing the efficacy of co-stimulatory signals, enhancing Antibody Dependent Cellular Cytotoxicity (ADCC) and the homing of immune cells to the lungs after the respiratory infection [10,78, 79, 80, 81, 82, 83].

Examples of relevant potential action mechanisms of antibodies against *M. tuberculosis* were discussed by Glatman-Freedman [40].

6. Potential uses of antibodies against TB

Future applications of antibody formulations for the control of TB may include several possibilities including treatment, prevention and diagnosis.

6.1. Treatment

Antibody based therapy could potentially be useful in several scenarios. They could be used to shorten the standard treatment period of patients with uncomplicated TB when coupled with standard chemotherapy. However, they would be particularly important in the treatment of patients infected with Multidrug Resistant (MDR) and Extensively Drug Resistant (XDR) strains, in combination with the standard treatment.

6.2. Prophylactic use

Prophylactic use of antibodies could be applied in recent contacts of TB patients, with special attention to risk groups [84]. In this regard, successful prophylactic use of antibodies in exposed individuals has been shown in the case of several other pathogens such as varicella, tetanus, Respiratory Synsicial Virus (RSV), rabies and Hepatitis B [85, 86]

6.3. Vaccines

The induction of specific protective antibody responses by vaccination, either alone or as an addition to the stimulation of cell mediated immunity could be a novel strategy for the development of new generation of prophylactic and therapeutic vaccines against TB.

The prevailing past dogma that discounted the role of antibodies in host protection against TB has resulted in a limited study of B cell immunodominant epitopes as targets for protective immunity [87].

6.3.1. Polysaccharide conjugate vaccines

Polysaccharide conjugate vaccines are considered to elicit specific protective antibody responses against a variety of pathogens [88]. However, the polysaccharide conjugate vaccine against *Salmonella typhi* [89] demonstrates the feasibility of this kind of vaccines for the prevention of infectious diseases caused by intracellular pathogens. In the case of *M. tuberculosis*, several authors reported the use of polysaccharide conjugated vaccine candidates [61, 90, 91, 92].

All these vaccine candidates induced the production of specific IgG [61, 90, 91, 92] and some of them conferred variable levels of protection [61, 91] which validate this strategy as one of the potential avenues for the development of new generation of vaccines against tuberculosis

6.3.2. Identifying other B-cell immunodominant epitopes

With the development of bioinformatics tools for bacterial genome analysis, it has been possible to predict *in silico* microbial regions that trigger immune responses relevant for protection and vaccine development. A candidate experimental vaccine based on proteoliposomes from *M. smegmatis* is currently in development [93].

In one study, bibliographic search was used to identify highly expressed proteins in active, latent and reactivation phases of TB [94]. The subcellular localization of the selected proteins was defined according to the report on the identification and localization of 1044 *M. tuberculosis* proteins using two-dimensional, capillary high-performance liquid chromatography coupled with mass spectrometry (2DLC/MS) method [95] and using prediction algorithms. Taking into consideration the cell fractions potentially included in the proteoliposome, from the previously identified proteins, the ones located in the cell membrane and cell wall, as well as those which are secreted and homologous to those of *M. smegmatis* were selected.

The regions of the selected proteins containing promiscuous B and T cell epitopes were determined [94]. Thus the *M. smegmatis* proteoliposomes were predicted to contain multiple

B and T epitopes which are potentially cross reactive with those of *M. tuberculosis*. It is important to note that there could be conformational B epitopes and additional epitopes related with lipids and carbohydrates included in the proteoliposomes that could reinforce the humoral cross reactivity.

Considering the results of the *in silico* analysis, proteoliposomes of *M. smegmatis* were obtained and their immunogenicity was studied in mice [93]. In addition to cellular immune effectors recognizing antigens from *M. tuberculosis*, cross reactive humoral immune responses of several IgG subclasses corresponding with a combined Th1 and Th2 pattern against antigenic components of *M. tuberculosis* were elicited. These findings were in concordance with the *in silico* predictions [93, 94]. It is interesting to note that differences in the pattern of humoral recognition of lipidic components was dependent on the characteristics of the adjuvant used, which could have relevance for the development of vaccines which includes lipidic components [93]. Currently studies are underway to evaluate the protective capacity of *M. smegmatis* proteoliposomes in challenge models with *M. tuberculosis* in mice.

Bioinformatics tools for prediction of T and B epitopes were also employed for the design of multiepitopic constructions, which were used to obtain recombinant BCG strains. Based on this prediction, B cell epitopes from ESAT-6, CFP-10, Ag87B and MTP40 proteins were selected and combined with T cell epitopes of the 87B protein and fused to Mtb8.4 protein [96].

A significant IgG antibody response against specific B cell epitopes of ESAT-6 and CFP-10 was obtained in mice immunized with the recombinant strain. After studying the specific response of spleen cells by lymphoproliferation assay and detection of intracellular cytokines in CD4 + and CD8 + subpopulations, the recognition of T epitopes was also observed. The response showed a Th1 pattern after immunization with this recombinant strain (Mohamud, R, et al. manuscript in preparation). In another series of experiments, recombinant BCG strains expressing several combinations of multiepitopic constructions were used to immunize BALB/c mice subcutaneously and challenged intratracheally with the *M. tuberculosis* H37Rv strain. Recombinant BCG strains expressing T epitopes from 87BAg fused to Mtb8.4 protein and BCG expressing a HSP62 T cell epitope plus different combinations of B cell epitopes from 87BAg, Mce1A, L7/L12, 16 kDa, HBHA, ESAT6, CFP10 and MTP40 and combinations of B cell epitopes alone produced significant reductions in lung CFU compared to BCG (Norazmi MN, et al. manuscript in preparation).

6.3.3. Diagnosis

Although no serological assays are currently recommended for diagnosis of TB [97], largely due to the possibility of false results and thus incorrect treatments, for many other pathogens, serological diagnostic tests has been of great value, particularly in poor countries. In some cases, antibody responses can constitute useful correlates of protection [98]. In the specific case of TB, several studies of the antibody response have been reported [99]. There is a substantial amount of variability in antibody response to TB [100]. This variability has been attributed to several factors. Some of these factors are associated with the pathogen (strain variation, microenvironment and growth state of bacteria) and others are related to the host, primarily previous exposure to related antigens and host genetics [99].

However, it is important to consider that only a small fraction of the genomic regions of *M. tuberculosis* encoding proteins has been explored. Currently, novel immunoassay platforms are being used to dissect the entire proteome of *M. tuberculosis*, including reacting protein microarrays with sera from TB patients and controls [101,102]. These studies could lead to the discovery of new antigens that may constitute suitable diagnostic markers and tools for the identification of protection correlates.

7. Concluding remarks

The cumulative work reviewed above with regard to the use of antibody formulations and vaccines suggest that antibodies if present at the right moment at the site of infection could provide protection against *M. tuberculosis*. This concept leads the way to the development of a new generation of vaccines. Such vaccines could work by eliciting specific IgA and/or IgG antibodies that could recognize and intercept the pathogen at the port of entry, primarily the mucosal surfaces, inactivating bacterial components essential for the microbial survival in the host, activating complement for direct lysis of the cells, opsonizing bacteria to promote their capture by phagocytic cells and inducing stimulation of specific cellular immune responses [103, 104].

Various antibody formulations could potentially be used as immunotherapeutic agents in combination with the conventional treatment and in the management of patients affected by Multidrug Resistant (MDR) and Extensively Drug Resistant (XDR) strains.

The study of the role of specific antibodies in the defense against tuberculosis opens new possibilities for future development of new vaccines, diagnostics tools and therapies against this pathogen. It is likely that new discoveries will arise from the ongoing studies in this area that will expedite the introduction of new strategies in the fight against tuberculosis.

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Chapter 5

Influence of the Interferon–Gamma (IFN–γ) and Tumor Necrosis Factor Alpha (TNF–α) Gene Polymorphisms in TB Occurrence and Clinical Spectrum

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1. Introduction

Tuberculosis (TB) is a major public concern and is the most important single infectious cause of mortality and morbidity worldwide. According the World Health Organization (WHO) records, in 2009, there were an estimated 9.4 million new cases, 14 million prevalent cases, and approximately 1.7 million deaths by TB [1]. Additionally, approximately one third of the world's population is infected with the causative bacterium, *Mycobacterium tuberculosis* (*Mtb*), and is at risk for developing active tuberculosis. Interestingly, while approximately 9 million people develop active TB each year, the majority remain asymptomatically (latently) infected with the pathogen presumably due to a protective immune response. Without intervention, approximately five to ten percent of those latently infected will develop overt disease and the potential to transmit *Mtb* to others [2].

Familial clustering data, twin studies and complex segregation analysis have all suggested a strong genetic component in the human susceptibility to the chronic mycobacterial diseases [3-7] but also a complex picture of geographic heterogeneity in genetic effects on the different

mycobacterial infections is involved [8, 9]. Several non-HLA genes have been implicated in TB susceptibility. However, the discrepant data reported may be attributed to a number of different factors, such as the types of studies, ethnicity, genetic background, and clinical status of patients with tuberculosis that may be associated with a particular genetic profile. The interaction among lung cells with pro and anti-inflammatory mediators during the infection with *Mtb* have been deeply investigated [10]. Among involved cytokines, the key role of interferon-gamma (IFN-γ) and tumor necrosis factor (TNF-α) in eliciting an inflammatory response against *Mtb* have been emphasized [11-13].

In human studies, the crucial role of TNF-α in protective host immunity against reactivation of latent TB was highlighted by the observation that the relapse and severe course of TB is over-represented in rheumatoid arthritis patients following the use of anti-TNF-α antibodies [14]. Concerning the IFN-γ, it is well established that deficiency in IFN-γ gene expression is associated with severe impairment of resistance to infections, in particular those that are normally killed by activated macrophages [15, 16]. Low synthesis of this cytokine has been associated with active tuberculosis [17]. However, on the contrary of TNF-α, the Interferon gamma conding gene (*IFNG*) is highly conserved and few single nucleotide polymorphisms (SNPs) are found in the intragenic region. Several case-control studies to evaluate association of SNPs in these genes with TB have produced mixed results, with little consensus in most cases on whether any TNF polymorphisms are actually associated with active TB disease [18, 19].

In the present study we aimed to analyse the existing promoter variability of the *IFNG* and *TNF-α* genes by partial mapping of this region in samples from Brazilians, followed by an association study of the identified SNPs and TB outcome after infection with *Mtb*.

2. Method used

2.1. Study population

In a case-control design, five hundred consanguineously unrelated individuals admitted at the University Hospital Complex: Thoracic Institute/ClementinoFraga University Hospital from Federal University of Rio de Janeiro-UFRJ were enrolled in this study after signing informed consent approved by the local Ethics Committee of HUCFF-UFRJ.

Demographic, clinical, and microbiological data as well as the HIV status of the subjects (age > 18 years old) were collected. Active TB cases (n=265) were defined as those after a positive culture confirmation in clinical specimen or with clinical, radiographic and laboratory improvement according to the American Thoracic Statements.They comprised 265 TB patients to be used for the descriptive genetic analysis. For the association study, TB-HIV comorbity was considered as an exclusion criteria and sample size was reduced as follow: 140 TB patients, being 121 with pulmonary TB (PTB) and 19 extrapulmonary forms of TB (TBE). The mean age of TB patients was ± 51 years (range 18-84 years) including 73 males and 67 females.

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For the control group, a complete questionnaire to document TB risk factors since baseline testing was used. Individuals were eligible as controls if they had no previous TB history, consanguinity and negative HIV status. In formations concerning Tuberculin Skin Test (TST) response were available for all controls. They comprised 235 individuals, to be used for descriptive genetic analysis. For the association study, after application of the exclusion criteria, 154 individuals were included in this group, of which, 96 were TST positive (TST+) and 58 TST negative (TST-). The mean age in this group was ± 50 (range 18 - 82 years) and included 55 males and 99 females.

Sample Collection and handling

A volume of 3 mL of venous blood was collected from each volunteer and stored at -20°C. Genomic DNA was isolated from 100 μL of frozen whole blood using the FlexiGene DNA Kit (Qiagen Inc., USA), according to the manufacturer's specifications. After extraction, DNA samples were stored at -20°C.

2.2. *IFNG* and *TNF–α* genotyping

Genotyping of the proximal portion of the promoter region in *TNF-α* and *IFNG* genes was achieved by direct sequencing of PCR products. Two sets of primers for PCR amplification and sequencing of *IFNG*, DNA fragment of 863bp, (IFN-EF: 5′ GGAACTCCCCCTGG-GAATATTCT 3`, IFNER: 5′AGCTGATCAGGTCCAAAGGA3′, IFNIF: 5 ′CGAAGTGGGGAGGT ACAAAA 3′ and IFNIR: 5′ CCCAGGAAACTGCTACTCTG 3′), and *TNF-α*, DNA fragment of 855bp (TNFEF: 5′CAGGACCTCCAGGTATGGAA3′, TNFER: 5′ TAGCTGGTCCTCTGCTGTCC3′, TNFIF: 5′CCTGCATCCTGTCTGGAAGT 3′ and TNF-IR: 5′TTTCAACCCCTGTGTGTTCG 3′) were designed by using the Primer3 software [20].

For PCR-mediated DNA amplification of *IFNG*, 100 ng of genomic DNA were added to a 50μL reaction mixture containing 200ng of each primer (IFN-EF and IFN-ER), 0.2mM of each dNTPs, 2.0mM $MgCl_2$ and 1U *Taq* DNA polymerase (Invitrogen by Life Technologies, USA) and submitted to an initial denaturation at 94°C for 5 min., followed by 35 cycles of 1 min. at 94°C, 1 min. at 65.3°C and 1 min at 72°C. Final extension was performed for 5 min. at 72°C. Likewise, for amplification of the 855pb *TNF-α* fragment, 100ng of genomic DNA were added to a 25μL reaction mixture containing 200ng of each primer (TNF-EF and TNF-ER), 2mM $MgCl_2$, 0.2mM of each dNTPs and 0.5U of *Taq* gold DNA polymerase (PE Applied BioSystems) and submitted to initial denaturation at 94°C for 15 min, followed by 35 cycles of 1 min. at 94°C, 1 min. at 65.9°C and 1 min at 72°C with a final extension at 72°C for 5 min. Evaluation of PCR products was done by electrophoresis on 1.2% agarose gel followed by ethidium bromide staining.

For sequencing, PCR products were purified with ChargeSwitch Kit (Invitrogen Life Technologies), according to the manufacturer's recommendations. Sequencing of the amplified fragments was performed in both DNA strands using a combination of the internal and external primers using ABI PRISM Big Dye Terminator v. 3.1 Kit (PE Applied BioSystems), according to the manufacturer's recommendations, on an ABI PRISM 3730 DNA Analyser (PE

Applied BioSystems). All singletons and even new/rare mutation identified were confirmed by re-amplification and re-sequencing.

2.3. Computational analysis

The SNPs identification in each individual sample was achieved after alignment of the generated sequences with the GenBank reference sequences AF3757790 and AB088112 for *IFNG* and *TNF-α* respectively. Transcription starting site sequence definition adopted for both genes considered as starting point, the first nucleotide immediately preceding position (-1) out of mRNA. Sequence analysis was carried out through SeqScapev. 2.6 software (Applied Biosystem). Haplotype reconstruction was achieved through the use of PHASE Vs. 2.1.1 software [21, 22].

2.4. Statistical analysis

Pair-wise linkage disequilibrium was tested for the loci studies. The Hardy-Weinberg equilibrium using χ^2 test. Statistics were performed in XLSTAT 2008.7 (Addinsoft Software Inc - New York USA). The magnitude of the associations was estimated by odds ratio values and the coefficient of associations. All tests were performed at the 0.05 level of significance by Epi Info version 3.5.1 2008 (Centers for Disease Control and Prevention, USA).

3. Results

In this work, a partial mapping of the promoter regions of *IFNG* and *TNF-α* genes was performed by direct PCR sequencing approach in 265 TB patients and 235 healthy controls residents in Rio de Janeiro, Brazil. Sequencing approach allowed the identification of new SNPs and consequently new haplotypes for both genes. Expected genotype frequencies were calculated from respective single allele frequencies and were consistent with Hard Weinberg Equilibrium using χ^2test.

3.1. Partial mapping of the *IFNG* promoter region in samples from Brazilians residents in Rio de Janeiro

Sequence analysis of the proximal portion of *IFNG* promoter region (863 bp upstream of the transcription starting site) revealed the presence of seven SNPs, of which, four were new, and located at positions (-787C>T, -599C>G, -517C>T, and -255A>G). The three remaining SNPs, already deposited in GenBank-Entrez SNP database, were located at positions (-785C>T, -200G>T, reported as (-183 and -179) and -172A>G (reported as -155). Table 1 show the allele and genotype frequencies of the identified SNPs in the whole studied population (500 samples). All SNPs were found in a very low frequency, sometimes as a singleton (-255A>G). In this case, the SNP was confirmed by new PCR amplification and re-sequencing. The two more frequent SNPs were the ones located at positions -599C>G and -200G>T, both with 1.4%. No homozygosity was identified in these positions.

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Locus *IFNG*	Genotype	Subjects (n = 500)	Absolute Frequency	Allele frequency
	CC	495	0.99	
-787*	TC	5	.001	0.05
	(f) T	5	_	
	CC	495	0.99	
-785	CT	5	0.01	0.05
	(f) T	5	_	
	CC	487	0.974	
-599*	CG	12	0.024	
	GG	1	0.002	0.014
	(f) G	2	-	
	CC	497	0.994	
-517*	CT	3	0.06	0.003
	(f)T	3	-	
	AA	499	0.998	
-255*	AG	1	0.002	0.001
		1	-	
	(f) G			
	GG	486	0.972	
-200	GT	14	0.028	0.014
	(f) T	14	-	
	AA	498	0.996	
-172	AG	2	0.004	0.002
	(f) G	2		

Table 1. Genotype and allele frequencies of SNPs within *IFNG* promoter in Brazilians from Rio de Janeiro.

3.2. *IFNG* haplotypes characterization

Haplotype reconstruction was achieved from genotyping data by using Phase Vs. 2.1.1 software. A total of eight different haplotypes were characterized with basis on the combination of the seven SNPs identified within the *IFNG* promoter. Table 2 shows the frequencies of the identified haplotypes in the total population. The haplotype 4 was the, more frequent among the whole samples analyzed.

Haplotypes	-787	-785	-599	-517	-255	-200	-172	Frequency
1	C	C	C	C	A	G	A	0.916
2	C	**T**	C	C	A	G	A	0.010
3	C	C	G	C	A	G	A	0.024
4	C	C	C	C	A	**T**	A	0.028
5	**T**	C	C	C	A	G	A	0.010
6	C	C	C	C	**G**	G	A	0.002
7	C	C	C	C	A	G	**G**	0.004
8	C	C	C	**T**	A	G	A	0.006

Table 2. Characterization of the identified haplotypes within *IFNG* proximal promoter region in Brazilians from Rio de Janeiro (n=500).

3.3. Partial mapping of the *TNF–α* promoter region in samples from Brazilians residents in Rio de Janeiro

The partial mapping of the proximal portion (855 bp upstream of the transcription starting site) of *TNF-α* promoter was also performed by direct sequencing of PCR products. Upon analysis of the generated sequences seven SNPs, all described in the literature, and were identified in a total of 500 samples. Table 3 shows the allele and genotype frequencies. With the exception of the most studied SNPs (-238 -308, and -376) presenting frequencies higher than 3%, all others were present in less than one percent.

3.4. *TNF–α*haplotypes characterization

A total of fourteen different haplotypes were characterized. Except for the wild-type, haplotype 1, the higher frequent was the haplotype 3, presenting a mutant variation only at -308 position. As expected, the rare combination presenting polymorphisms only at positions -238 and -308 was present in the sample studied although in a low frequency (Table 4).

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Locus	Genotype	Subjects (n=500)	Absolute Frequency	Allele Frequency
	GG	495	0.990	-
-646	GA	5	0.010	-
	A	5	_	0.005
	AA	495	0.990	-
-572	AC	5	0.010	-
	C	5	_	0.005
	CC	499	0.998	_
-422	CT	1	0.002	_
	T	1	_	0.001
	GG	471	0.942	_
-376	GA	28	0.056	_
	AA	1	0.002	_
	A	30	_	0.030
	GG	418	0.836	_
-308	GA	77	0.154	_
	AA	5	0.010	_
	A	87	-	0.087
	GG	489	0.978	_
-244	GA	11	0.022	_
	A	11	_	0.011
	GG	453	0.906	_
-238	GA	44	0.088	_
	AA	3	0.006	_
	A	50	-	0.050

Table 3. Genotype and allele frequencies of SNPs within *TNF-α* promoter in Brazilians from Rio de Janeiro.

Haplotype	-646	-572	-422	-376	-308	-238	-244	Frequency
1	G	A	C	G	G	G	G	0.710
2	G	A	C	**A**	G	G	G	0.006
3	G	A	C	G	**A**	G	G	0.146
4	G	**C**	C	G	G	G	G	0.006
5	G	A	C	G	G	G	**A**	0.020
6	G	A	C	G	G	**A**	G	0.040
7	**A**	A	C	G	G	G	G	0.008
8	G	A	**T**	G	G	G	G	0.002
9	G	A	C	**A**	G	**A**	G	0.044
10	G	A	C	**A**	**A**	**A**	G	0.060
11	G	A	C	**A**	**A**	G	G	0.002
12	G	A	C	G	**A**	G	**A**	0.002
13	G	A	C	G	**A**	**A**	G	0.004
14	G	**C**	C	G	**A**	G	G	0.004

Table 4. Haplotypes description and frequencies within *TNF-α* promoter in Brazilians from Rio de Janeiro.

3.5. Association of the *IFNG* SNPs and TB outcomes

Association of the identified SNPs variations within the analyzed region of*IFNG* with different TB outcomes (susceptibility *per se*, protection, severity and susceptibility to latent *M. tuberculosis* infection) was assessed based in the comparison of allele, genotype and haplotype frequencies between the stratified groups. The groups used for each evaluation were as follow: a) susceptibility *per se* to TB (TB patients versus TST+ controls), b) disease severity (PTB versus TBE) and c) susceptibility to the latent infection (healthy controls TST+ versus TST-).

As previously stated, for this analysis, the sample size was reduced in groups, (patients and controls) because of the exclusion criteria of TB-HIV co-infection and consanguinity. After exclusions, because of the very low frequency of the -255 A>G and -172 A>G these SNPs were also excluded.

Results of the association study upon comparison of genotype frequencies of the five remaining SNPs between TB patients (TBP/EPTB) versus TST+ controls are shown in Table 5. Only the SNP -200G>T presented a significantly higher frequency of the GT genotype in the control group indicating an association of this genotype with protection to the occurrence of active TB ($\chi^2 = 3.86$, $p = 0.033$, OR = 0.18 CI = 0.03 -1.00). Evaluation of the identified SNPs with the other outcomes did not show any association (data not shown).

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Loci	Genotype	Patientes (N=140)	Controls TST+* (N= 96)	χ^2	*p-value*	OR	IC
-787	CC	138	96	1.383	0.515	#	#
	CT	2	0				
-785	CC	140	94	2.942	0.16	#	0.00<2.79
	CT	0	2				
-599	CC	135	93	0.035	NS	1.15	0.23<6.23
	CG	5	3				
-517	CC	140	93	2.29	0.066	#	0.00<1.52
	CG	0	3				
-200	GG	138	89	3.86	0.033	0.18	0.03<1.00
	GT	2	7				

Table 5. Genotype distribution of the *IFNG* SNPs among TB patients and healthy controls (TST+).

Given that the SNP -200 *IFNG* was the only one that was associated with any of the studied outcomes at genotype level, allele frequency was also tested for the same outcomes. Table 6 shows the comparison of the -200T variant between the stratified groups. The results confirm the association with protection to the occurrence of active TB and, additionally to TBP. Association of the -200T variant was also seen to occurrence of latent infection (p=0.035).

Different outcomes	Groups		SNP IFNG -200		
			*P-valor**	OR	IC
Occurrence of TBactive	Pacientes 0.0071	TST+ 0.036	0033	0.19	0.03<1.01
Occurrence pulmonary TB	TBP 0.0082	TST+ 0.036	0.043	0.22	0.033<1.17
disease severity	TBP 0.0082	TBE 0.00	1.00	#	#
latent infection	TST+ 0.036	TST- 0.000	0.035	#	#

Table 6. Distribution of allele frequencies of-200T variant mutant groups according to the different outcomes.

Finally, the more prevalent *IFNG* polymorphisms (-599C>G and-200G>T) were tested against demographic variables, such as, gender and age. No significant association was found after stratified analysis at allele, genotype or haplotype levels (data not shown).

3.6. Association of the *TNF–α* SNPs and TB outcomes

Table 7 summarizes the distribution and comparison of genotype frequencies of each individual SNP among TB patients and TST+ controls. No significant difference was observed. The evaluation of the possible association of different genotypes of *TNF-α* gene with susceptibility to the occurrence of TBP or TBE was also carried out separately, however, no association was found (data not shown).

Locus	Genotype	Patients (N=140)	Controls TST+ (N= 96)	*p-valor*	OR	IC
-646	GG	139	95			
	GA	1	1	1.00	0.68	0.02<25.33
-572	AA	138	95			
	CA	2	1	1.00	1.38	0.10<38.92
-422	CC	140	96			
	CT	0	0	-	-	-
-376	GG	127	93			
	GA	11	3	0.11	3.17	0.81<14.46
	AA	2	0			
-308	GG	118	83			
	GA	19	11	0.78	1.19	0.54<2.67
	AA	3	2			
-244	GG	136	92			
	GA	4	4	0.71	0.68	0.14<3.31
-238	GG	123	88			
	GA	15	8	0.47	1.50	0.58<3.97
	AA	2	0			

Table 7. Genotype distribution of the *TNF*-α SNPs among PTB patients and healthy controls (TST+).

Comparison of the *TNF-α* SNPs frequencies between TBP and TBEis shown in (Table 8). Only the -572A>C (CA genotype) presented a significant difference between these groups, being absent among the 121 TBP subjects, (RR = 8.12, CI = 5.20 <12.67 and *p*-value = 0.0175). The results indicate a risk for disease severity. This association was confirmed upon allele frequency evaluation (RR = 7.72, CI = 5.69 <10.47 and $p = 0.0179$) data not shown.

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Locus	Genotype	PTB N=121	TBE N=19	*p-value*	RR	IC
-646	GG	120	19	1.00	0.00	0,00<115.2
	GA	1	0			
-572	AA	121	17	0.0175	8.12	5.20<12.67
	AC	0	2			
-422	CC	121	19	-	-	-
	CT	0	0			
-376	GG	111	17	0.506	1.25	0.33<4.79
	GA	10	1			
	AA	0	1			
-308	GG	101	17	0.392	0.63	0.16<2.54
	GA	18	1			
	AA	2	1			
-244	GG	117	19	0.554	#	#
	GA	4	0			
-238	GG	106	17	0.585	0.85	0.22<3.37
	GA	14	1			
	AA	1	1			

Table 8. Genotypes distribution of the *TNF*-α SNPs among TBP and TBE.

The association between the *TNF-α* genotypes with latent infection was also evaluated. No significant difference was found (data not shown).

The final evaluation of independent SNPs with the different TB outcomes was performed based in the allele frequencies comparison for the most common *TNF-α* SNPs (-376G>A, -308G>A, -244G>A and -238G>A). The SNP -376G>A, allele variant -376A, showed a significant association with susceptibility to the occurrence of active TB ($p = 0.035$, OR = 3.57, CI = 0.95 < 15.72) and severity ($p = 0.038$ and RR = 2.68) (Table 9). All other outcomes showed no significant association with any of the variants tested, (data not shown).

Different outcomes	Study Group		SNP TNF-α -376A		
			p-valor	OR	IC
Occurrence of activeTB	Patients	TST+	0.035	3.57	0.95<15.72
	(*fa*) 0.054	0.016			
Occurrence of PTB	PTB	TST+	0.201	2.72	0,68<12.62
	(*fa*) 0.041	0.016			
Severity of disease	PTB	TBE	0.038	2.68*	1.22<5.86
	(*fa*) 0.041	0.052			
Latent infection	TST+	TST-	0.90	0.623	0.12<7.86
	(*fa*) 0.016	0.017			

Table 9. Distribution of allele frequency of the *TNF*-α -376A variant and association analysis with different outcomes studied.

Table 10 shows the distribution of the 14 identified haplotypes in the different groups used for the association study. No significant difference was observed in the haplotypes frequencies between groups (data not shown) and their distribution was quite homogeneous.

Haplotype	General TB n= 140	PTB n=121	EPTB n=19	Controls PPD+ n=96	Controls PPD- n=58
1	95(67.9%)	82(67.8%)	0	70(72.9%)	40(69%)
2	2(1.4%)	1(0.8%)	0	0	0
3	19(13.6%)	17(14.1%)	2(10.5%)	11(11.5%)	7(12.1%)
4	1(0.7%)	0	1(5.26%)	0	1(1.72%)
5	3(2.1%)	3(2.5%)	0	4(4.2%)	2(3.4%)
6	7(5 %)	6(5%)	1(5.3%)	5(5.2%)	5(8.6%)
7	1(0.7%)	1(0.8%)	0	1(1.1%)	1(1.7%)
9	9(6%)	8(6.6%)	1(5.3%)	3(3.1%)	2(3.4%)
11	1(0.7%)	1(0.8%)	0	1(1.1%)	0
12	1(0.7%)	1(0.8%)	0	0	0
13	1(0.7%)	1(0.8%)	0	0	0
14	1(0.71%)	0	1(5.26%)	1(1.04%)	0

Table 10. Frequency of *TNF*-α haplotypes in the different groups studied

After the genotyping of all samples and evaluation of the possible association with the different TB outcomes, the most frequent polymorphisms (-376G>A; -308G>A; -244G>A and -238G>A) were tested in a stratified analysis against the demographic variables gender and age. No significant differences were found for gender or age (data not shown).

4. Discussion

It is well known that to *M. tuberculosis*, ethiologic agent of human TB can cause a broad spectrum of effects ranging from no infection to different clinical disease phenotypes [2, 16, 23-25]. However, the reasons for individual or ethnic differences in acquiring infection, active disease, disease severity, and different clinical outcomes have not been completely clarified. It has long been realized that many human diseases arise from the complex interplay between environmental exposures and host genetics susceptibilities [26]. In addition, several genetic factors have also been associated with different outcomes: host susceptibility *per se* the occurrence of active TB, disease severity and / or protection for the occurrence of active disease [27-33].

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The establishment of an efficient immune response involves many different molecules, among which, cytokines and their receptors play an extremely important role. Thus, any genetic alteration leading to changes in the regulation of gene expression may reflect this response. It is known that the interindividual variation in the production of these molecules is directly related to the genetic "background". Literature data have clearly demonstrated that genetic variability of the genes encoding these molecules can affect the regulation of gene expression positively or negatively influencing the final yield of the molecule in question. In the last decade, several single nucleotide polymorphisms (SNPs) in the regulatory region of different cytokine genes have been described and associated with susceptibility, severity or protection for a growing number of diseases of different etiologies including tuberculosis [7, 34-35].

Among the possible genetic variations associated with an increased risk of developing TB, there are several polymorphisms, mainly SNPs, in genes coding for cytokines, cytokine receptors and several other molecules such as vitamin D receptor, NRAMP1 (SLC11A1), HLA genes, etc.

The immune defense against *M. tuberculosis* is complex and involves the interaction between T $CD4^+$, T $CD8^+$ lymphocytes, macrophages, and monocytes along with the production of cytokines, such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) [36].

Convincing evidence indicating the importance of IFN-γ in particular, in the control of mycobacterial infections has been found in both experimental and clinical studies [37-38].

Among the mainly important cytokines involved in TB progress after infection with *M. tuberculosis*, TNF-α plays a key role. It is also a potent proinflammatory cytokine acting in protection against intracellular pathogens [39-40].

The genetic variability of *TNF-α* and *IFNG* has been described in the last decade [41-46] including association studies with tuberculosis. However, the frequencies of the polymorphisms already described varies according to the ethnicity of the population studied, hampering the better interpretation of the value of association studies. Unfortunately, most of these are performed in ethnically homogeneous populations, and therefore, many of the associations described for a particular allelic variant in a certain gene may not represent genetic risk factor in other populations. In Brazil, a country characterized by ethnically mixed population, there are few data regarding the frequency of single nucleotide polymorphisms in these genes (*IFNG*,*TNF-α*) and the few existing studies refers to one or two SNPs only. In view of the importance of the promoter region with respect to regulation of gene expression, the major goal of this work was to proceed a partial mapping of the promoter region of IFN*G* and *TNF-α* genes (approximately, 800bp upstream of the transcription starting site) through PCR-sequencing approach in samples from TB patients and healthy controls from Rio de Janeiro, Brazil. Subsequently, based on frequencies of the different SNPs found individually for each gene, we performed an association study with different TB outcomes.

4.1. Polymorphisms in the promoter region of IF*NG* and its association with TB

Characterization of the important portion within the *IFNG*was firstly identified two decades ago bydeletion analysisstudies [47-48]. According to authors, it comprises a highly conserved

region from positions -117 to -47 and contains two sub regions that can be complexes with proteins. The sub-proximal region (-90 to -65) shows strong homology to the IL-2 promoter [49]. Several transcription factors activate transcription of *IFNG* by binding to this region. Conversely, several others inhibit factors binds in other regions affecting transcription. Hence, the interest in investigating the polymorphic sites within *IFNG* gene promoter, particularly considering the importance of this cytokine in eliciting the immune response.

Here, analysis of the generated sequences identified seven polymorphic sites, four of which were new. The transition C →T, was identified at position-787 from the transcription starting site in five subjects, all heterozygous. The second C → T transition, previously described in the data base of SNPs at position-785 was also found in five individuals, all heterozygous, however, no reference to this SNP was found in the literature. The other three SNPs not yet described, were C to G transition at position -599; C to T at position -517 and A to G at position -255. Finally, two additional SNPs, transition from G to T at position -200 and A to G at position -172, already described and well characterized [45] were found in our population.

One of the main problem found during this mapping was the confirmation of the identified SNPs based on literature data and from different SNPs data bases available online because of the lack of standardization regarding to the reference nucleotide to define the promoter region (transcription starting site nt +1). Many authors describe the SNPs identified in relation to the site of translation or use reference sequences containing sequencing errors leading to misclassification of SNPs (eg SNP-200G>T, originally described as -183 [45], later called as -179 [50] and finally, confirmed in this study as -200). The current name, confirmed in this study is based on the correction of the reference sequence used in previous studies and now available online. These types of errors greatly hampered the beginning of the sequence analysis regarding the identification of novel SNPs.

The frequency of each polymorphism was determined in the study population. As noted in Table 1, the allele frequencies for all the identified SNPs were less than one percent, except for the variants -599G and -200T, both in a frequency of 1.4%.

Functionally, it is known that polymorphisms (-200 and -172) can affect transcription of the *IFNG*. The region from -213 to -200 induces transcription factor through (AP-1) [51]. A polymorphism at this site (position -200, for example) must change the connection of AP-1 and the promoter activity in T cells. The polymorphism -172 is near to the nuclear factors-activated T-cells site (NFAT site) (-186TAAAGGAAA-178) and should affect the stability of this region [50].

The variant IFNG -200T is highly inducible by TNF-α and binds constitutively to nuclear extracts obtained from T cells, whereas the allele -200G does not respond to TNF-α [50; 52]. The induction of transcriptional activity, when the T allele is present, increases protection against tuberculosis. Our results corroborate these data, since the *IFNG* -200T variant showed to be associated with protection the occurrence of active TB in our study group (P = 0.033, OR= 0.18, CI= 0.03 to 1.00).

According Bream,JH et al., 2002 [50], the promoter region of *IFNG* is highly conserved, suggesting that these cytokine production variations are probably due to difference in binding

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to regulatory factors instead of polymorphisms in the gene, which is consistent with our results. Only seven SNPs were found in our population, five of which were at a low frequency. The polymorphisms found with a higher frequency were the -200G>T and -599C>G, the latter being located between two putative binding sites of transcription factors. As this is not yet a SNP described in the literature, functional studies are needed to better understand their functional role. The SNP -200 is of great interest for association studies. However, this polymorphism was not found in Caucasians or Indian populations, suggesting that different selective forces may be operating in different ethnic or racial groups. These data corroborate the evidence that IFN-γ is very important in the immune response and that mutations that interfere with their production may influence the outcome of active tuberculosis as shown by authors [28,31,53-54], and therefore, a selective force lead the gene to be so conserved.

4.2. Polymorphisms within the promoter region of *TNF–α* and their association with TB

TNF-α is a proinflammatory and immunoregulaty cytokine which plays a key role in the initiation, regulation and perpetuation of host defense against infections, but is fatal in excess. As this molecule plays an important role against a variety of pathogens involving different patterns of risks and benefits, it is expected that several genetic elements are involved in its control and production.

The levels of circulating TNF-α are regulated at transcriptional and post-transcriptional levels and several polymorphisms within the promoter region of TNF-α have been associated with altered circulating levels of this cytokine.

In humans, the *TNF-α* gene is located within the complex involving the human leukocyte antigens (HLA), a highly polymorphic region on chromosome 6p21.3 and hence, many of *TNF-α* polymorphisms are in linkage disequilibrium with the HLA genes. Because of differences in the distribution of HLA alleles we might expect variations in associations between polymorphisms of *TNF-α* and various conditions in different geographical areas.

The human genome analysis showed that the level of variations in the genome is approximately one SNP/1.71Kb [55]. However, the *TNF-α* promoter has higher density of SNPs. Despite this level of variation, the regions involved in gene regulation are highly conserved in humans [56-58].

In our work, we perform the mapping of the first 800pb promoter region, through direct sequencing of the amplified PCR product in 500 DNA samples from individuals living in the metropolitan area of Rio de Janeiro, Brazil and found seven polymorphisms previously described in the literature, most of which well characterized. However, according allele frequencies, only the variants -376A, -308A, -238A and -244A were present in more than one percent (0.030, 0.087, 0.011 and 0.050) respectively. The influence of these SNPs in binding of transcription factors have not been fully explored, most of the studies are focused on the association of one or two SNPs with different diseases. The SNPs -376, -308 and -238 have been the most studied but, the results of functional studies performed so far for SNPs -308 and -238 are controversial. It is believed that the variant -308A is associated with an increased transcription rate, leading to an increased production of TNF-α [59] and the variant-238A with a

decreased rate of transcription. Regarding the SNP-376G>A different studies show that this polymorphism is located in a region of multiple interactions between proteins and DNA, and that the minor allele acts in the recruitment of proteins OCT1 for this region. According Knight et al (1999) [60] there is a significant interaction between variant -376A and the OCT-1 protein, this variant binds the proteins while the variant -376G does not. The authors report also by tests with the reporter gene system, that this mutant variant moderately increases the basal levels of TNF-α and associate the same with a relative risk of 4 to cerebral malaria. The problem is that the linkage disequilibrium is strong in this area and it is difficult to study the function of an isolated SNP. In some Caucasian populations -376A allele variant is liked to -308G and -238A [60-61], what is not observed in the African Gambia. Thus, association between the linked allelic variants on TNF-α production and diseases has been studied. According, Hajeer& Hutchinson (2001), the combined allele variants -238G, -308A and -376G are associated with high TNF-α levels [62].

A large number of studies have investigated the association between polymorphisms in the promoter region of the gene for TNF-α and tuberculosis. Results vary according to the different populations studied, finding no association [63-72] or a positive association [26, 29, 73-75]. In our analysis of the single SNP association, TB was associated with the -376G>A. In this case, we observed an association of the minor allele -376A with the outcome of susceptibility *per se* the occurrence of active TB (p= 0.035, OR 3.57, IC 0.95 <15.72) and an increased relative risk for the occurrence of extrapulmonary TB (p= 0.038, OR 2.68, IC 1.22 < 5.86). The association of this allele variant with the occurrence of TB and an increased relative risk for the development of extrapulmonary TB is intriguing. Given the influence of this allele with increased expression of TNF-α, one would expect an association with the protection. One possible explanation for this observation may be the small sample size in the stratified groups. The large confidence intervals (CI) for both outcomes could be a reflection of the small sample size.

The PCR-sequencing approach (gold standard) used for the mapping these genes practically discard the possibility of genotyping errors and all mutants found for all SNPs evaluated were confirmed twice by new PCR and resequencing. Another possibility would be due to the strong linkage disequilibrium observed in this region of the gene promoter of TNF-α. It is possible that other allelic variant (eg, 238A), as opposed to the functional role of variant -376A is canceling the same level of control of gene expression.

An important aspect of this study relates to the ethnic characteristics of the studied population. Brazilian population is characterized by mixture of ethnicities and the results obtained here contribute for a global understanding of the influence of genetic factors in TB outcomes. Usually, most of the studies on this field are made with ethnically homogeneous populations. A study conducted by Baena et al., 2002 [76] clearly shows the importance of ethnic difference in the association study of SNPs in *TNF*-α promoter with disease. According authors, the -857 SNP is a marker for Amerindians. In that study, SNPs in *TNF*-α promoter were also used to identify markers of ancestry, understanding that this region was well characterized previously with primates and humans. Several studies [77-81] have shown that some polymorphisms as -238; - 244 and -308 are in association with the HLA genes and in addition, the SNPs -308 [77]; -863 and -857 [81], are markers of Caucasians. The -238 SNP was found in three populations

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studied, although it has also been found in Caucasians [78] and Asian [80]. Instead, the -376 SNP was not detected in any of the non-African analyzed and were found with the -238.

These data demonstrate the importance of taking into account the "background" of the frequencies of SNPs of TNF-α in studies of gene-disease association.

Association studies of genetic factors with infectious diseases are difficult to conduct because of the multi factorial nature of these diseases that includes host, pathogen and environmental variables in different proportions for each disease. This multi factorial nature of TB stresses the importance to look for haplotypes in the association studies. The fact that our population is so mixed allowed us to find mutations that do not exist in other populations, such as the -308, for example, which is relatively rare in Asians and American Indians. Data from the genotyping of a large number of SNPs for different samples revealed that the human genome has a block structure haplotype [82-83] and configuration of a haplotype sometimes is more important than a single SNP genotype to determine phenotype [84]. Moreover, the construction of a haplotype block is useful for identifying SNPs that isolates would not influence the phenotype [85].

5. Conclusions

In conclusion, this study showed that the proximal part of the promoter region of *IFNG* is highly conserved, as seen in previous publications and the identified SNPs were in very low frequency. The -200T allele variant was associated with protection occurring active TB, and pulmonary TB. In addition, this variant was also associated with latent infection. Concerning *TNF-α*, the high genetic variability was confirmed, but only the -376G>A SNP showed an association with susceptibility *per se* to TB occurrence and increased risk for the occurrence of extrapulmonary TB.

The data presented here shows the reality of a population with characteristics of high ethnic miscegenation, provides the different SNPs identified enabling the realization of real sample calculation for any association studies that may be idealized with these targets and other conditions for this population and finally, provides haplotype that can be used in other studies of association with other diseases.

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Chapter 6

Tuberculosis Pharmacogenetics: State of The Art

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Additional information is available at the end of the chapter

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1. Introduction

The interindividual variability in the metabolism of xenobiotics and drug response is extensive and many factors are involved with this variation including genetic composition, gender, age, co-administration of medication, individual physiology, pathophysiology and presence of other environmental factors (alcohol consumption, smoking, eating habits).

To produce their therapeutic effects, the drug must be present in appropriate concentrations at its site of action. Although the therapeutic concentrations are dependent on the given dose, they will also depend on the magnitude and rate of absorption, distribution, biotransformation, and excretion. Pharmacokinetics studies the course and distribution of drug and its metabolites in different tissues, covering the mechanisms of absorption, transport, metabolism and excretion. In addition, pharmacodynamics concentrates on the biochemical and physiological effects of drugs and their mechanism of action. Proteins involved in drug effects are defined as target molecules and include not only (direct) receptors, but also proteins associated with mechanism of action such as e.g. signal transducer proteins [1].

After its administration, a drug is absorbed and then distributed throughout the body, requiring the coordinated functioning of various proteins, including metabolic enzymes, trafficking proteins, receptor proteins, and others. Medication can enter the body as either active drugs or as inactive prodrugs. Most drugs are metabolized in the liver to make them more soluble for subsequent elimination through the kidneys or intestines. Prodrugs require metabolic conversion, also called biotransformation, to liberate the active compound. Complete biotransformation of any one drug typically requires several different enzymes. [2]. Genetic variability has been described to have effect on drug absorption and metabolism and its interactions with the receptors. This forms the basis for

slow and rapid drug absorption, poor, efficient or ultrarapid drug metabolism and poor or efficient receptor interactions [3]. The consequences of such variations can lead to adverse drug reaction and/or terapeutic failure.

In this context, pharmacogenetics is the study of genetic variations associated with individual variability in drug response, including differences in efficacy, drug-drug interactions, and the relative risk of an adverse response to drugs. It includes the study of genetic polymorphisms that could affect the expression or activity of drug transporters, drug metabolizing enzymes and drug receptors [2-4].

It's estimated that 99.9% of the human genome sequence between individuals is identical and genetic differences in polulations are called mutations if they are present in less than 1% and polymorphisms when present in at least 1% of a population. A single-nucleotide polymorphism (SNP) involves a replacement of one nucleotide base with any one of the other three and occuring at approximately one out of every 1,000 bases in the human genome [5].

A mutation or polymorphism in genes that encode metabolic enzymes, carriers or receptors can affect the drug pharmacokinetics and pharmacodinamics leading to undesired therapeutic effects. The identification of these genetic markers which predicted if a person responds well or not to a specific drug could help to select the right medication in right dosage, maximizing the eficacy and preventing or reducing the adverse drug reactions.

2. Problem statement

TB is an important global public health problem but has cure in almost 100% of the new cases if correct quimiotherapy is applied. The American Thoracic Society (ATS) treatment guidelines recommend an initial phase for TB treatment which consists of rifampicin 10 mg/kg (maximum 600 mg), isoniazid 5 mg/kg (maximum 300 mg), pyrazinamide 15–30 mg/kg (maximum 2 g), and ethambutol 15–20 mg/kg (maximum 1.6 g) given daily for 8 weeks, followed by a continuous phase of isoniazid 15 mg/kg (maximum 900 mg) and rifampicin 10 mg/kg (maximum 600 mg) administered 2–3 times/week for 18 weeks [6]. The use of fixed-dose combination (FDC) tablets containing anti-TB drugs has been recommended by the World Health Organization (WHO) as an additional measure to improve treatment adherence by reducing the number of tablets to be taken. The principal disadvantages of combining three or more drugs in one tablet include (a) the possibility of overdosage or underdosage resulting from a prescription error, (b) changes in the bioavailability of rifampicin and (c) difficulties in determining which drug is responsible for adverse effects [7].

Isoniazid (INH) is an important drug in the TB treatment and was introduced in chemotherapic scheme since 1952. It is the hidrazine of isonicotinic acid and shows cytotoxic activity for *Mycobacterium tuberculosis* both in rest (during latency) and proliferation phases. This drug enters easily in macrophague cells to kill bacilli in multiplication and is specific for mycobacteria [1].

INH-induced adverse reactions include fever, nausea, vomiting, hepatotoxicity, skin reactions, gastrointestinal and neurological disorders. Only in the early 1970s, the occurrence of severe liver injury as a side effect of this drug was recognized, resulting in the death of some patients [8]. Among the first-line anti-TB drugs, INH is the main associated with drug-induced hepatotocixity with a frequency ranging from 1 to 30% in different populations [9]. Other drugs causing liver injury are mainly reported in combination with INH [10, 11]. Drug-induced hepatotoxicity is defined as a serum alanine aminotransferase (ALT) level three times greater than the upper limit of normal (ULN) with clinical symptoms or five times the ULN without symptoms. In both cases treatment should be interrupted and, generally, a modified or alternative regimen is introduced [9]. Because these adverse reactions do not only affect morbidity and mortality rate but also lead to treatment interruptions, failure and relapse, adverse reactions contribute to the spread of the disease and the emergence of multidrug resistence (MDR).

Adverse Drug Reactions (ADRs) are common causes of hospitalization and lead to large costs to society. There are two main financial burdens due to illnesses caused by ADRs: that of treating and that of avoiding them [12]. The occurrence of serious and fatal ADRs has been extensively studied in hospitalized patients and a meta-analysis of prospective studies in approximately forty hospitals in the United States of America (USA) suggests that 6-7% of hospitalized patients suffer from serious ADRs and 0.32% of patients develop fatal ADRs [13]. This results in approximately 100,000 deaths annually in the U.S. and an annual cost of over a hundred billion dollars to the society due to prolonged hospitalization and reduced productivity [3, 13]. Furthermore, it has been estimated that ADRs are responsible for up to 7% of all admissions in hospitals in the United Kindown (UK) and 13% in medical clinics in Sweden [3], which shows the magnitude of this problem in the context of chemotherapy and drug development. Additionally, in France, a 10-year study in the Liver Unit of Hôpital Beaujon in Paris showed that among all patients hospitalized with acute hepatitis, 10% were due to adverse reaction to drugs and the prevalence of drug hepatotoxicity in patients older than fifty years exceeded 40%. In Japan and other Eastern countries, drugs are responsible for about 10-20% of cases of fulminant hepatitis [14].

Liver injury is the most common ADR and the main complication during chemotherapy since liver is the central organ for the biotransformation and excretion of most drugs and xenobiotics [14-17]. There are basically six mechanisms involving primarily the hepatocyte injury. The reactions of mono-oxygenase cytochrome P450 (CYP450) with certain drugs generate toxic metabolites that bind to intracellular proteins, leading to calcium homeostasis pump dysfunction with consequent disruption of actin fibers and cell lysis. Some drugs affect transport proteins in the cell membrane interrupting the flow of bile and then causing cholestasis. Several reactions involving CYP P450 can promote binding of the drug to the enzyme, with consequent exposure of this complex on the cell surface for recognition by T cells and antibody production as part of the autoimmune response. Finally, certain drugs may promote hepatic injury mediated by programmed cell death (apoptosis) or being capable of inhibiting respiration and/or mitochondrial beta-oxidation [17].

Xenobiotics are usually lipophilic and this facilitates their transport in association with lipoproteins in the blood stream and their penetration of lipid membranes and entrance into organs. However, physicochemical properties of drug molecules difficult their removal from the organism by biliary or renal excretion and therefore, these substances require enzymatic conversion to water soluble compounds [1]. The xenobiotics metabolization, often through multiple pathways, can generate metabolites that are more toxic than the substrate and through their interaction with target macromolecules such as DNA, RNA, proteins and receptors, generate the toxic effects. The organ affected is generally that reponsible for drug metabolization or excretion of metabolites [1].

The enzyme systems responsible for the biotransformation of many drugs are located in the endoplasmic reticulum of the liver (microsomal fraction). Such enzymes are also present in the kidneys, lungs and gastrointestinal epithelium, although at a lower concentration [1]. The metabolic modification in biotransformation usually takes place in two consecutive steps and results in the loss of biological activity. Phase I reactions convert the xenobiotic into a metabolite with higher polarity by oxidation, reduction or hydrolysis and generates a pharmacologically inactive or less active, or in the case of a pro-drug, more active molecule. This metabolite is than either eliminated or go through Phase II reactions (so-called synthesis or conjugation reactions), involving binding to a primary metabolite or endogenous substrate such as glucuronate, sulfate, acetate, amino acids or glutathione (tripeptide). Such enzymatic reactions include glucuronidation, methylation, sulfation, acetylation, conjugation with glutathione and conjugation with glycine [1].

The risk for developing hepatotoxicity is associated both with genetic and acquired factors. The acquired factors include: age, gender, nutritional habits, drug abuse, pregnancy and extrahepatic disease. Genetic variations in isoenzymes involved in drug biotransformation can result in abnormal reactions leading to toxic effects [14,17]. In the case of INH in particular, advanced age is a risk factor for hepatotoxicity whereas deficiency in the ability of N-acetylation represent a genetic risk factor for liver injury.

INH is administered orally and rapidly absorbed through the gastrointestinal tract passing through the liver by the portal venous system before reaching the general circulation where is metabolized by a process known as the first pass effect with reduction of its biodiponibility. About 75% to 95% of the INH is excreted by the kidneys during the first 24 hours, mainly as the metabolic forms acetyl-isoniazid and isonicotinic acid [1].

In the liver, INH is metabolized to acetylisoniazid by N-acetyltransferase 2 (NAT2), followed by hydrolysis to acetylhydrazine and then oxidized by cytochrome P4502E1 (CYP2E1) to hepatotoxic intermediates [18, 19]. These metabolites can destroy hepatocytes either by interfering with cell homeostasis or by triggering immunologic reactions in which reactive metabolites that are bound to hepatocyte plasma proteins may act as haptens [17]. The other metabolic pathway to generate toxic metabolites is direct hydrolysis of INH to hydrazine, a potent hepatotoxin. NAT2 is also responsible for converting acetylhydrazine to diacetylhydrazine, a nontoxic component [18, 20, 21] (Figure 1). Glutathione S-transferase (GST), an important phase II detoxification enzyme, is thought to play a protective role as an intracellular free radical scavenger, which conjugates glutathione with toxic metabolites that are generated

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from CYP2E1 [22]. Sulphydryl conjugation facilitates the elimination of metabolites from the body and reduces the toxic effect [23] (Figure 1).

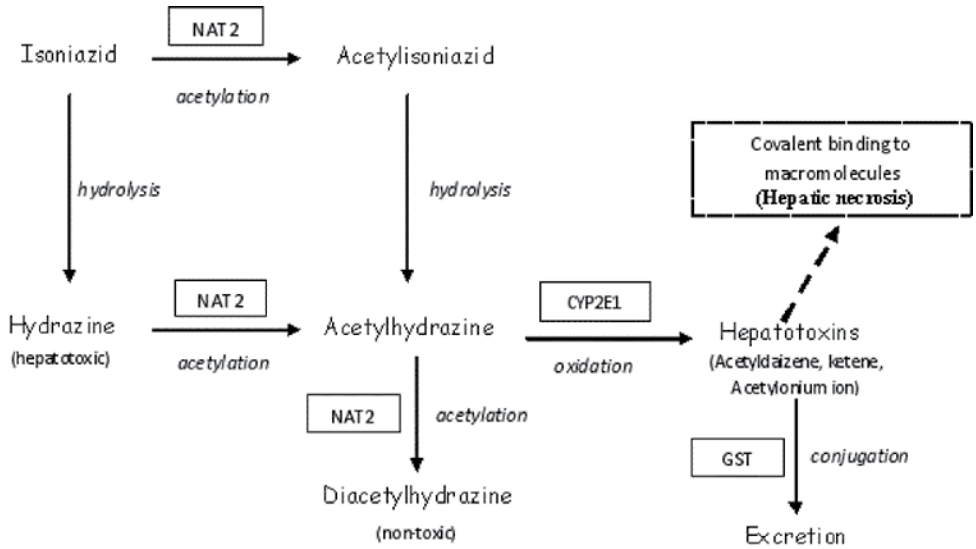

Figure 1. Schematic representation of the INH metabolism. The major enzymes involved in this pathway are indicated in boxes [20, 24].

In the last few years, an increasing number of studies have suggested that genetic polymorphisms in *NAT2*, *CYP2E1* and *GST* genes would be associated with susceptibility to drug-induced hepatotoxicity during TB treatment. The present work focused in an overview of the role of such polymorphisms in occurence of liver injury induced by anti-TB drugs, and by INH in particular.

3. State of the art

3.1. N-acetyltransferase 2

NAT2, the main enzyme responsible for the metabolism and inactivation of INH in humans, is a Phase II enzyme that catalyzes the transfer of the acetyl group from the cofactor acetyl coenzyme A (acetyl-CoA) to the nitrogen terminal of the drug. Variations in activity of NAT2 were discovered over 50 years ago when observing interindividual differences in the metabolism of INH and the level of drug-induced toxicity in TB patients. NAT2 is encoded by the *NAT2* gene and according family genetic studies, variability of *NAT2* was directly related to the emergence of different phenotypes of acetylation [25].

The molecular study of human N-acetyltransferases revealed the presence of three genetic loci, two very homologous encoding the enzymes NAT1 and NAT2, and a third including the

pseudogene *pNAT* (Figure 2). These loci are located on chromosome 8 between 170-360Kb at 8p22 [26]. The *pNAT* is a pseudogene containing a premature stop codon, and is not transcribed. *NAT1* and *NAT2* genes consist of 873 bp, are intronless, and encode proteins of 34 kDa. Protein sequence homology between both enzymes is 81% while that between their respective genes is 87%. Both enzymes have N-acetylation, O-acetylation and NO-transfer in different xenobiotics and carcinogens but differ considerably in their tissue distribution and expression levels during embryonic development [26-28].

Both *NAT1* and *NAT2* are polymorphic genes and SNPs in their coding region can alter the enzymatic activity [29, 30] and are the basis of the three major genetically determined phenotypes, being rapid, intermediate and slow acetylators, which are inherited as a codominant trait [31, 32]. The reference *NAT2*4* allele (without mutations / wild-type) and 66 variants were identified and classified in human populations depending on the combination of up to four SNPs present throughout the *NAT2* coding region [33]. So far, over 30 SNPs have been identified in this region, including several rare mutations described in different populations [34]. Among these, the seven most frequent are the 191 G>A (R64Q), 282 C>T (silent), 341 T>C (I114T), 481 C>T (silent), 590 G>A (R197Q), 803 A>G (K268R) and 857 G>A (G286T) SNPs identified in different human populations [35]. *NAT2* alleles containing the 191G>A, 341T>C, 590G>A or 857G>A SNPs are associated with slow acetylator *NAT2* alleles [33].

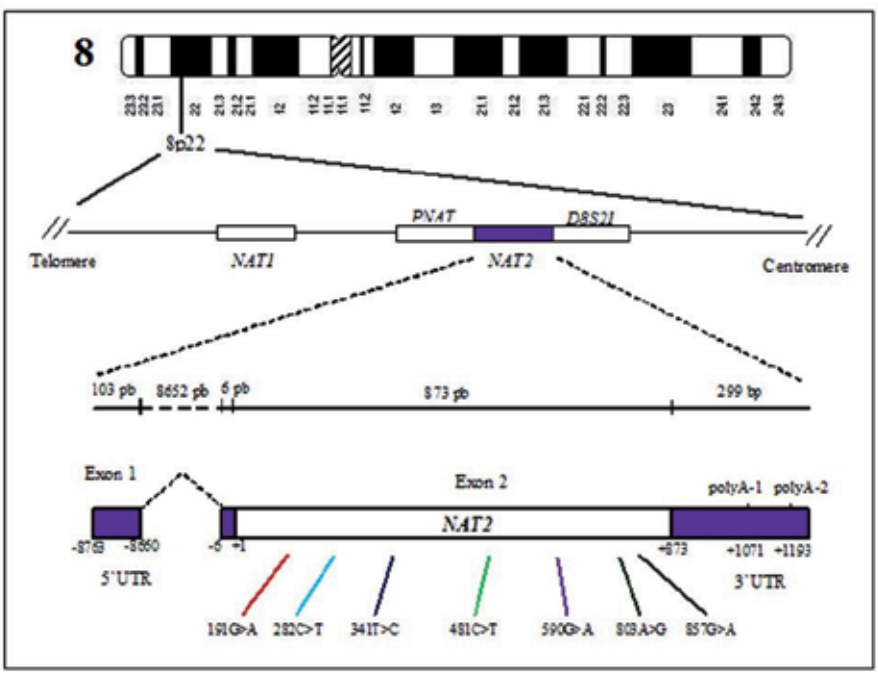

Figure 2. Schematic representation of *NAT* genes on human chromosome 8p22. Distribution of the seven most common SNPs in *NAT2*. D8S21 represents a polymorphic marker situated in the *NAT2* locus [26, 36].

Presence of different SNPs in *NAT2* can be easily determined by genotyping procedures such as PCR-RFLP [37], allele specific PCR [38] or direct sequencing [39]. To achieve the *NAT2* genotype of each individual and predict the phenotype, the haplotype of both chromosomes is usually reconstructed using the statistic software (PHASEv2.1.1[40, 41]). Using haplotype data, many studies have reported the frequencies of the different acetylation profiles among ethnically different populations showing the high diversity around the world. In Asians and Ameridians, the fast acetylator phenotype is more frequent [42-44] whereas in Euro-descendants slow acetylators account for 50% of the study population [37,45]. The molecular basis for such discrepancy is that the most common *NAT2* allele in Euro-descendants is very rare in Asians and may represent a different selective advantage within the gene pools of these separate populations. Description of new alleles of *NAT2* is still ocurring in recent studies [34].

In an attempt to establish an association between acetylation profiles and development of disease, cohort or case-control studies have been performed using of genotyping and phenotyping tools. Evidence was found for an association between the slow acetylator predicted phenotype and developing urinary bladder cancer, while rapid acetylators seem more susceptible to development of colon cancer. For a review, see [27, 46].

For many years, INH has been considered the main cause of hepatotoxicity during TB treatment and association studies between the acetylation phenotypes and susceptibility to liver-related ADRs have been performed. Two early studies conducted in oriental populations investigated the association of the acetylator phenotype with INH induced hepatotoxicity and observed an increased risk of developing hepatotoxicity by INH among the slow acetilators [47, 48]. This observation was confirmed in several other studies performed in different populations [49-52].

Several studies reported the absence of a relationship between acetylation status and hepatotoxicity during TB treatment [53-55] but some, suggested the rapid acetylators as more susceptible to side effects [55, 56]. Reasons for these different findings range from genotyping methods to ethnicity. In some studies, NAT2 acetylation phenotypes were determined by an enzymatic method leading to possible misclassification of the acetylation status [53, 56, 57]. Indeed, it is difficult to compare the accuracy of different NAT phenotyping methods or different cut-off points using the same phenotyping method. In addition, for genotyping, investigators sometimes select a small number of SNPs to define the acetylation status [54, 55]. Since the frequencies of *NAT2* alleles are different among worldwide populations and new alleles are been identified in some countries, investigators need to characterize such alleles in their own study population in order to choose appropriate SNPs for genotyping and classify the acetylation status of individuals, otherwise overestimation of slow acetylators may be obtained, contributing to a spurious results in the association study.

Recently, a study with an admixed population showed that *NAT2* is a genetic factor for predisposition to anti-TB drug-induced hepatitis. In this case, *NAT2* genes were well characterized by direct sequencing and their genotypes achieved by haplotype reconstruction using the PHASE software. In addition, functional unknown genotypes were disconsidered and others confounding variables for hepatotocixity were taken into account. The incidence of elevated levels of serum transaminases was significantly higher in slow acetylators than those

of the rapid/intermediate type. These results corroborate with the current hypothesis that the acetylator status may be a risk factor for the hepatic side effects of isoniazid [58].

Finally, a meta-analysis was conducted to solve the problem of inadequate statistical power and controversial results based on accumulated data with small sample size [59]. Data from 14 studies performed between 2000 and 2011 were pooled and showed that TB patients with a slow acetylator genotype had a higher risk of anti-tuberculosis drug induced hepatotocixity than patients with rapid or intermediate acetylation ($p < 0.001$). Moreover, subgroup analyses indicate that both Asians and non-Asians slow acetylators develop anti-tuberculosis drug induced hepatotocixity more frequently. Additionally, there were statistically significant associations between NAT2*5/*7, NAT2*6/*6, NAT2*6/*7 and NAT2*7/*7 and the risk of anti-TB drug induced hepatotocixity [59].

As a final consideration, NAT acetylates more slowly not only isoniazid but also acetylhydrazine, the immediate precursor of toxic intermediates, to the harmless diacetylhydrazine [60, 61]. This protective acetylation is further suppressed by INH competition. Therefore, slow acetylators may be prone to higher accumulation rates of INH toxic metabolites. Another important route to generate toxic intermediates is the direct hydrolysis of unacetylated INH [62], producing hydrazine that also induces hepatic injury [62, 63]. Pharmacokinetic studies showed that the serum concentration of hydrazine was significantly higher in slow acetylators than in rapid acetylators, probably due to the high INH concentration. The high amount of INH disposed of through this pathway is likely to lead to enhanced hydrolysis to hydrazine, since the rate of metabolic conversion of INH to acetylisoniazid is lower in slow than in rapid acetylators [64, 65]. All of these drug-disposal processes may support the finding that slow acetylators are prone to INH-induced hepatitis. We therefore conclude that screening of patients for the *NAT2* genetic polymorphisms can prove clinically useful for the prediction and prevention of anti-tuberculosis drug induced hepatotoxicity.

3.2. CYP450

Cytochromes P450 (CYP450) are hemoproteins and form the most important enzymatic group for Phase I biotransformation. The main activity of isozymes of CYP450 system is oxidation and they are located in the smooth endoplasmic reticulum, mainly in liver cells. However, these mono-oxygenases are also localized in the intestine, pancreas, brain, lung, kidney, bone marrow, skin, ovary and testicles [66]. The CYP450 proteins are clustered into families and subfamilies according to the similarity between the amino acid sequences: where family members have ≥ 40% identity in amino acid sequence, members of the same subfamily share ≥ 55% identity [67].

The CYP450s are responsible for the metabolization of several endogenous substrates and the synthesis of hydrophobic lipids such as cholesterol, steroid hormones, bile acids and fatty acids. Moreover, some enzymes of P450 complex metabolize exogenous substances including drugs, environmental chemicals and pollutants as well as products derived from plants. The metabolism of exogenous substances by CYP450 usually results in detoxification of the xenobiotic; however, the reactions triggered by such enzymes can

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lead to generation of toxic metabolites that contribute to the increased risk of developing cancers and other toxic effects [68].

The complete sequencing of the human genome revealed the presence of about 115 genes of CYP450, including 57 active genes and 58 pseudogenes [67]. They belong to families 1-3 and are responsible for 70-80% of Phase I-dependent metabolism of clinically used drugs. Other families of CYPs are involved in metabolism of endogenous components [66]. The CYP2 constitutes the largest family of isoenzymes and comprises one third of all human CYPs. Genes encoding these enzymes are polymorphic and the frequency distribution of allelic variants in different ethnic groups differs. Overall, four phenotypes based on genotypes can be identified: (i) poor metabolizers who present low enzymatic activity, (ii) intermediate metabolizers, usually heterozygous for a defective allele, (iii) rapid metabolizers, who have two normal alleles and (iv) ultrarapid metabolizers, who have several gene copies [69].

The enzyme CYP2E1 is expressed mainly in the liver but can be found in other organs such as kidney, gastrointestinal tract and brain and involved in oxidation of substrates such as ethanol and the metabolism of many drugs and pre-carcinogens. Besides ethanol, CYP2E1 can be induced by various drugs such as INH but also by hydrocarbons, benzene, chloroform and various organic solvents [70].

The activity of CYP2E1 is also modulated by polymorphisms in several locations of its gene and more activity of this enzyme may increase the synthesis of hepatotoxins. Two polymorphisms upstream of the *CYP2E1* transcriptional start site are characterized by *Pst* I and *Rsa* I digestion and appear to be in complete linkage disequilibrium (Figure 3). These two polymorphisms are located in a putative HNF-q binding site and thus may play a role in the regulation of *CYP2E1* transcription and subsequent protein expression [71]. Genotypes of *CYP2E1* are classified as being *1A/*1A, *1A/*5 or *5/*5 by *Rsa* I based restriction analysis. The polymorphism detectable by *Dra* I (7632 T>A) is located in intron 6 and characterizes the allelic variant *CYP2E1*6*. The other polymorphism is an insertion/deletion of 96 bp (*CYP2E1*1D* and *1C* alleles) that regulates the expression of the gene [72]. Some studies have shown that allelic variants *CYP2E1 *5*, *6* and *1D* would increase enzyme activity [71, 73]. However, other authors did not confirm any relationship with these polymorphisms with CYP2E1 activity [74].

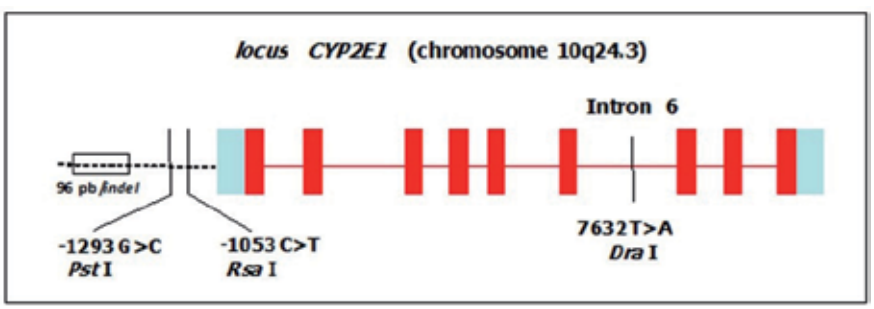

Figure 3. Polymorphic and corresponding restriction enzyme cutting sites at *CYP2E1* [24].

Several studies have described the involvement of polymorphisms in *CYP2E1* in cancer development but results are controversial. The studies showed that the frequency of SNP -1053 C>T in the promoter region varies significantly in different ethnic groups. The mutant allele is present with a frequency of 2-8% in Euro-descendants but varies in Asia from 25 to 36% [75].

In 2003, Huang and coworkers showed an association of the wild-type genotype *1A/*1A with risk of developing liver damage induced by isoniazid in adult TB patients, regardless of their profile of acetylation (OR 2.52; 95% CI 1.26 to 5.05) [76]. Later, Vuilleumier and colleagues showed association between this CYP and isoniazid-induced hepatotoxicity, without hepatitis, during chemoprophylaxis for TB (OR 3.4; 95% CI 1.1 to 12; p = 0.02). The risk of having high levels of liver enzymes was 3.4-fold higher when compared with all other *CYP2E1* genotypes [55]. Another study on Indian children with TB showed association between risk of hepatotoxicity and polymorphisms in *CYP2E1*, despite of low sample size [77]. However, a study with on a Korean population found no relationship between hepatic adverse effects with genotype *1A/*1A of *CYP2E1* during anti-TB treatment [51]. Lack of association between this CYP and antituberculosis drug-induced liver injury was also observed in Brazil [58]. The discrepancy of these results may be due to differences in the frequencies of *CYP2E1*1A* and *CYP2E1*5* alleles among the populations and the different criteria to define hepatotoxicity used.

Finally, CYP2E1 converts acetyl hydrazine into hepatotoxins like acetyldiazene, ketene and acetylonium ion. The reaction of acetyl hydrazine (at high levels) with CYP2E1 leads to covalent binding of these secondary metabolites with intracellular proteins (Figure 1). As a consequence, intracellular changes occur resulting in loss of ionic gradients and decrease of ATP levels and consequent disruption of actin followed by cell lysis. Further studies in different populations and with a larger sample size are needed to determine the true influence of CYP2E1 gene polymorphisms on the occurrence of liver injury during treatment for TB.

3.3. Glutathione S-transferases

Glutathione S-transferases constitute a superfamily of multifunctional ubiquitous enzymes that play an important role in cellular detoxification by protecting macromolecules against reactive electrophilic attack. The GSTs are Phase II enzymes that catalyze the nucleophilic attack of glutathione (GSH) into components that contain an electrophilic carbon, nitrogen or sulfur atom. The combination of the GSH with these compounds often leads to formation of less reactive and more water soluble products, more easily excreted by the body [23, 78].

Glutathione transferases are of great interest to pharmacologists and toxicologists, since they are drug targets for the treatment of asthma and cancer, in addition to metabolize drugs, insecticides, herbicides, carcinogens and products of oxidative stress. Polymorphisms in *GST* genes are often correlated with susceptibility to various cancers, as well as alcoholic liver disease [23, 78-81].

In humans, eight gene families of soluble (or cytosolic) GSTs have been described: alpha (α) located on chromosome 6, mu (μ) on chromosome 1, theta (θ) on chromosome 22, pi (π) on chromosome 11; zeta (ζ) on chromosome 14, sigma (σ) on chromosome 4; kappa (κ) (chromo-

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somal location not given) and omega (Ω) on chromosome 10 [80]. This classification is based on amino acid sequences, substrate specificity, chemical affinity, protein structure and enzyme kinetics. These enzymes are highly expressed in the liver and constitute up to 4% of total soluble proteins but can be seen in several other tissues [82]. GSTs have an overlap of specific substrates and the deficiency in one isoform can be compensated by other isoforms. Glutathione S-transferase mu (GSTM), glutathione S-transferase theta (GSTT) and glutathione S-transferase Pi (GSTP) have been the most studied isoform [83-88].

The subfamily GST mu is encoded by five genes arranged in tandem (5_-*GSTM4-GSTM2-GSTM1-GSTM5-GSTM3*-_3), forming a 100 kb gene cluster on chromosome 1p13.3 (Figure 4). Polymorphisms have been identified and clinical consequences of genotypes resulting from combinations of alleles *GSTM1*0*, *GSTM1*A*, and *GSTM1*B* have been widely investigated [78, 81, 89, 90]. Individuals who possess the homozygous null for *GSTM1* (GSTM1*0/GSTM1*0) do not express this protein. Thus, the absence of this gene can cause an increased accumulation of reactive metabolites in the body, increasing the interaction with cellular macromolecules and tumor initiation process. *GSTM1*A* and *GSTM1*B* differ in only one base in exon 7 and encode monomers that form active dimers. The catalytic activity of these enzymes are very similar [91].

The *GSTM1* gene is flanked by two almost identical 4.2-kb regions. *GSTM1*0* originates from homologous recombination between the two repeat regions which results in a 16 Kb deletion containing the entire gene *GSTM1* (Figure 4). *GSTM1* is precisely excised leaving the adjacent *GSTM2* and *GSTM5* genes intact [78]. In a study of liver specimens of 168 autopsied Japanese subjects, observed was that the *GSTM1*0* null allele was more frequent in livers with hepatitis and hepatocellular carcinoma compared to control livers [92].

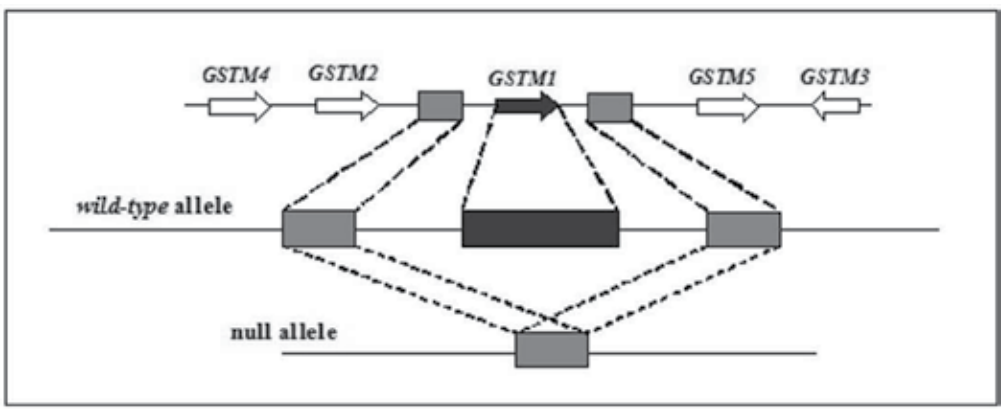

Figure 4. Structural localization of 100 kb gene cluster encoding the GST mu subfamily (chromosome 1p13.3). The figure indicates the homologous recombination event that can happen causing the null allele (*GSTM1*0* - no *GSTM1*). Figure adapted from [78].

The subfamily GST theta consists of two genes, *GSTT1* and *GSTT2*, located on chromosome 22q11.2 and separated by approximately 50 Kb (Figure 5). Analysis of the 119 Kb portion

containing these genes revealed two regions flanking *GSTT1*, HA3 and HA5, with more than 90% homology. HA3 and HA5 contain two identical 403-bp repeats and the occurence of *GSTT1*0* allele is probably caused by homologous recombination between the two regions [78]. In humans, *GSTT1* is also expressed in erythrocytes and probably plays a global role in early detoxification of xenobiotics and carcinogens.

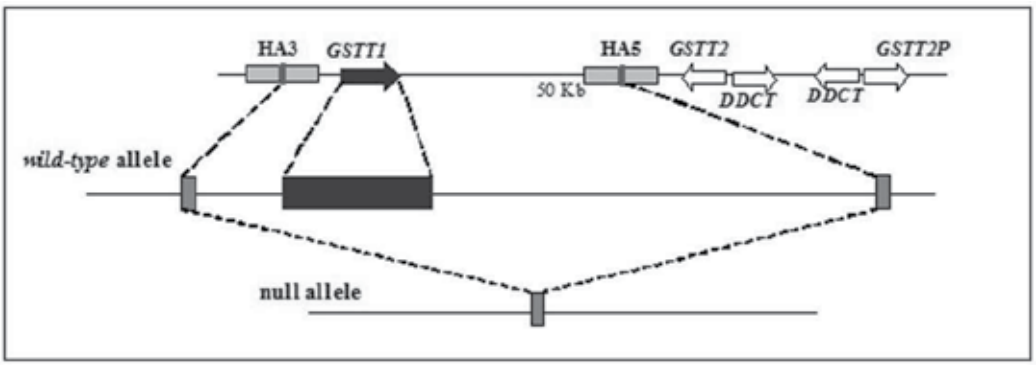

Figure 5. Structural localization of gene cluster encoding the GST subfamily theta (chromosome 22q11.2). The *GSTT1* null allele (*GSTT1*0*) arises by homologous recombination of the left and right 403-bp repeats, which results in a 54-kb deletion containing the entire *GSTT1* gene. Figure adapted from [78].

Deficiencies in the GST activity due to the null genotypes of *GSTM1* and *GSTT1* may modulate susceptibility to the development of hepatotoxicity induced by drugs and xenobiotics. Furthermore, it was observed that the frequencies of *GSTT1*0* and *GSTM1*0* alleles vary within different ethnic groups [78, 82]. Liver injury induced by INH has been associated with the depletion of glutathione content and reduction of GST activity in an animal model for hepatotoxicity by anti-TB drugs [22].

In 2001, Roy and colleagues demonstrated that individuals, homozygous for the null *GSTM1*, had a relative risk of 2.12 for developing hepatotoxicity induced by anti-TB drugs. However, these authors found no association of the *GSTT1* null genotype with this side effects [54]. Similarly, another study in the Thai population found that only the *GSTM1* null genotype increases the risk of liver injury (OR 2.23, 95% CI 1.07 to 4.67) [93]. The opposite was observed by Leiro and colleagues: individuals with the *GSTT1* null genotype had an increased risk of developing hepatotoxicity induced by anti-TB drugs and no significant association was observed between GSTM1*0/*0 genotype and liver injury [94]. These studies suggest a protective effect of glutathione S-transferases to the hepatotoxic effects of isoniazid.

On the other hand, recent studies in different population showed no relationship between GSTM1*0/*0 or GSTT1*0/*0 genotypes and liver injury during anti-TB treatment [58, 95, 96]. In a population-based prospective antituberculosis treatment coort in China, a more robust case-control study was conducted and there was no statistically significant association between null genotypes and hepatotoxicity induced by anti-TB drugs [97].

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These controversal results may be due to the small sample size in many studies and the different frequencies of the null genotypes. New populations should be evaluated with large sample size to see which of these polymorphisms can be used as genetic markers for the risk of side effects during anti-TB treatment.

4. Conclusion

The concept of personalized medicine is not really new, but it has been receiving increasing attention in recent years for improval of drug regulation and medical guidelines. There is considerable interindividual variability in metabolism, partly due to human differences on a genetic level. Genetic polymorphisms in drug-metabolizing enzymes can affect enzyme activity and may cause differences in treatment response or drug toxicity, for example, due to an increased formation of reactive metabolites. Such polymorphisms may explain differences in incidence of anti-TB drugs induced hepatotoxicity between different populations.

Genotyping cannot completely predict the phenotype on an individual level because of to the additional contribution of epigenetic, endogenous and environmental factors. However, pharmacogenetics is able to add important information in many cases where therapeutic drug scheme is inappropriate or not sufficient. Nowadays, we can cite three examples of personalized medicine application in clinical practice, (i) AIDS treatment (abavir / skin hypersensitivity / *HLA-B*5701*), (ii) anticoagulation (warfarin / bleeding / *CYP2C9*) and (iii) treatment of acute lymphoblastic leukemia (azathioprine / treatment resistence / *TPMT*) [98].

Although limited information exists regarding isoniazid concentrations that cause toxic reactions, it has been proposed to adjust isoniazid dosage depending on individuals acetylator status: a lower dosage for slow acetylators to reduce the risk of liver injury and a higher isoniazid dosage for fast acetylators to increase the early bactericidal activity and thereby lower the probability of treatment failure [50]. However, more robust clinical prospective studies are needed to evaluate the real contribution of these different polyporphirms in the occurence of liver side effects during anti-TB treatment. Future studies should include larger sample size, different ethnic population, simultaneous analysis of different genetic markers, different degrees of liver injury and consideration of possible confounding factors.

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Chapter 7

Pathophysiology of Tuberculosis

Ruiru Shi and Isamu Sugawara

Additional information is available at the end of the chapter

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1. Introduction

1.1. Inflammatory process of tuberculosis

When many infectious units of 1-3 bacilli are inhaled, a phenotypically hardy bacillus is likely to be among them. In addition, the alveolar macrophages apparently vary in their capacity to destroy bacilli [1]. Staining for acid-fast bacilli is very useful for demonstrating *M. tuberculosis* (A). Fig. 1 reveals histologic manifestation of tuberculosis over the time course. Histologically, tuberculosis displays exudative inflammation (B), proliferative inflammation (D) and productive inflammation (C) depending on the time course. Using animal experiments and an inhalation exposure system, the pathologic condition of the infected animals was followed up for one year. Exudative inflammation was observed for the first 10 days. Thereafter, granulomas, which corresponded to foci of proliferative inflammation, were formed. Cavity formation was not recognized in animal tuberculosis, except for rabbits. Using rabbit models, Dr. Arthur Dannenberg described the pathology of tuberculosis in detail [2, 3]. There are five stages: onset, symbiosis, early stages of caseous necrosis, interplay of cell-mediated immunity and tissue damaging delayed-type hypersensitivity, and liquefaction and cavity formation. In stage 1, tubercle bacilli are usually destroyed or inhibited by the mature resident alveolar macrophages that ingest them. If bacilli are not destroyed, they grow and eventually destroy the alveolar macrophages. In stage 2, bacilli grow logarithmically within the immature nonactivated macrophages. These macrophages enter a tubercle from the bloodstream. This stage is termed symbiosis because bacilli multiply locally without apparent damage to the host, and macrophages accumulate and divide. In stage 3, the stage at which caseous necrosis first occurs, the number of viable bacilli becomes stationary because their growth is inhibited by the immune response to tuberculin-like antigens released from bacilli. Stage 4 is the stage that usually determines whether the disease becomes clinically apparent. Cell-mediated immunity plays a major role in this situation. The cytotoxic delayed- type hypersensitivity immune response

kills these macrophages, causing enlargement of the caseous center and progression of the disease. If good cell-mediated immunity develops, a mantle of highly activated macrophages surrounds the caseous necrosis. In stage 5, bacilli evade host defenses. When liquefaction of the caseous center occurs, the bacilli multiply extracellularly, frequently attaining very large numbers. The high local concentration of tuberculin-like products derived from these bacilli causes a tissue-damaging delayed-type hypersensitivity response that erodes the bronchial wall, forming a cavity.

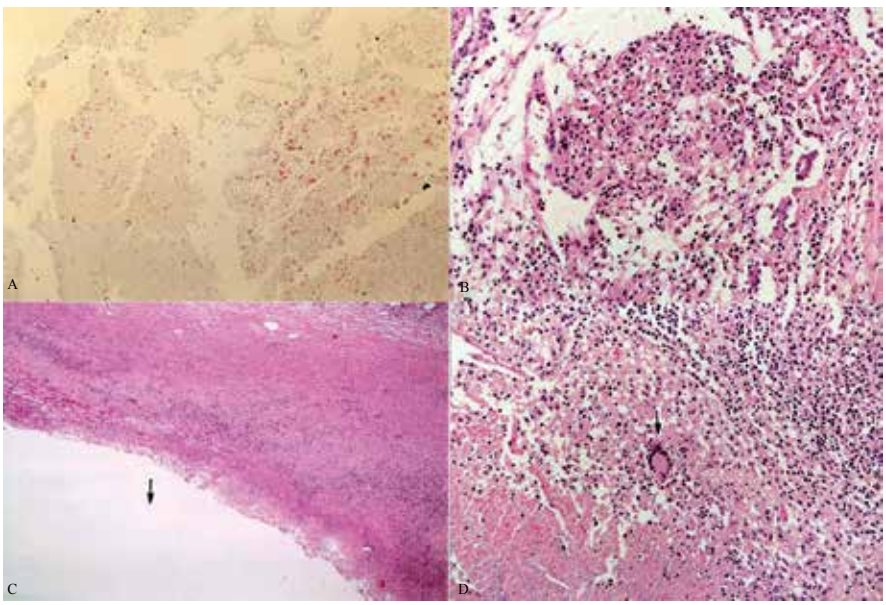

Figure 1. Histologic appearance of tuberculosis A. Staining for acid-fast bacilli, B. exudative stage, C. productive stage with cavity formation (→), D. proliferative stage with a multinucleated giant cell.

2. Clinical manifestations

As the cellular processes occur, tuberculosis may develop differently in each patient, according to the status of the patient's immune system. Stages include latency, primary disease, primary progressive disease, and extrapulmonary disease. Each stage has different clinical manifestations [4]. *M. tb* organisms can be enclosed but are difficult to completely eliminate [5]. Persons with latent tuberculosis have no signs or symptoms of the disease, do not feel sick, and are not infectious [5]. However, viable bacilli can persist in the necrotic material for years or even a lifetime [6], and if the immune system later becomes compromised, as it does in many critically ill patients, the disease can be reactivated. Primary pulmonary tuberculosis is often asymptomatic. Although it essentially exists subclinically, some self-limiting findings might be noticed. Associated paratracheal lymphadenopathy may occur because the bacilli spread from the lungs through the lymphatic system. Active tuberculosis develops in only 5% to 10% of persons

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exposed to *M. tb*. Fig. 2 shows typical chest X-ray before (A) and after (B) chemotherapy. Fatigue, malaise, weight loss, low-grade fever, night sweats, cough, sputum, are the main symptoms. The sputum may also be streaked with blood. Hemoptysis can be due to destruction of a patent vessel located in the wall of the cavity [7]. Extrapulmonary disease occurs in more than 20% of patients. The most serious location is the central nervous system, where infection may result in meningitis, which could be fatal in most cases. Another fatal form is infection of the blood stream by mycobacteria, this form is called disseminated or military tuberculosis. The most common extrapulmonary tuberculosis is lymphatic tuberculosis. Other possible locations include bones, joints, pleura, and genitourinary system [4].

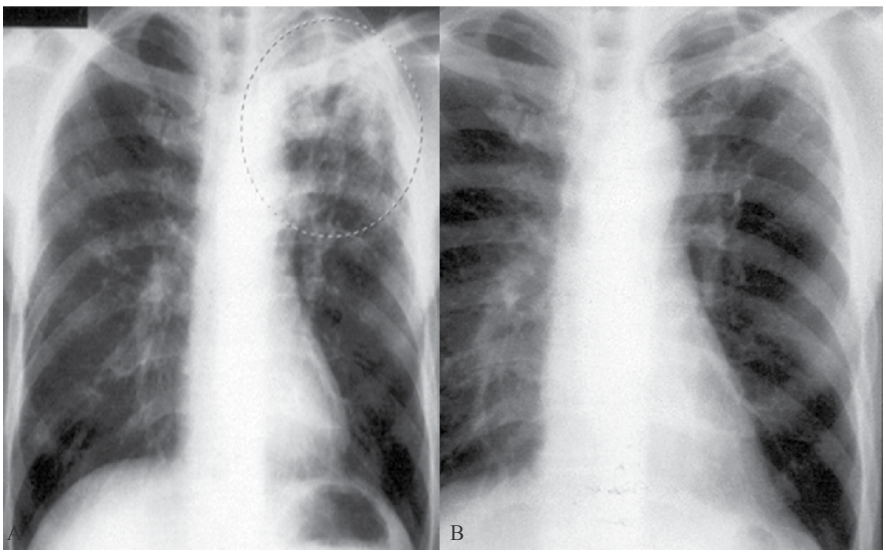

Figure 2. Chest X-ray of pulmonary tuberculosis and cured Tuberculosis A. before chemotherapy with rifampicin, isoniazide, ethambutol and pyrazinamide, B. after chemotherapy. Apical shadow (dotted circle) disappears.

3. T cell activation against *Mycobacterium tuberculosis*

In human, a TB index case may infect a contact person through cough and expectoration, so the lung is the primary route of infection and often the main tissue exhibiting TB. Infectious droplet nuclei are deposited in the alveolar spaces of the contact person where *Mycobacterium tuberculosis (M. tb)* can be phagocytosed by alveolar macrophages, epithelial cells, dendritic cells (DC) and neutrophils [8, 9]. Alveolar macrophages and DC are then believed to transport *M. tb* to local lymph nodes where T cell activation occurs and expand. Activation of the phagocytic host cell is much required to limit growth of *M. tb*; as in the absence of activation, disease outcome is extremely poor. Effective phagocyte activation requires a specific cellular response, as infected hosts lacking specific components of the acquired response have a poor outcome [10]. While acquired cellular protection is expressed rapidly

following systemic challenge with *M. tb*, it is less rapid in the lung. Slow expression of protection in the lung allows mycobacteria to grow and modulate the infection site. Until recently it has not been clear whether the slow response to aerosol delivery of bacteria resulted from limited availability of antigen or inhibition of antigen-presentation by *M. tb*. Several studies show that the first T cell activation occurs in the draining lymph node (DLN) of the lung 8–10 days following initial challenge. The activation of T cells correlated temporally with the arrival of bacteria and availability of antigen in the DLN, however conditions for T cell activation were unique to the draining lymph nodes as the presence of antigen-producing bacteria in the lung and spleen did not result in initial activation of T cells [11, 12]. While delivery of lipopolysaccharide (LPS) to the MTB-infected lung failed to accelerate T cell priming [11], increasing the bacterial dose did accelerate the response modestly suggesting that both antigen burden and refractory cells serve to slow the response. So, protective memory cells will not become activated until they see antigen, i.e. more than 8 days post infection. Once T cells become activated they differentiate into effector T cells that migrate to the lung. By day 14 of infection, when activated T cells first arrive in the lung, bacteria are within alveolar macrophages, myeloid DC and neutrophils [11]. T cells can recognize antigen within the mycobacterially-infected lung but the antigen presentation is not optimal. It takes time for the protective T cells to reach sufficient numbers to stop bacterial growth. T cells can be divided into two subsets, Th1 and Th2, on the basis of the cytokines they produce. In tuberculosis, Th1 plays a major role in defense against tuberculosis. Th1 cells suppress Th2 cells. CD4 + T cells have unambiguously been identified as the most important lymphocyte subset for mediating protection.CD4 T lymphocytes differentiate in the peripheral tissues to adopt a variety of fates such as the Th-1 cells, which produce interferon (IFN)-γ to down-regulate Th2 responses and Th-2 cells, which produce interleukin (IL)-4. CD8 T lymphocytes produce predominantly IFN-γ. Though CD4 response is greater than the CD8 response, the latter can provide protection in the absence of CD4 help [13]. During active TB there is a local pulmonary immune response characterized by α/β T cells and strongly enhanced *M. tuberculosis* antigen-specific Th1 responses, with large amounts of locally secreted IFN-γ [14].

4. Animal models of tuberculosis

A wide variety of animal models have been used to test new vaccines and drugs [15]. Mice can harbor high numbers of *M. tb* within lung tissue without showing clinical signs [16]. Mice do not cough nor form cavitary lesions, making them a poor model for transmission studies [17]. Fibrous capsules are not observed histologically, which can affect the validity of antibiotic studies, as *M. tb* would be more easily accessed by drugs in the mouse lung. In addition, because of their short life span, mice are poor models for the study of latent infection. Rat TB also showed similar pathophysiology to murine TB [31]. Guinea pigs develop robust DTH response to mycobacterial antigens and, after infection with *M. tb*, reproduce many of the aspects of human infection, such as caseous and mineralized granulomas, primary and hemato-genous pulmonary lesions, fibrous capsule formation, and dissemination [19],

however, pulmonary lesions in guinea pig contain a high proportion of granulocytes, particularly eosmophils, which are not common features of human disease [20]. The rabbit is the only common laboratory animal in which the disease closely resembles the typical chronic cavitary type found in the majority of human beings [21, 22]. Rabbits infected with *M. tb* mount a moderate DTH response and form caseous granulomas and cavitary lesions [23-25]. Rabbits, including currently available inbred strains, are relatively resistant to *M. tb*, however, requiring the inhalation of 500 to 3000 bacilli to form one grossly visible tubercle at 5 weeks postinfection [23]. Most rabbits will also overcome disease completely, with few culturable bacilli [24]. This model is useful in the study of latent or paucibacillary TB states, however, without the use of antibiotics as in the Cornell model. Rabbits do need to be experimentally immunosuppressed as they will not spontaneously reativate disease [26]. There are minimal immune reagents, however, for this model, and the larger size of rabbits makes them more costly to use. There are inbred strains of rabbits, such as the Lurie and Thorbecke rabbits, which are more susceptible to *M. tb* infection. This susceptibility has been linked to suppressed macrophage antimycobacterial activity, decreased MHC Class 2 expression, and impaired development of type 4 hypersensitivity [27]. Other animal models, such as nonhuman primates, which are susceptible to *M. tb* and full spectrum of granuloma types can be observed [28], have not been widely used. Using mycobacterial inoculation into trachea, at necropsy, all unvaccinated monkeys (*Macaca fascicularis* and *Macaca mulatta*) exhibited extensive bilateral lung pathology characterized by the presence of multiple granulomas. These granulomas exhibited conglomeration to larger caseous areas, especially in the hilar region [55].

5. Alveolar macrophages in tuberculosis

When tubercle bacilli reach alveoli, they are phagocytosed by resident alveolar macrophages. Though tubercle bacilli are killed byalveolar macrophages, tubercle bacilli can also kill macrophages through apoptosis. What is the fate of tubercle bacilli once they enter the phagosomes of macrophages? Alveolar macrophages of aerially infected guinea pigs were collected by bronchoalveolar lavage. At 12 days after infection, one out of 10,000 alveolar macrophages of various sizes contained many tubercle bacilli [31]. This indicates that certain alveolar macrophages permit M. tuberculosis to replicate in the phagosomes, although most of tubercle bacilli are killed by activated alveolar macrophages. It will be of great interest to examine the survival mechanism of *M. tuberculosis* at the single-cell level, but we still do not know why macrophages targeted by tubercle bacilli cannot kill the bacilli.

IFN-γ knockout mice were infected with avirulent H37Ra or BCG Pasteur, multinucleated giant cells were recognized in the granulomatous lesions. The lesions also contained tubercle bacilli and consisted of multinucleated cell clusters, being immunopositive with anti-Mac-3 antibody. The alveolar macrophages were transformed into multinucleated ginat cells. We subsequently infected various cytokine-konockout mice with *M. tb*, but no Langerhans' multinucleated giant cells were recognized in the granulomas. Therefore, it seems that formation of multinucleated giant cells requires optimal combinations and concentrations of various cytokines, and the level of IFN-γ, at least, has to be significantly low.

6. Roles of cytokines, neutrophils, NK cells, NKT cells and γδT cells

IFN-γ and TNF have long been implicated as regulators of T cell responses in mycobacterial disease [29]. The technique of gene targeting (knockout) has swept through biomedical research. IFN-γ, TNF-α, IRF-1, NF-IL6, NF-κB p50, STAT 1 and STAT 4 knockout mice succumbed to *M. tuberculosis* infection over time. There appears to be a cytokine and transcription factor hierarchy in experimental tuberculosis. The results indicate that these molecules play major roles in defense against the disease, IFN-γ and TNF-α being the leading players in this respect [30].

The role of neutrophils in the development of tuberculosis remained unknown for a long time. We utilized LPS-induced transient neutrophilia in the lungs [31]. LPS (50μg/ml) was administered intratracheally to male Fischer rats, which were then infected with *M. tuberculosis* via an airborne route. Intratracheal injection of LPS significantly blocked the development of pulmonary granulomas and significantly reduced the number of pulmonary colony-forming units (CFU). Treatment with amphotericin B (an LPS inhibitor) or neutralizing anti-rat neutrophil antibody reversed the development of pulmonary lesions. LPS-induced transient neutrophilia prevented early mycobacterial infection. The timing of LPS administration was important. When given intratracheally at least 10 days after aerial infection, LPS did not prevent the development of tuberculosis. Neutrophils obtained by bronchoalveolar lavage killed M. tuberculosis bacilli. These results indicate clearly that neutrophils participate actively in defense against early-phase tuberculosis.

Natural killer (NK) cells are innate lymphocytes which are a first line of defense against infection. NK cells can kill autologous infected cells without prior sensitization, and are believed to play a pivotal role in innate immunity to microbial pathogens. In mouse model, NK cells are activated and produce IFN-γ during the early response to pulmonary tuberculosis [31] and NK cell-produced IFN-γ regulates the anti-mycobacterial resistance mediated by neutrophils [32]. However animal models do not give a clear answer to whether NK cells is important in *M. tb* infection in vivo. Depletion of NK cells had no effect on bacterial replication in the lung of immunocompetent mice [33], suggesting that NK cells may be redundant in the presence of intact adaptive immunity. Surprisingly, IFN-γ knockout mice, which are impaired in their ability to clear mycobacteria, cleared them as effectively as wild-type mice when NK cells were depleted, suggesting that NK cells can inhibit protective immunity [34].

Human NK cells use the NKp46, the natural cytotoxicity receptors (NCRs) and NKG2D receptors to lyse *M. tuberculosis*-infected monocytes and alveolar macrophages [35], through damage of infected cells and secretion of cytokines, such as IFN-γ [36]. Inhibitory receptors of NK cells include killer immunoglobulin-like receptors (KIRs) and the NKG2A:CD94 dimer and NK cell activation can also be triggered by loss of inhibitory ligands from the cell surface. In addition, NK cells can also be activated by cytokines, including type I interferons, IL-12 and IL-18. NK cells are a potent and early source of cytokines, particularly IFN- γ, but they can also produce Th2-associated cytokines, such as IL-5 and IL-13, and the regulatory cytokine IL-10 [37]. NK cell NKp46 expression and cytotoxicity are reduced in freshly isolated peripheral blood mononuclear cells (PBMCs) from tuberculosis patients, which may be attributable to

suppression by monocytes and IL-10. Recent studies have found that NK cells produce IL-22 [38], which was induced by IL-15 and DAP-10, an adaptor protein that is known to be involved in NK cell activation, in response to *M. tuberculosis*. Rohan Dhiman *et al*. also found that IL-22 can restrict growth of *M. tuberculosis* in macrophages by enhancing phagolysosomal fusion [39]. Nonetheless to fully understand the importance of NK cells in *M. tb* infection it may be necessary to differentiate their contributions at different stages of disease.

Certain T subsets, such as NKT cells and $\gamma\delta$ T cells, have features of innate immune cells including a partially activated phenotype, a rapid response following detection of infected cells, and the modulation of other cell types. Together with NK cells, these cell subsets are functionally defined as innate lymphocytes.CD1d-restricted invariant NKT (iNKT) cells are a conserved subset of T cells that express an invariant T cell receptor (TCR) α chain (Vα24-Jα18 in humans, and Vα14-Jα18 in mice) paired with TCR β chains encoded by one or a few Vβ gene segments (Vβ11 in humans, and predominantly Vβ2, 7 and 8 in mice). These cells show different phenotypes and functions [40].Many iNKT cells are CD4+, and they have been mainly associated with the induction of Th2 cytokines such as IL-4, IL-5, IL-13. This subset is believed to play a prominent role in suppression of autoimmune or chronic inflammatory diseases, and in promoting allergic conditions such as asthma. Few iNKT cells are CD8+, and most of those express only the CD8α subunit, which means that they likely express only CD8$\alpha\alpha$ homodimers. An additional fraction of iNKT cells are negative for both CD4 and CD8 (DN T cells). They have been found to produce predominantly IFN-γ and other Th1-associated cytokines. Studies of human iNKT cells have shown that they have the ability to kill *M. tuberculosis* organisms within infected macrophages, possibly through their production of the peptide granulysin [41]. Jin S. Im *et al*. [42] found that the percentages of iNKT cells among total circulating T cells in TB patients were not significantly different compared to those in healthy controls. However, TB patients showed a selective reduction of the proinflammatory CD4−CD8− (DN) iNKT cells with a proportionate increase in the CD4+ iNKT cells. The mouse model of tuberculosis has been used by Sada-Ovalle et al to find that iNKT cells have a direct bactericidal effect on *M. tuberculosis*, and protect mice against aerosol *M.TB* infection [43]. Their activation requires CD1d expression by infected macrophages as well as IL-12 and IL-18. In addition, pharmacological activation of iNKT cells with the synthetic ligand aGalCer often enhances host resistance to infection. iNKT cell use several mechanisms to modify host immunity. These include induction of DC maturation, secondary activation of effector cells (NK cells) or recruitment of inflammatory cells to the site of infection [44, 45]. Thus, by being an early producer of IFN-γ and suppressing intracellular bacterial growth, iNKT cells function as an important part of the early immune response against *M. tb* that affect both the innate and the adaptive arms of the immune response.

Antigen-specific γ/δ T cells represent an early innate defense that may play a role in antimycobacterial immunity. Studies done in humans and animal models have demonstrated complex patterns of γ/δ T cell immune responses during early mycobacterial infections and chronic TB. Like α/β T lymphocytes, γ/δ T cells carry antigen TCR that vary in the physical properties of their ligand-binding sites. γ/δ T cells are frequently activated by a variety of pathogens including *M. tb* [46]. Mice lacking γ/δ T cells succumb more rapidly than control

mice following intravenous challenge with virulent *M. tb*; however, such a difference has not been observed following infection by the aerosol route. γ/δ T cells constitute a whole system of functionally specialized subsets that have been implicated in the innate responses against tumors and pathogens, the regulation of immune responses, cell recruitment and activation, and tissue repair [47]. Human alveolar macrophages and monocytes can serve as antigen presentation cells (APCs) for γ/δ T cells. Furthermore, the predominance of Vγ9Vδ2 T cells in TB disease has been confirmed [48]. When MTB-activated CD4+ and γ/δ T cells from healthy tuberculin-positive donors were analyzed for cytokine production in response to MTB-infected monocytes, both groups secreted large amounts of IFN-γ [49]. Previous studies have also demonstrated an increased proliferative activity of Vγ9Vδ2 T cells from patients with TB [50], but reduced production of IFN-γ, compared with that of healthy tuberculin-positive donors [51]. Additionally, Dieli *et al.* reported that decrease of Vγ9Vδ2 T cell effector functions involves not only IFN-γ production but also expression of granulysin [52]. Fig. 3 shows interaction of cells and cytokines involved in tuberculosis.

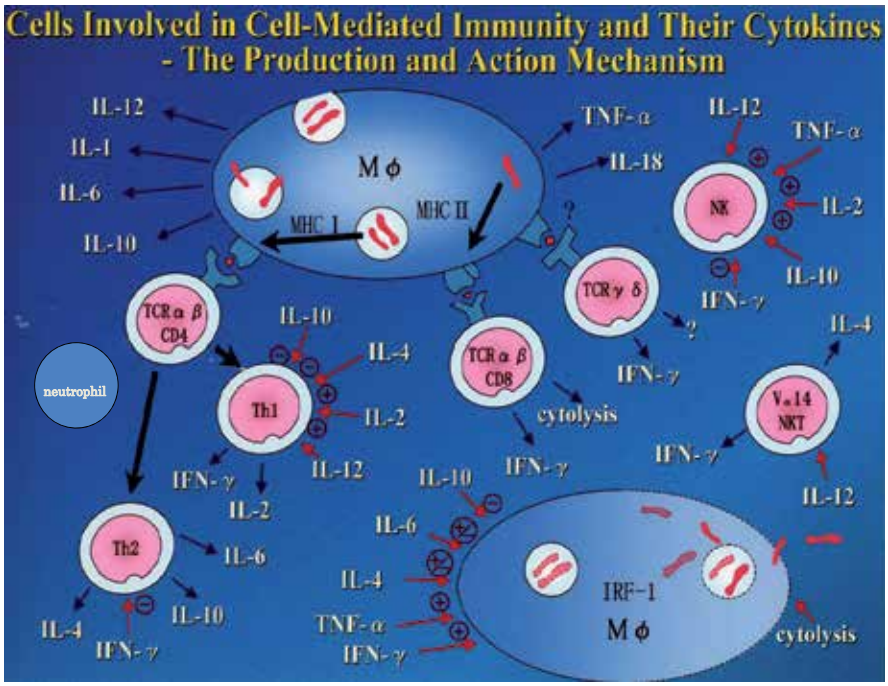

Figure 3. Cytokine and cellular network in tuberculosis +. Production of cytokine, -. No production of cytokine.

7. Conclusion

Tuberculosis is an international public health problem. It is becoming evident that *M. tb* infection is a dynamic state with a wide spectrum of pathology. An improved understanding

of the immunopathogenesis of TB can facilitate the design of effective vaccines, new drug candidates and evaluation of their efficacy [53]. Understanding latent tuberculosis can also be the key to improve diagnostic and novel treatment strategies [54].

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Section 2

Diagnosis and Management of Tuberculosis

Chapter 8

Laboratory Diagnosis of Tuberculosis - Latest Diagnostic Tools

Gunes Senol

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/54248

1. Introduction

Early diagnosis of tuberculosis and drug resistance improves survival and by identifying infectious cases promotes contact tracing, implementation of institutional cross-infection procedures, and other public-health actions. There have been many advances in methodology for tuberculosis diagnosis [1-3].

For every stages of diagnosis, there are new approaches. New tests are available by level of laboratory and phase of application.

2. Microscoby

Microscopy has been a diagnostic tool for TB for over a century, and still currently the most rapid diagnostic method. Standard light microscopy (LM) and fluorescent microscopy (FM) are common methods. The recent development of light emitting diodes (LED), with the appropriate fluorescent light output for FM and low power consumption, has led to the development of simple, robust LED FM microscopes, requiring minimal mains or battery power and no dark room requirement. The WHO has recommended rolling it out as an alternative to LMs in resource-limited settings, based on studies that have shown comparable performance of LM and standard FM systems [4,5].

3. Culture and drug resistance testing

3.1. Phenotypic methods

Significant effort has been invested into further development of simple, alternative phenotypic methods such as the nitrate reductase assay (NRA), thin-layer agar (TLA), colour test (Color Test), the microscopic observation drug susceptibility assay (MODS), the colorimetric redox indicator (CRI) method and phage-based assays, most of which can be set up directly on specimens [6,7,8]. These methods can detect MTB and resistance to INH and RMP. While MODS, NRA and CRI have been endorsed by the WHO, current evidence was considered to be insufficient for recommending the use of TLA or phage-based assays [8].

MODS is an extensively validated method that has almost perfect agreement with conventional DST for INH, RMP and MDR-TB (100%, 97% and 99%). The results are available within a median of 7 days; the method is cheap, non-commercial and works well on all types of primary specimens as well as on isolates. However, it requires relatively long, detailed staff training. [6,7,9,10]

TLA recently demonstrated a good performance of the MDR-/XDR-TB colour test for the identification of MTB complex and detection of resistance to INH, RMP and ciprofloxacin in cultures [11].

3.2. Genotypic methods

Molecular techniques are aimed at the nucleic acid of the mycobacterium as the analyte. Ribosomal rRNA is useful genetic target for the identification of organisms, since it often contains spesific sequences and is present in the cells and media in high quantity due to the growth of the mycobacteria. There are various applications of molecular tecniques for the detection and identification of MTB.

PCR is the common format of nucleic acid amplification tests (NAAT); other methodologies include ligase chain reaction, strain displacement amplification, loop-mediated isothermal amplifi cation (LAMP) and transcription mediated amplification. More recently, real-time (RT) PCR technologies based on fluorescent- probe detection or melting-curve analysis have been developed [12-16].

These molecular techniques also aimed detecting resistance genes. Example includes; DNA probe and DNA sequencing of MTB gene such as catalase (katG) or RNA polymerase (rpoB). Mutations in these genes have been associated with resistance to isoniazid and rifampicin respectively. The using of molecular primers in real- time PCR reaction can differentiate between the presence of the wild- type sequence and mutated sequence associated with drug resistance. Molecular tests are rapid (within few hours), highly sensitive and specific, but expensive, requires expertise and may not differentiate active infection as DNA from a dead organism during antibiotic treatment can be detected and amplified by PCR [17]. Genotypic methods are not routinely used in the mycobacterium laboratory; they are essentially for research purposes [18,19].

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Line-probe assays and XPERT MTB/RIF: Line probe assays (LPAs) are actual molecular tests. Three main LPAs for the rapid diagnosis of TB and/or rapid detection of RMP resistance and MDR-/XDR-TB are currently available on the market: INNO-LiPA Rif. TB (Innogenetics, Belgium), GenoType®MTBDR/MTBDR*plus* and Geno-Type® MTBDR*sl* (both Hain Lifescience, Germany). These assays are based on the targeted amplification (PCR) of specific fragments of the MTB genome, followed by hybridisation of PCR products to oligonucleotide probes immobilised on membranes. INNO-LiPA Rif TB detects only RMP resistance, GenoType MTBDR/MTBDR*plus* detects both RMP and INH resistance, and GenoType MTBDR*sl*detects resistance to flouroquinolons, injectable second-line drugs and ethambutol. These tests are designed for detection the MTB isolates in respiratory specimens. Xpert® MTB/RIF (Cepheid Inc, USA) is a fully automated RT-PCR based assay for the detection of TB bacteria and resistance to RMP in direct clinical specimens [20].

4. Lysis-centrifugation blood culture system

The recovery of mycobacterium from peripheral blood and bone marrow samples may be improved by lyses- centrifugation blood culture method. In this method, blood is put into a tube containing an anticoagulant and an agent to effect rupture of both erythrocytes and neutrophils. Following centrifugation of the tube, the sediment is inoculated into the appropriate culture media. This method has increased both the yield and shortened the time of recovery of mycobacteria from blood cultures [21].

5. Phage Amplification Technique (PAT)

This is a bacteriophage based test to detect MTB in sputum. Non-pathogenic mycobacteria (sensor cells) were used for control bacteria in the test. The phage replicate, infect and lyses the sensor cells leaving zones of clearing (holes) in the agar. The areas of clearing indicates that the patient sputum contain viable MTB. It is fast with a turnaround time of 2 days. It is cheap, requires few equipments, sensitive (detection as low as 100 tubercles per ml of sputum). It can be adapted for sensitivity testing. Limitations are applicability to sputum specimen only and technically demanding [22].

6. Immunological methods

Immunodiagnostic tests can provide indirect evidence current or past infections of MTB. Exception of tuberculin skin test, immunodiagnostic tests are of limited application due to cross reactivity and poor sensitivity.

6.1. Detection of antibodies

Although the detection of antibodies against MTB in the blood is a relatively simple and costeffective method, recent meta-analyses and systematic reviews concluded that commercial serological tests provided inconsistent results [23,24]. As the overall test performance and data quality of these assays were poor, the WHO currently recommends against their use for the diagnosis of pulmonary and extrapulmonary TB.

Antibodies against lipoarabinomannans, A60, 38Kd and 16 Kd are mostly studied [25].

6.2. Detection of antigens

Lipoarabinomannan (LAM) was identified as a promising target for antigen detection for TB diagnosis due to its temperature stability and could be detected in urine. LAM-based assays are currently being developed by a number of commercial companies, and preliminary results indicate their potential applicability in the rapid diagnosis of TB by detecting LAM in a variety of body fluids, including urine [26]. LAM-based assays are included in the WHO TB diagnosis re-tooling programme [27] and forma part of a Foundation for Innovative New Diagnostics (FIND) funded TB Project [19,28].

MTB antigen detection provides direct evidence of TB. Such as LAM, 65Kd, 14 Kd antigens were widely used. It is very quick and easy to perform. Main limitation is low sensitivity (detect high levels of antibody). It does not rule out TB in patients with poor antibody response as in HIV and malnutrition and not specific due to cross reactivity with other species of mycobacteria in the environment [26].

7. Interferon-Gamma Release Assays (IGRA)

Because of the difficulties with the tuberculin test interpretation, the interferon-gamma assay test was developed. Two available formats of the interferon-gamma release assays are; the Quantiferon-TB Gold and T Spot-TB test. The IGRA assay is based on the ability of the MTB antigens, which includes the Early Secretory Antigen Target 6 (ESAT-6) and Culture Filtrate Protein 10 (CFP-10) to stimulate host production of interferon –gamma. These antigens are not present in NTM or in BCG vaccine, so, these tests can distinguish latent TB infection from BCG immunization and NTM infections. Requiring a single visit to draw a blood sample and result available within 24 hours are main advantages. It does not boost immune response measured by subsequent tests which can happen with tuberculin skin test. It does not cause to readers bias as in tuberculin skin test and not affected by prior BCG vaccination. Blood must be processed within 12 hours while leukocytes are still viable. There are limited data for sensitivity of IGRAs in children younger 17 years of age and immunocompromised patients e.g. HIV/AIDS, diabetics, treatment with immunosuppressive drugs [29].

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8. The future of TB diagnostics

The rapid technological evolution in the laboratory diagnosis of TB, especially in the application of molecular biology h as diminished the time required for identification and susceptibility testing. Continuous effort endeavor for increasing reproducibility, improvment of performance and cost containment. WHO founded an organisation (FIND-Foundation for Innovative New Diagnostics) for researching fast, relible and inexpensive tests as given Table 1 [28].

	Concept phase	**Feasibility phase**	**Devolapment phase**	**Evaluation phase**	**Demonstration phase**	**Implementation phase**
Reference laboratory level	-	-	-	-	-	Liquid culture & DST Rapid speciation Line probe assay (1st line drugs)
Disrtict/peripheral level	-	Rapid colorimetric DST	-	Line probe assay /2nd line drugs)	LAMP TB	LED florecence microscopy Xpert MTB/RIF
Community level	LFI sensitivity increase	Antibody detection Antigen detection	Beta lactamase detection	-	-	-

LFI sensitivity increase: Alternative quantitative fluorescence (LFI) sensitivity increase

Table 1. WHO projects for TB diagnostic tests. LAMP TB: Loop mediated isothermal amplification (LAMP) for TB

9. Assays being developed/evaluated

Another new approach to diagnosis of TB is biosensing technologies. Variety of portable, rapid, and sensitive biosensors with immediate "on-the-spot" interpretation have been developed for MTB detection based on different biological elements recognition systems and basic signal transducer principles. Combination of nanotechnology and biosensing technology has very promising.

Transrenal DNA detection provides a challenging new target for molecular TB diagnosis. No commercial assays are currently available, largely due to the difficulties in the development of TB detection/read-out assays.

Combined high-resolution melting (HRM) curve analysis using a closed-tube RT-PCR is potentially an ideal screening method with a positive predictive value (PPV) of 100% and neg-

ative predictive value (NPV) of at least 99.9%, for screening large specimen numbers in any TB laboratory.

New amplification methodologies and refinements of 'molecular beacon' approaches, such as linear-after-the exponential PCR, offer future improvements, particularly in drug resistance analysis [30-32].

MTB urease is a bacterial virulence factor. Isotopcally labelled urea as substrate, Urea tracer has detected in exaled breath using portable infrared spectrofotometer. Signal correleted with bacterial load. [33]

A biophotonic detection platform has been developed that utilizes reporter enzyme fluorescence to detect β-lactamase produced by MTB. This innovative new technology is now being adapted for point of care (POC) use [28]

10. Conclusions and further work

This is an exciting time for new TB diagnostics. This is in part a reflection of the funding and application of good science, a clear understanding of unmet needs, a commercial sector that is considering new approaches to a global market, and the complexity of and limited progress in new drug and vaccine development, which has encouraged more academic and industrial partners to participate in diagnostic development.

Overall, the technology for the diagnosis of TB and RMP resistance in pulmonary specimens is well advanced, with high specificity and increasingly high sensitivity. Rapid, high-specificity molecular assays for TB identification and drug resistance cannot replace the standard diagnostic methods, such as microbiology, clinical and radiological assessments, and conventional DST for active TB in pulmonary (particularly sputum smear-negative) and extrapulmonary TB specimens. Implementation of all of these tools in routine laboratory practice requires the implementation of appropriate quality assurance systems.

The performance of molecular tools of extra-pulmonary specimens varies and should be considered separately for each specifi c specimen type. Evidence for the use of these assays to identify TB and detect drug resistance in TB-HIV co-infected individuals is limited. There is a need for designed studies among children, including HIV-positive children. There also remains a need to increase the sensitivity of TB detection among all patients, but especially among immunocompromised patients and children [20].

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Chapter 9

Diagnostic Evaluation of Tuberculosis

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Additional information is available at the end of the chapter

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1. Introduction

Tuberculosis is still one of the leading causes of death by infectious diseases with 2 million deaths per year and 9.2 million new cases of tuberculosis disease annually [1-3]. Besides, more than 2 milliard people are infected with latent tuberculosis infection (LTBI) [1-3]. Despite continuous effort in the prevention, monitoring and treatment of tuberculosis, the disease remains a major health problem in many countries [4-6], particularly in developing countries like Indonesia [7]. National tuberculosis programs and other programs conducted by foreign organizations still fail to eliminate the transmission and incidence of tuberculosis. Transmission is even on the rise in developing countries despite the availability of effective therapies for tuberculosis, whereas the spread and the incidence of tuberculosis in Europe and North America are under control. Several reasons may be responsible for this failure, such us the difficulty of providing adequate anti-tuberculosis medication in many developing countries due to cost issues, the emergence of multi-drug resistant (MDR) strains of *M. tuberculosis*, and the dramatically high co-incidence of tuberculosis in HIV-infected patients [2, 7]. Another important issue is delay of diagnosis due to the lack of a proper method to identify tuberculosis agents [1, 8].

Smear is the cheapest and most widely available detection method for *M. tuberculosis*. In this technique, the diagnosis of tuberculosis is based on identification of acid-fast bacilli (AFB) in a patient`s sputum [9, 10]. Many staining techniques are available for AFB smear, the most common one of which is the modified Ziehl-Neelsen stain. Unfortunately, the sensitivity and the specificity of those techniques are low due to difficulty in the identification and differentiation of the various species of *M. tuberculosis* [10]. Two studies found that the AFB smear was positive in only half of patients with subsequent culture positive for *M. tuberculosis* [9, 10]. Another worldwide available detection method is the conventional culture method on

Lowenstein-Jensen (LJ) medium. This method is the gold standard in the identification of *M. tuberculosis* and still serves as the reference method due to its high sensitivity (89%) and specificity (98%) [4, 7, 9, 10]. However, this technique requires equipments or materials that are often unavailable in resource-poor settings. In addition, this technique is time consuming; the results only can be obtained after 6–12 weeks. In addition, the incidence of other bacterial contamination on culture tends to be high [7, 11]. Even a modern culture method such as the BACTEC MGIT 960 culture system, which uses the modified Middlebrook 7H9 broth and a fluorescent signaling system, allows for earlier detection of growth, but still takes at least 10 days to give any result [9].

The goal of tuberculosis control programs is to identify and to cure as many cases as possible; therefore the critical role of early diagnosis is obvious [11]. Under-diagnosis may lead to further spread of the disease because undiagnosed patients can spread the disease unnoticeably [11]. Accurate and early diagnosis is the first important step to effective management. Several new methods for the identification of tuberculosis are available, which including serologic tests and also various molecular methods developed as a result of major advances in understanding the genetic aspects of tuberculosis [8, 9, 11]. Those detection methods can be grouped into two types First, by detection of mycobacteria or its components directly; second by measurement of immunologic responses to mycobacterium infection [9]. In this chapter we present a short review of some these promising detection methods used in the laboratory to identify tuberculosis.

2. Direct detection methods

The genus mycobacterium consists of almost 100 different species, which all appear similar on AFB staining and culture [7, 10, 12]. Many of these can be isolated from humans, although many also can be found in the environment including in animals. It is not easy, however, to distinguish between pathogen and saprophyte species. Each mycobacterium isolate must be evaluated individually regarding its potential to cause a disease; therefore identification of mycobactera is a lengthy and tedious effort. Since the introduction of nucleic acid amplification assays as diagnostic tool for mycobacteria identification, several probes/gene amplification systems for tuberculosis have been developed for rapid and specific identification of *M. tuberculosis* and other mycobacteria [12, 13]. These techniques allow for the confirmation of identity of isolates, direct detection of gene sequences from the clinical specimens and also for molecular detection of drug resistance [12]. Many previous publications have shown the sensitivity and specificity of several molecular detection assays such as BDProbeTec ET, (Becton Dickinson), COBAS AMPLICOR (Roche), Amplified *M. tuberculosis* Direct Test AMTDT (Gen Probe, USA) for identification of mycobacteria[9].

The use of nucleic-acid probe identification systems was a one step ahead in the rapid identification of mycobacterium species of *M. tuberculosis* complex, *M. avium* complex, *M.*

avium, M. intracellulare, M. kansasii, and *M. gordonae* and also other nontuberculous mycobacteria (NTM) in culture because the result can be obtained after 2 hours [10, 12]. But the sensitivity and specificity of this probe technology will only approximate 100% if there are more than 100 *mycobacteria* present in the sample, except for *M. kansasii* (87%) [12]. Thus, these probes are not sensitive enough to be used directly in clinical specimens like sputum. Also, it still needs to be confirmed by other conventional detection methods such as biochemical test and molecular tests to able to identify the species identity within the *M. tuberculosis* complex, such as for *M. microti, M. bovis, M. bovis of* BCG,, *M. canettii,* and *M. africanum* [10]. There has been extensive research to design an identification system for ribosomal RNA/DNA fingerprinting and for development of probes that targeting specific rRNA, ribosomal DNA, spacer and flanking sequences of various types of mycobacterium species including *M. tuberculosis, M. leprae, M. avium, M. gardonae,* etc [12, 13]. Those rRNA targeting probes are 10-100 fold more sensitive than DNA targeting. However, since the lowest detection limit is still around 100 organisms. it still needs more evaluation before it can be applied to clinical specimens [12].

Several techniques based on polymerase chain reaction (PCR) and isothermal amplification assay have been developed [7-10, 12]. Various researchers have described the rapid detection of *M. tuberculosis* by PCR, and many have reported a high sensitivity in detecting *M. tuberculosis* in clinical samples by means of DNA amplifications [7, 14]. Such techniques involve amplification of specific gene regions followed by hybridization with species specific primers, and also frequently followed by sequencing and or restriction fragment length polymorphism (RFLP) analysis [12]. RFLP is still most widely used in clinical microbiology laboratories due to its simplicity and lower costs than PCR Sequencing [12]. Multiplex PCR has been used to detect *M. tuberculosis* complex bacteria and other mycobacterium. This technique is based on the amplification of the most widely used specific insertion sequences IS6110 and 16S [7-9]. Based on our experience, multiplex PCR has sensitivity up to 81.62% with negative predictive value up to 79.51% [7]. Nevertheless, taking into account the "simple and economical" issue this technique is probably not suited for most of the countries with a high tuberculosis burden [11]. Other rapid molecular amplification detection method which is being used in our laboratory is multiplex PCR-reverse cross blot hybridization, which can be modified to identify multiple species of mycobacteria at one time by using a specific probe for each species. Compared to the culture and microscopic method, this technique had a sensitivity of 86.03%, negative predictive value of 82.41% and it can be applied to detect NTM [7]. The multiplex PCR reverse cross blot hybridization technique is more complicated than conventional multiplex PCR; but it can detect considerably more NTM species such as *M. avium, M. intracellulare, M. kansasii, M. fortuitum, M. chelonae, M. genavense and M. smegmatis* (Fig. 1) [7].

In term of accuracy and duration time that it needs to get a result, Raman spectroscopy is one of the most promising techniques. This vibrational spectroscopy-based detection method can detect and differentiate various molecular compositions of microorganism [15-18] and therefore is suitable to identify the species and strains of microorganism. Buijtels et al., demonstrated that Raman spectroscopy differentiated between *M. tuberculosis* with NTM with accuracy up to 100% and with 92.5% correct species identification. This

technique is also are much faster; results can be obtained within 3 hours since positive automated cultured system is obtained [18]. In view of the importance of early diagnosis to prevent further spread of tuberculosis in the community, this time efficiency is the most significant contribution of Raman spectroscopy.

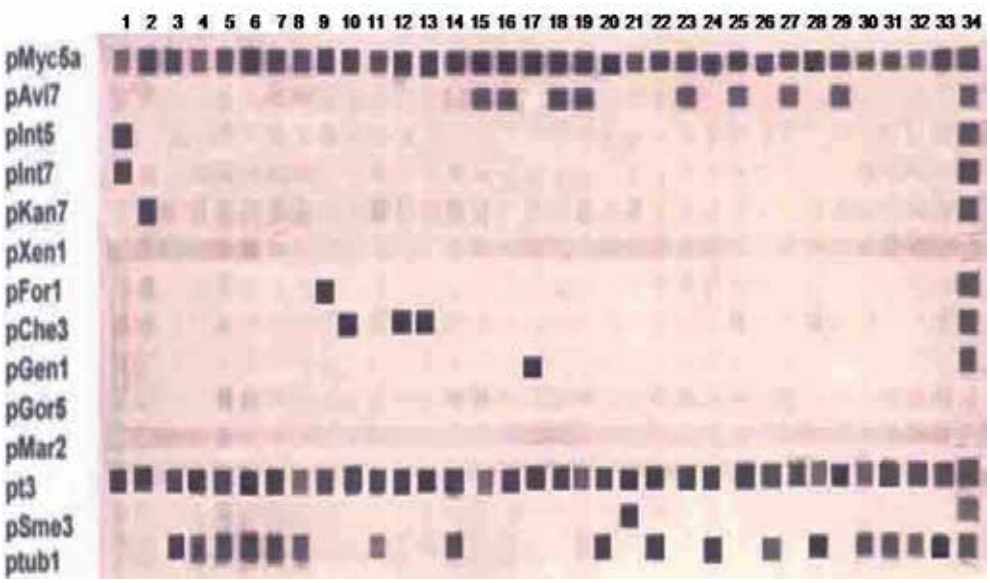

Figure 1. Multiplex PCR reverse cross blot hybridization assay is able to detect various species of mycobacteria simultaneously. Each column (Col) represents certain species of mycobacteria; Col 1, *M. intracellulare*; Col 2, *M. kansasii* ; Col 3-8, 11, 14, 20, 22, 24, 26, 28, 30-33, *M. tuberculosis*; Col 9, *M. fortuitum*; Col 10, 12, 13, *M. chelonae*; Col 15, 16, 18, 19, 23, 25, 27, 29, *M. avium*; Col 17, *M. genavense*; Col 21, *M. smegmatis* ; 34, pool PCR product of mycobacteria. [7]

3. Indirect detection methods

Even those remarkable molecular detection methods are not yet up to the mark when it comes to in the identification of tuberculosis, particularly latent tuberculosis infection (LTBI). Approximately 2 milliard people are silent tuberculosis patients, i.e. they have been infected by *M. tuberculosis* but show no tuberculosis symptoms [1, 2]. LTBI has been defined by evidence of a cellular immune response to *M. tuberculosis* derived antigens. It may be the result of incomplete elimination of *M. tuberculosis* by the host's adaptive immune system, resulting in asymptomatic infection with almost undetectable bacilli [2]. Thus, the diagnosis of LTBI currently depends on detecting the host's immune response to the infection [2]. Affected individuals have little risk

of progression from LTBI to active tuberculosis, but any disruption of their cellular immunity – such as in HIV co-infection cases – can considerably increase this risk [2]. Currently, the diagnosis of LTBI is commonly made with the tuberculosis skin test (TST), which is based on the delayed hypersensitivity to purified protein derivative (PPD). Unfortunately, patients sensitized to environmental nontuberculous mycobacteria or patients vaccinated with the bacillus Calmette–Guérin (BCG) vaccine may have a false positive result. On the other hand, a false negative result may occur in immunosuppressed patients and also in children [2]. This immunologic response is often not conclusive as antibodies and delayed type hypersensitivity response persist long after infection or after the diseases has disappeared [12]

Interferon Gamma Release Assays (IGRAs) have been introduced in the clinical setting for the diagnosis of LTBI [19-21]. These more specific whole-blood tests are based on the principle of measuring host interferon-y (IFN-y) released by T-cells specific to *M. tuberculosis* as a marker. IFN-y is stimulated by *early secretory antigen target 6* (ESAT-6) and *culture filtrate protein 10* (CFP-10). These are not present in the BCG or in the most of the NTM [2]. There are two types of IGRAs: The enzyme-linked immunospot assay (ELISpot)-based IGRA, where individual IFN-y producing T-cells responding to *M. tuberculosis* antigens stimulation are counted [22], and the QuantiFERON-TB Gold In-Tube test, an ELISA-based IGRA where the IFN-y produced by those T-cells is measured after stimulation with *M. tuberculosis* antigens [2]. Pai et al. showed that the sensitivity of the ELISpot and ELISA-based approach was around 90% and 70%, respectively, and that the specificity of both was 93% [2, 20]. As there is still no gold standard for the diagnosis of LTBI, these assays potentially may serve as routine diagnosis test other than TST to identify people with LTBI [2].

Cytokine-based detection methods could be useful not only in the detection of LTBI cases but also of active tuberculosis cases. However, considering the high number of LTBI in the community, a single cytokine identification method such as IGRAs is not sufficient to detect active tuberculosis. For this reason the identification of multiple tuberculosis biomarkers-cytokines seems to be a promising strategy. Several studies have shown the potential usefulness of TNA-a, IL-2, IP-10, MIG along with IF-g simultaneously [23-26]. Using a multiplex microbead-based assay, Wang et al. showed significant differences in expression of these cytokines/chemokines between active tuberculosis patients and healthy controls. Regarding active pulmonary tuberculosis the sensitivity of IFN-y, IP-10 and MIG was 75.3% and the specificity was 89.7%. They also demonstrated the potential usefulness of this multiplex microbead-based assay for the detection of new tuberculosis cases by documenting a sensitivity of 96.3% [23].

Untill now, smear and culture methods are still the gold standard to detect mycobacteria. Based on our experience, combination of conventional and advanced detection methods would greatly improve the sensitivity and specificity of the assays. Detection of the *mycobacteria* species are quite difficult with culture, therefore we using multiplex PCR as the first confirmation assay to detect the species while it also as confirmation test for negative results from either smear or culture assay. Hence, to overcome the limitation of multiplex PCR in species detection, multiplex PCR- reverse cross blot hybridization assay would further expand the range of *mycobacteria* species detection (Fig. 2).

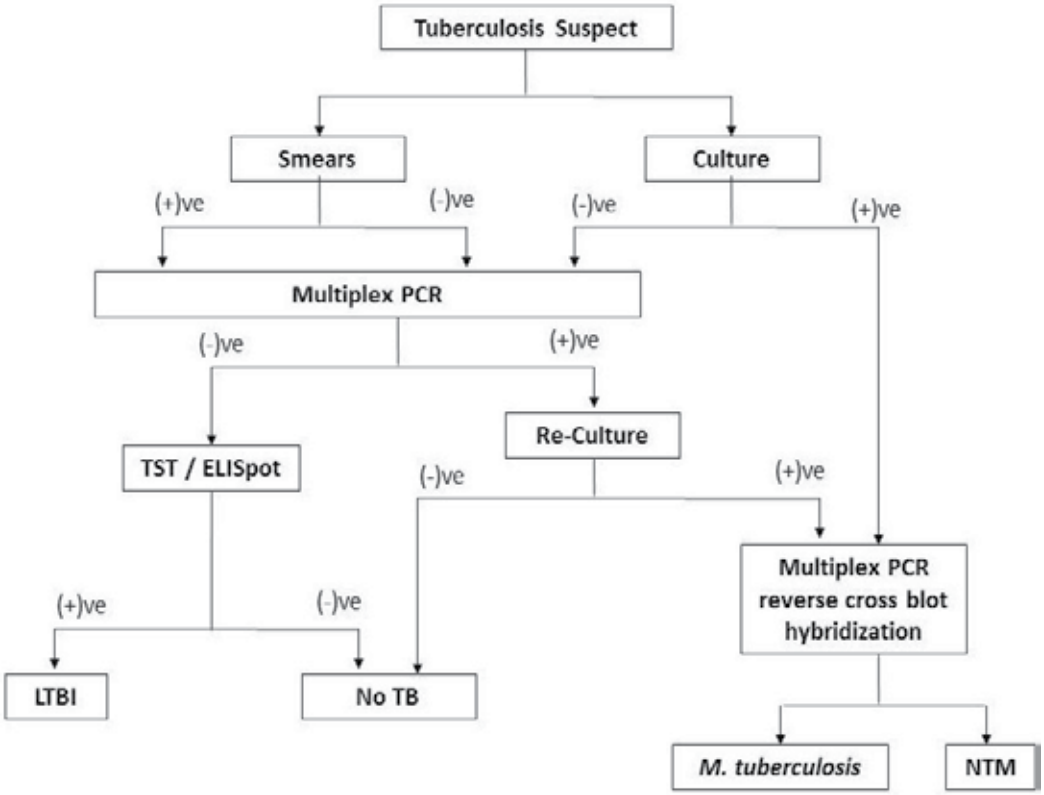

Figure 2. Microbiologic diagnosis of tuberculosis. Multiplex PCR are used to confirm smears results and negative result of culture assay. Patients with negative multiplex PCR result would be proceed for ELISpot or Tuberculin skin test (TST) to detect latent tuberculosis (LTBI), while specimen from patient with positive culture result would get final confirmation by Multiplex PCR reverse cross blot hybridization assay to further detect the mycobacterium species

4. Conclusion

Conventional methods for the diagnosis of tuberculosis, such as the smear and culture methods have some limitations, particularly the low specificity and sensitivity as well as the time-consuming nature. Now these limitations have been overcome in some novel and rapid detection methods. Various gene amplification techniques have demonstrated their usefulness in the identification of mycobacteria and its various species. The rapid detection of *M. tuberculosis* by probes, PCR or other molecular techniques and some newest serologic assays offer good opportunities to improve the diagnosis and therapy of tuberculosis [2, 7-9, 12, 13].

However despite the availability of diagnostic tools for laboratory identification of tuberculosis at high sensitivity and specificity, the "simple and economically" aspect of those new methods is still a matter of consideration. The question is whether they can be used in simple

clinical settings and whether they are economically affordable for developing countries, in most of which tuberculosis is still rampant [11].

Summary

Tuberculosis still remains a major health problem in many developing countries, despite continuous long-standing vaccination and surveillance programs, and worldwide availability of effective anti-tuberculosis drugs. Early detection is of major importance in the control of tuberculosis. The emergence of multidrug resistant *Mycobacterium tuberculosis* and the association of HIV with tuberculosis outbreaks in community both illustrate that rapid diagnosis is essential. Therefore, a fast and reliable diagnosis of tuberculosis would greatly improve the control of the tuberculosis. Regrettably, current conventional laboratory diagnostic methods of tuberculosis are still time-consuming. The rapid development of novel diagnostic methods for the identification of mycobacteria and its species bring new hope, however, for the diagnosis and management this infectious disease. Meanwhile those techniques still seem to clash with simplicity and economically affordable issues.

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Chapter 10

First– and Second–Line Drugs and Drug Resistance

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Additional information is available at the end of the chapter

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1. Introduction

Tuberculosis (TB) is caused by infection with *Mycobacterium tuberculosis*, which is transmitted through inhalation of aerosolized droplets. TB mainly attacks the lungs, but can also affect other parts of the body. TB is highly contagious during the active stage of the disease and can infect an individual through inhalation of as few as 10 *Mycobacterium tuberculosis* (MTB) bacteria. After inhalation, these bacteria are mainly captured by the alveolar macrophages, but they can evade the host immune system and remain in the dormant stage for a long period of time, at which point they can reactivate to a virulent form under immune-compromised conditions of the host. This is possible because *M. tuberculosis* can persist in slow growing as well as in fast growing stages which makes treatment challenging. Almost all of the antibiotics that can be used to treat TB work when the bacteria are actively dividing. In the intensive phase of TB treatment, the antibiotics mainly kill rapidly growing bacteria, which causes rapid sputum conversion, and the eradication of clinical symptoms. However, in order to kill the persistent or slow growing strains of MTB, the continuation phase of the treatment is essential. TB can be treated effectively by using first line drugs (FLD) isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), ethambutol (EMB) and streptomycin (SM). However, this first line therapy often fails to cure TB for several reasons. Relapse and the spread of the disease contribute to the emergence of drug resistant bacteria. The emergence of multidrug resistant TB (MDR-TB), i.e. which is resistant to at least isoniazid (INH) and rifampicin (RIF), is of great concern, because it requires the use of second-line drugs that are difficult to procure and are much more toxic and expensive than FLDs [1]. Therefore, the detection and treatment of drug susceptible or single drug resistant TB is an important strategy for preventing the emergence of MDR-TB [2]. *M. tuberculosis* strains with extensively drug resistant-TB (XDR-TB), that is resistant to either isoniazid or rifampicin (like MDR tuberculosis), any fluoroquinolone, and at least one of three second-line antituberculosis injectable drugs—*i.e.*, capreomycin, kanamycin, and amikacin have also been reported [3].

First- and second-line drugs, minimum inhibitory concentrations (MICs) and mechanisms of drug resistance are presented in Table 1 [4]. Antituberculosis drugs are mainly divided into two parts.

1. First-line antituberculosis drugs- Isoniazid (INH), rifampicin (RIF), ethambutol (EMB), pyrazinamide (PZA) and streptomycin (SM).
2. Second-line antituberculosis drugs- Sub divided into two
   - i. Fluoroquinolones- Ofloxacin (OFX), levofloxacin (LEV), moxifloxacin (MOX) and ciprofloxacin (CIP).
   - ii. Injectable antituberculosis drugs- Kanamycin (KAN), amikacin (AMK) and capreomycin (CAP).
   - iii. Less-effective second-line antituberculosis drugs- Ethionamide (ETH)/Prothionamide (PTH), Cycloserine (CS)/Terizidone, P-aminosalicylic acid (PAS).

Drug	MIC (mg/L)	Gene	Role of gene product
Isoniazid	0.02–0.2 (7H9/7H10)	*katG*	catalase/peroxidase
		inhA	enoyl reductase
		ahpC	alkyl hydroperoxide reductase
Rifampicin	0.05–0.1 (7H9/7H10)	*rpoB*	β-subunit of RNA polymerase
Pyrazinimide	16–50 (LJ)	*pncA*	PZase
Streptomycin	2–8 (7H9/7H10)	*rpsL*	S12 ribosomal protein
		rrs	16S rRNA
		gidB	7-methylguanosine methyltransferase
Ethambutol	1–5 (7H9/7H10)	*embB*	arabinosyl transferase
Fluoroquinolones	0.5–2.0 (7H9/7H10)	*gyrA/gyrB*	DNA gyrase
Kanamycin	2–4 (7H9/7H10)	*rrs*	16S rRNA
		eis	aminoglycoside acetyltransferase
Amikacin	2–4 (7H9/7H10)	*rrs*	16S rRNA
Capreomycin	2-4 (7H9/7H10)	*rrs*	16S rRNA
		tylA	rRNA methyltransferase
Ethionamide	2.5–10 (7H11)	*inhA*	enoyl reductase
p-aminosalicylic acid	0.5 (LJ)	*thyA*	thymidylate synthase A

Table 1. First- and second-line drugs, MICs and mechanisms of drug resistance

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2. First-line antituberculosis drugs

2.1. Isoniazid

Isoniazid (INH) is one of the most effective and specific antituberculosis drugs, which has been a key to treatment since its introduction in 1952 [5]. *M. tuberculosis* is highly susceptible to INH (MIC 0.02–0.2 μg/ml). INH is only active against growing tubercle bacilli, and is not active against non-replicating bacilli or under anaerobic conditions. INH enters the mycobacterial cell by passive diffusion [6]. The most significant adverse reactions associated with isoniazid administration are hepatotoxicity and neurotoxicity.

Resistance to isoniazid is a complex process. Mutations in several genes, including *katG*, *ahpC*, and *inhA*, have all been associated with isoniazid resistance. INH is a prodrug that is activated by the mycobacterial enzyme KatG [7]. INH-resistant clinical isolates of *M. tuberculosis* often lose catalase and peroxidase enzyme encoded by *kat*G [8], especially in high-level resistant strains (MIC > 5 μg/ml) [9]. Low-level resistant strains (MIC < 1 μg/ml) often still possess catalase activity [9]. Although mutations in *katG* have been shown to be responsible for INH resistance [10], it is not clear whether the regulation of *katG* expression plays a role in INH resistance. The *katG* gene encodes a bifunctional catalase-peroxidase that converts INH to its active form [7]. Activated INH inhibits the synthesis of essential mycolic acids by inactivating the NADH-dependent enoyl-acyl carrier protein reductase encoded by *inhA* [11].

A study by Hazbo´n et al. [12] analysed 240 alleles and found that mutations in *katG*, *inhA* and *ahpC* were most strongly associated with isoniazid resistance. A decrease in or total loss of catalase/peroxidase activity as a result of *katG* mutations are the most common genetic alterations associated with isoniazid resistance [7]. Ser315Thr is the most widespread *katG* mutation in clinical isolates, but there are many mutations that result in inactivation of catalase-peroxidase, with MICs ranging from 0.2 to 256 mg/L.

Mutations in *inh*A or its promoter region are usually associated with low-level resistance (MICs = 0.2 −1 μg/ml) and are less frequent than *kat*G mutations [10, 12]. INH-resistant *M. tuberculosis* harboring *inh*A mutations could have additional mutations in *kat*G, conferring higher levels of INH resistance [13]. The most common *inhA* mutation occurs in its promoter region (-15C → T) and it has been found more frequently associated with mono-resistant strains [14].

In *M. tuberculosis*, *ahpC* codes for an alkyl hydroperoxidase reductase that is implicated in resistance to reactive oxygen and reactive nitrogen intermediates. It was initially proposed that mutations in the promoter of *ahpC* could be used as surrogate markers for the detection of isoniazid resistance [15]. However, several other studies have found that an increase in the expression of *ahpC* seems to be more a compensatory mutation for the loss of catalase/peroxidase activity rather than the basis for isoniazid resistance [4, 16].

2.2. Rifampicin

Rifampicin (RIF) was introduced in 1972 as an antituberculosis drug and has excellent sterilizing activity. Rifampicin acts by binding to the β-subunit of RNA polymerase (*rpoB*) [17], the en-

zyme responsible for transcription and expression of mycobacterial genes, resulting in inhibition of the bacterial transcription activity and thereby killing the organism. An important characteristic of rifampicin is that it is active against actively growing and slowly metabolizing (non-growing) bacilli [18]. RIF produces relatively few adverse reactions. It may cause gastrointestinal upset. Hepatotoxicity occurs less frequently than with isoniazid administration.

Rifampicin MICs ranging from 0.05 to 1 μg/ml on solid or liquid media, but the MIC is higher in egg media (MIC = 2.5–10 μg/ml). Strains with MICs < 1 μg/ml in liquid or agar medium or MICs < 40 μg/ml in Lowenstein-Jensen (LJ) medium are considered RIF-susceptible. The great majority of *M. tuberculosis* clinical isolates resistant to rifampicin show mutations in the gene *rpoB* that encodes the β-subunit of RNA polymerase. This results in conformational changes that determine a low affinity for the drug and consequently the development of resistance [19]. Mutations in a 'hot-spot' region of 81 bp of *rpoB* have been found in about 96% of rifampicin-resistant *M. tuberculosis* isolates. This region, spanning codons 507–533 (numbering according to the *Escherichia coli rpoB* sequence), is also known as the rifampicin resistance-determining region (RRDR) [17]. Mutations in codons 531, 526 and 516 (Ser531Leu, His526Tyr, and Asp516Val) are the most frequently reported mutations in most of the studies [20, 21]. Some studies have also reported mutations outside of the hot-spot region of *rpoB* in rifampicin-resistant *M. tuberculosis* isolates [22].

2.3. Pyrazinamide

Pyrazinamide (PZA) is an important first-line antituberculosis (anti-TB) drug that is used in short-course chemotherapy and is one of the cornerstone drugs in the treatment of MDR-TB [23]. One key characteristic of pyrazinamide is its ability to inhibit semidormant bacilli residing in acidic environments [23]. Pyrazinamide is a structural analogue of nicotinamide and is a pro-drug that needs to be converted into its active form, pyrazinoic acid, by the enzyme pyrazinamidase/nicotinamidase (PZase) [24]. PZA is only active against *M. tuberculosis* at acid pH (e.g., 5.5) [25]. Even at acid pH (5.5), PZA activity is quite poor, with MICs in the range of 6.25–50 μg/ml [26]. Hypersensitivity reactions and gastrointestinal upset may occur with PZA administration.

PZase is encoded in *M. tuberculosis* by the gene *pncA* [27]. Mutations in the *pncA* gene may cause a reduction in PZase activity which may be the major mechanism of PZA resistance in MTB [28, 29]. The mutations of the *pncA* gene in PZA-resistant MTB isolates has been well characterized, however the correlation varies between different geographical areas including missense mutations, one or more base insertions or deletions, and complete deletion [28-32]. Despite the highly diverse and scattered distribution of *pnc*A mutations, there is some degree of clustering of mutations within different regions of the *pncA* gene such as at amino acid residues 3–17, 61–85 and 132–142 has been reported [33, 34]. The highly diverse mutation profile in the *pncA* gene observed in PZA-resistant strains is unique among drug-resistance genes in *M. tuberculosis* [28]. While the reason behind this diversity is still unclear, it is thought that this could be due to adaptive mutagenesis or due to deficiency in DNA mismatch repair mechanisms [23]. Most PZA-resistant *M. tuberculosis* strains (72–97%) have mutations in *pnc*A; [28, 29, 34, 35] however; some resistant strains do not have *pnc*A mutations.

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2.4. Ethambutol

Ethambutol (EMB) [dextro-2,2′-(ethylenediimino)di-1-butanol], which is an essential first-line drug in tuberculosis treatment, plays an important role in the chemotherapy of drug-resistant TB [36]. EMB is also an important antimycobacterial drug as it enhances the effect of other companion drugs including aminoglycosides, rifamycins and quinolones. The most common side effects observed with ethambutol are dizziness, blurred vision, color blindness, nausea, vomiting, stomach pain, loss of appetite, headache, rash, itching, breathlessness, swelling of the face, lips or eyes, numbness or tingling in the fingers or toes. Patients taking ethambutol should have their visual acuity and color vision checked at least monthly.

The MICs of EMB for *M. tuberculosis* are in the range of 0.5–2 μg/ml. EMB is a bacteriostatic agent that is active for growing bacilli and has no effect on non-replicating bacilli. EMB interferes with the biosynthesis of cell wall arabinogalactan [37]. It inhibits the polymerization of cell-wall arabinan of arabinogalactan and of lipoarabinomannan, and induces the accumulation of D-arabinofuranosyl-P-decaprenol, an intermediate in arabinan biosynthesis [38, 39].

Arabinosyl transferase, encoded *by embB*, an enzyme involved in the synthesis of arabinogalactan, has been proposed as the target of EMB in *M. tuberculosis* [40] and *M. avium* [41]. In *M. tuberculosis*, *embB* is organized into an operon with *embC* and *embA* in the order *embCAB*. *embC, embB* and *embA* share over 65% amino acid identity with each other and are predicted to encode transmembrane proteins [40]. Mutations in the *embCAB* operon, in particular *embB*, and occasionally *embC*, are responsible for resistance to EMB [40]. Point mutations of the *embABC* gene commonly occurred in *embB* codon 306 [40, 42, 43], and mutations in the *embB306* codon have been proposed as a marker for EMB resistance in diagnostic tests [44]. However, point mutations in the *embB306* locus occur in only 50 to 60% of all EMB-resistant clinical isolates [42, 45-47], and *embB306* mutations can also occur in EMB-susceptible clinical isolates [46, 47]. Five different mutations were uncovered in this codon (ATG→ GTG/CTG/ATA, ATC and ATT), resulting in three different amino acid shifts (Met→ Val, Leu, or Ile) [43]. Although the association between *embB306* mutation and ethambutol resistance or broad drug resistance has been observed in several groups' studies with either clinical or laboratorial isolates [48, 49], the exact role of *embB306* mutations play in the development of ethambutol resistance and multidrug resistance in *M. tuberculosis* is not fully understood. About 35% of EMB-resistant strains (MIC <10 μg/ml) do not have *embB* mutations [39, 45], suggesting that there may be other mechanisms of EMB resistance. Further studies are necessary to identify the potential new mechanisms of EMB resistance.

2.5. Streptomycin

Streptomycin (SM), an aminocyclitol glycoside antibiotic, was the first drug to be used in the treatment of TB, in 1948 [50]. SM kills actively growing tubercle bacilli with MICs of 2–4 μg/ml, but it is inactive against non-growing or intracellular bacilli [23]. The drug binds to the 16S rRNA, interferes with translation proofreading, and thereby inhibits protein synthesis [51, 52]. Ototoxicity and nephotoxicity are associated with SM administration. Vestibular dysfunction is more common than auditory damage. Renal toxicity occurs less frequently than with

kanamycin or capreomycin. Hearing and renal function should be monitored in patients getting SM.

Mutations associated with streptomycin resistance have been identified in the genes encoding 16S rRNA (*rrs*) [53] and ribosomal protein S12 (*rpsL*) [54-57]. Ribosomal protein S12 stabilizes the highly conserved pseudoknot structure formed by 16S rRNA [58]. Amino acid substitutions in RpsL affect the higher-order structure of 16S rRNA [51] and confer streptomycin resistance. Alterations in the 16S rRNA structure disrupt interactions between 16S rRNA and streptomycin, a process that results in resistance [59]. Mutations in *rps*L and *rrs* are the major mechanism of SM resistance [54, 56, 57], accounting for respectively about 50% and 20% of SM-resistant strains [54, 56, 57]. The most common mutation in *rps*L is a substitution in codon 43 from lysine to arginine [54, 56, 57], causing high-level resistance to SM. Mutation in codon 88 is also common [54, 56, 57]. Mutations of the *rrs* gene occur in the loops of the 16S rRNA and are clustered in two regions around nucleotides 530 and 915 [39, 54, 56, 57]. However, about 20–30% of SM-resistant strains with a low level of resistance (MIC < 32 μg/ml) do not have mutations in *rps*L or *rrs* [60], which indicates other mechanism(s) of resistance. A mutation in *gid*B, encoding a conserved 7-methylguanosine (m(7)G) methyltransferase specific for 16S rRNA, has been found to cause low-level SM resistance in 33% of resistant *M. tuberculosis* isolates [61]. A subsequent study showed that while Leu16Arg change is a polymorphism not involved in SM resistance, other mutations in *gid*B appear to be involved in low-level SM resistance [62]. In addition, some low-level SM resistance seems to be caused by increased efflux as efflux pump inhibitors caused increased sensitivity to SM, although the exact mechanism remains to be identified [62].

3. Second-line antituberculosis drugs

3.1. Fluoroquinolones

The fluoroquinolones (FQs) have broad-spectrum antimicrobial activity and so are widely used for the treatment of bacterial infections of the respiratory, gastrointestinal and urinary tracts, as well as sexually transmitted diseases and chronic osteomyelitis [63]. In contrast to many other antibiotics used to treat bacterial infections, the FQs have excellent in vitro and in vivo activity against *M. tuberculosis* [64, 65]. FQs include ciprofloxacin, ofloxacin, levofloxacin, and moxifloxacin. So, FQs are currently in use as second-line drugs in the treatment of TB. Adverse effects are relatively infrequent (0.5–10% of patients) and include gastrointestinal intolerance, rashes, dizziness, and headache. Most studies of fluoroquinolone side effects have been based on relatively short-term administration for bacterial infections, but trials have now shown the relative safety and tolerability of fluoroquinolones administered for months during TB treatment in adults.

The cellular target of FQs in *M. tuberculosis* is DNA gyrase, a type II topoisomerase consisting of two A and two B subunits encoded by *gyrA* and *gyrB* genes, respectively [66]. Mutations in a small region of *gyrA*, called quinolone resistance-determining region (QRDR) and,

less frequently, in *gyrB* are the primary mechanism of FQ resistance in *M. tuberculosis* [66, 67]. Analysis of QRDR alone by genotypic tests has been suggested as sufficient for rapid identification of vast majority of FQ-resistant *M. tuberculosis* strains as additional targeting of *gyrB* did not enhance the sensitivity significantly [67, 68].

Mutations within the QRDR of *gyr*A have been identified in clinical and laboratory-selected isolates of *M. tuberculosis*, largely clustered at codons 90, 91 and 94 [69-73], with Asp94 being relatively frequent [71, 74]. Codon 95 (Ser95Thr) contains a naturally occurring polymorphism that is not related to fluoroquinolone resistance, as it occurs in both fluoroquinolone-susceptible and fluoroquinolone-resistant strains [75]. A less common involvement is codon 88 [76]. For clinical isolates, *gyr*B mutations appear to be of much rarer occurrence [72, 73]. Generally, two mutations in *gyr*A or concomitant mutations in *gyr*A plus *gyr*B are required for the development of higher levels of resistance [69, 77].

3.2. Aminoglycosides (kanamycin, amikacin and capreomycin)

The aminoglycosides amikacin (AMK)/kanamycin (KAN) and the cyclic polypeptide capreomycin (CAP) are important injectable drugs in the treatment of multidrug-resistant tuberculosis. Although belonging to two different antibiotic families, all exert their activity at the level of protein translation. Renal toxicity occurs from these drugs. Regular monitoring of hearing and renal function is recommended.

AMK and KAN are aminoglycosides that have a high level of cross-resistance between them [78-80]. The cyclic polypeptide CAP is structurally unrelated to the aminoglycosides and thus is a potential candidate to replace AMK or KAN if resistance to either of them is suspected [81, 82]. It has been demonstrated that the risk of treatment failure and mortality increase when CAP resistance emerges among MDR-TB cases [83]. However, cross-resistance in *M. tuberculosis* between AMK/KAN and CAP has been observed in both clinical isolates and laboratory-generated mutants [79, 80, 84, 85].

AMK/KAN and CAP primarily affect protein synthesis in *M. tuberculosis* and resistance to these drugs is associated with changes in the 16S rRNA (*rrs*) [78, 80, 81, 85, 86]. The *rrs* mutation A1401G causes high-level AMK/KAN and low-level CAP resistance. C1402T is associated with CAP resistance and low-level KAN resistance. G1484T is linked to high-level AMK/KAN and CAP resistance [79, 80, 84, 86]. Low-level resistance to kanamycin has been correlated to mutations in the promoter region of the *eis* gene encoding aminoglycoside acetyltransferase, the enhanced intracellular survival protein, Eis [87].

Resistance to the cyclic peptide capreomycin has also been associated with mutations in *tlyA* [86]. The gene *tlyA* encodes a putative 2′-O-methyltransferase (TlyA) that has been suggested to methylate nucleotide C1402 in helix 44 of 16S rRNA and nucleotide C2158 in helix 69 of 23S rRNA in *M. tuberculosis* [81, 88]. Capreomycin binds to the 70S ribosome and inhibits mRNA–tRNA translocation [89]. It is believed that TlyA methylation enhances the antimicrobial activity of capreomycin [81] and that disruption of *tlyA* leads to cap-

reomycin resistance because the unmethylated ribosome is insensitive to the drug [81, 86, 88]. The identified mechanism of capreomycin resistance on the basis of in vitro selected mutants has found that *tlyA* mutations were common [80, 86] whereas infrequent in clinical isolates of *M. tuberculosis* [79, 80].

3.3. Ethionamide/prothionamide

Ethionamide (ETH, 2-ethylisonicotinamide) is a derivative of isonicotinic acid and has been used as an antituberculosis agent since 1956. The MICs of ETH for *M. tuberculosis* are 0.5–2 μg/ml in liquid medium, 2.5–10 μg/ml in 7H11 agar, and 5–20 μg/ml in LJ medium. Ethionamide and the similar drug prothionamide (PTH, 2-ethyl-4-pyridinecarbothioamide) act as prodrugs, like isoniazid. Which is activated by EtaA/EthA (a mono-oxygenase) [90, 91] and inhibits the same target as INH, the InhA of the mycolic acid synthesis pathway [92]. Once delivered into the bacterial cell, ethionamide undergoes several changes. Its sulfo group is oxidized by flavin monooxygenase, and the drug is then converted to 2-ethyl-4-aminopyridine. The intermediate products formed before 2-ethyl-4-aminopyridine seem to be toxic to mycobacteria, but their structures are unknown (may be highly unstable compounds). Mutants resistant to ethionamide are cross-resistant to prothionamide. ETH frequently causes gastrointestinal side effects, such as abdominal pain, nausea, vomiting and anorexia. It can cause hypothyroidism, particularly if it is used with *para*-aminosalicyclic acid.

3.4. *p*-Amino salicylic acid

p-Amino salicylic acid (PAS) was one of the first antibiotics to show anti-TB activity and was used to treat TB in combination with isoniazid and streptomycin [93]. Later, with the discovery of other more potent drugs including rifampicin, its use in first line regimens was discontinued. PAS is still useful as part of a treatment regimen for XDR TB although its benefit is limited and it is extremely toxic. Thymidylate synthase A, encoded by *thyA*, an enzyme involved in the biosynthesis of thymine, has been proposed recently as the target of PAS in *M. bovis* BCG [94]. Most common mutation in *thyA* was Thr202Ala, though few susceptible isolates also showed the same mutation [95]. However, its mechanism of action was never clearly elucidated. The most common adverse reactions associated with PAS are gastrointestinal disturbances.

3.5. Cycloserine

Cycloserine (CS) is an antibiotic that is used to treat TB. The exact mechanism of action of cycloserine is unknown, but it is thought to prevent the tuberculosis bacteria from making substances called peptidoglycans, which are needed to form the bacterial cell wall. This results in the weakening of bacteria's cell wall, which then kills the bacteria. Cycloserine possesses high gastric tolerance (compared with the other drugs) and lacks cross-resistance to other compounds. But it causes adverse psychiatric effects; [96, 97] which is its main drawback. So, psychiatric interrogation is necessary before prescribing cycloserine drug. Cycloserine is one of the cornerstones of treatment for MDR and XDR tuberculosis [96, 97, 98]. Terizidone (a combination of two molecules of cycloserine) might be less toxic [96, 97], although studies of this drug are scarce.

4. Conclusions

Despite all the advances made in the treatment and management, TB still remains as one of the main public health problems that have plagued mankind for millennia. The challenges posed by *M. tuberculosis* infection, through its interaction with the immune system and its mechanisms for evasion, require many more breakthroughs to make a significant impact on the worldwide tuberculosis problem. The introduction of MDR and XDR strains of *M. tuberculosis* poses several problems in mycobacterial genetics and phthisiotherapy. Among the response priorities, rapid detection of anti-tuberculosis drug resistance, use of appropriate regimens for treatment, and new drug development are of paramount importance. However, regarding the dynamics of TB transmission, and also in view of rational development of new anti-TB drugs, it is extremely important to extend our knowledge on the molecular basis of drug resistance and all its complexity. It is necessary to clarify the association between specific mutations and the development of MDR-TB or the association between drug resistance and fitness. This would allow better evaluation of the transmission dynamics of resistant strains and more accurate prediction of a future disease scenario. Adequate monitoring of drug resistance, especially MDR/XDR-TB in new patients and its transmission, molecular characterization of the drug-resistant strains, and analysis of patients' immune status and genetic susceptibility are also needed to address the problem of the fitness, virulence and transmissibility of drug-resistant *M. tuberculosis* strains.

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Section 3

Multi-Drug Resistant Tuberculosis

Chapter 11

Epidemiology of Multidrug Resistant Tuberculosis (MDR-TB)

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Additional information is available at the end of the chapter

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1. Introduction

An understanding of the epidemiology of multidrug resistant tuberculosis (MDR-TB) and the extensively drug-resistant tuberculosis (XDR-TB) is critical for effective control of the global burden of tuberculosis (TB) which is caused by the organisms belonging to the *Mycobacterium tuberculosis* complex. Epidemiology of MDR-TB and XDR-TB will be reviewed here.

The history of tuberculosis treatment has observed sequential development of resistance to anti-tuberculosis drugs over the decades. Para amino salicylic acid (PAS) and isoniazid (INH) were introduced to reduce the development of streptomycin (SM) resistance, which heralded the era of combination treatment for tuberculosis [1]. Within 20 years, resistance to both INH and SM was already a challenge in the use of INH, SM and PAS as the standard anti-tuberculosis regimen. With the discovery of rifampicin (RMP) in 1966 [2] and the expansion of its use between 1970 and 1990, patients who were already carriers of isoniazid (INH) resistant *Mycobacterium tuberculosis* strains became resistant to RMP. This was the start of a progressively growing problem, multi drug resistant tuberculosis (MDR-TB), which has reached epidemic proportions in some countries. In the last two decades, with the misuse of other drugs with anti-tuberculosis action, in particular the fluoroquinolones (FQs), the most effective among the second-line drugs, resistance has dramatically increased to extensively drug-resistant TB (XDR-TB) which is defined as resistance to at least RMP and INH (the definition of multidrug-resistant tuberculosis (MDR-TB)), in addition to any fluoroquinolone, and at least one of the three injectable anti-tuberculosis (TB) drugs capreomycin, kanamycin and amikacin [3].

1.1. Epidemiology

John Last has defined epidemiology as "The study of the distribution and determinants of health-related states or events in specified populations, and the application of this

study to the control of health problems" [4]. Epidemiologists are concerned not only with death, illness and disability, but also with more positive health states and, most importantly, with the means to improve health [5]. Epidemiological studies are classified as either observational or experimental. Various methods can be used to carry out epidemiological investigations: surveillance and descriptive studies are used to study distribution while analytical studies are used to study determinants.

The two mostly common terms used in epidemiology are the 'prevalence' and the 'incidence'. The incidence of disease represents the rate of occurrence of new cases arising in a given period in a specified population, while prevalence is the frequency of existing cases in a defined population at a given point in time [5]. These are fundamentally different ways of measuring occurrence, and the relation between incidence and prevalence varies among diseases [5]. [For a comprehensive study on epidemiology, please refer the World Health Organization (WHO) manual on Basic Epidemiology].

1.2. Epidemiology of tuberculosis

Despite the availability of highly efficacious treatment for decades, TB remains a major global health problem. In 1993, WHO declared TB a global public health emergency, at a time when an estimated 7–8 million cases and 1.3–1.6 million deaths occurred each year. In 2010, there were an estimated 8.5–9.2 million cases and 1.2–1.5 million deaths from TB [6]. According to the newest report, has observed a gradual decline in the absolute number of TB cases since 2006 and also in the incidence rates of TB since 2002 [6].

2. Global epidemiology of MDR-TB

2.1. Global epidemiology of MDR-TB (global tuberculosis control: WHO report 2011)

Globally, around 50 000 cases of MDR-TB were notified to WHO in 2010, mostly by European countries and South Africa. This represented 18% of the 290 000 (range, 210 000–380 000) cases of MDR-TB estimated to exist among patients with pulmonary TB who were notified in 2010. The proportion of TB patients estimated to have MDR-TB that were actually diagnosed was under 10% in all of the 27 high MDR-TB countries outside the European Region, with the notable exception of South Africa where 81% of estimated cases were diagnosed. In, 15 high MDR-TB burden countries in the European Region, the proportion of estimated cases that were diagnosed ranged from 24% (in Tajikistan) to over 90% of cases (in Belarus and Kazakhstan); no data were reported from Lithuania. In Russian Federation, which ranks third in terms of estimated numbers of cases of MDR-TB at the global level, the proportion of estimated cases that were diagnosed was 44% in 2010. The numbers of patients diagnosed with MDR-TB and started on treatment with recommended second-line drug regimens in the high MDR-TB burden countries in 2010, at just under 40 000, was less than the number of cases notified [6]

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2.2. Regimen surveys and definitions of patients registration groups for treatment of tuberculosis

'Regimen surveys' measure first-line and/or second-line drug resistance among a group of selected patients that cannot be considered representative of a patient population [7]. These surveys help to determine the predominant patterns of drug resistance, and are useful in providing guidance on appropriate regimens for MDR-TB treatment for particular patient groups. These include return cases after treatment failure, chronic cases and symptomatic contacts of MDR-TB cases. According to WHO, Regimen surveys should be conducted in the process of developing MDR-TB treatment programmes, or within selected centres or diagnostic units that regularly address high-risk cases.

The fourth edition of WHO *Guidelines for treatment of tuberculosis* defines patient registration groups by history of previous treatment [8]. [For a comprehensive study on definitions, please refer the document WHO/HTM/TB/2009.420].

2.2.1. New case

For the purpose of surveillance, a 'new case' is defined as a newly registered episode of TB in a patient who, in response to direct questioning denies having had any prior anti-tuberculosis treatment (for up to one month), and in countries where adequate documentation is available, for whom there is no evidence of such history. Determining the proportion of drug resistance among new cases is vital in the assessment of recent transmission.

2.2.2. Previously treated case

For the purpose of surveillance, a 'previously treated case' is defined as a newly registered episode of TB in a patient who, in response to direct questioning admits having been treated for TB for one month or more, or, in countries where adequate documentation is available, there is evidence of such history.

2.2.3. Primary resistance

Patients with TB resistant to one or more anti-tuberculosis drugs, but who have never been previously treated for TB, are said to have "primary resistance" (or "initial resistance") due to transmission of a drug-resistant strain.

2.2.4. Acquired resistance

Patients diagnosed with TB who start anti-tuberculosis treatment and subsequently acquire resistance to one or more of the drugs used during the treatment, are said to have developed "acquired resistance". In the past, resistance among previously treated cases (defined as cases with ≥ one month history of treatment) was used as a proxy for acquired resistance; however, this patient category is now known to also be comprised of patients who have been re-infected with a resistant strain, and patients who were primarily infected with a resistant strain and subsequently failed therapy or relapsed.

2.2.5. Cured

A patient who has completed a course of anti-TB treatment according to programme protocol and has at least five consecutive negative cultures from samples collected at least 30 days apart in the final 12 months of treatment. If only one positive culture is reported during that time, and there is no concomitant clinical evidence of deterioration, a patient may still be considered cured, provided that this positive culture is followed by a minimum of three consecutive negative cultures taken at least 30 days apart.

2.2.6. Failed

Anti-TB treatment will be considered to have failed if two or more of the five cultures recorded in the final 12 months of therapy are positive, or if any one of the final three cultures is positive. Treatment will also be considered to have failed if a clinical decision has been made to terminate treatment early because of poor clinical or radiological response or adverse events. These latter failures can be indicated separately in order to do sub-analysis.

3. Surveillance studies for the assessment of resistance rates and the detection of MDR-TB

MDR-TB poses a therapeutic challenge and is associated with increased mortality. Surveillance studies for the assessment of resistance rates and the detection of MDRTB are therefore crucial in order to optimize empiric drug therapy and to prevent the dissemination of resistant strains in a community [9]. The extent of the problem of MDR-TB has been examined by the WHO in cross-sectional surveys of drug resistance in either clinical series or whole-country cohorts [10]. Cross-sectional surveys almost certainly underestimate the burden and number of cases of MDR-TB because they do not take into account the numerical burden of TB in the high-burden countries [11]. When the exercise is repeated with a mathematical modeling design using drug-resistance estimates and the number of cases of TB, a more accurate picture of the global MDR-TB burden is claimed [12].

3.1. Global project of drug resistance surveillance

The WHO and the IUATLD (International Union against Tuberculosis and Lung Disease) have established a global project of drug resistance surveillance that is based on standard epidemiological methods and quality control through an extensive network of reference laboratories. The Global Project has served as a common platform for country, regional and global level evaluation of the magnitude and trends in anti-tuberculosis drug resistance quantified the growing global burden of MDR-TB and started to document the spread of XDR-TB. Since its launch in 1994, the Global Project has collected and analyzed data on drug resistance from surveys of sampled patients and from national surveillance systems from an ever increasing number of settings around the world [7].

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The review of Cohn et al, 1997 represented a comprehensive description of worldwide drug resistance surveys performed during the 1990s. According to the study, resistance to multiple drugs varied by geographic region and was more common when resistance was acquired rather than primary. The rate of multidrug resistance (and occasionally other drugs) was low in most surveys of primary resistance, ranging from 0 to 10.8% (median rate, 0.5%); however, for acquired resistance, the rate of multidrug resistance ranged from 0 to 48.0% (median rate, 12.2%). For surveys that did not distinguish between primary and acquired resistance, the range was 0.5% to 14.3% (median rate, 2.3%). In terms of antituberculous drug resistance, they found a great deal of variability between different countries, and within some countries, differences between regions or cities [13].

The review of Caminero et al of 2010 [14], broadly discuss the epidemiological data of the global report, issued in 2008. The report included drug susceptibility data from 90 726 patients in 83 countries and territories from year 2002 to 2007. The median prevalence of resistance in new cases of TB was 11.1% for any drug and 1.6% for MDR-TB. The prevalence of MDR-TB in new TB cases ranged from 0% in eight countries to 22.3% in Baku, Azerbaijan, and 19.4% in the Republic of Moldova. Of the 20 settings with the highest proportion of MDR-TB in new cases, 14 were located in countries of the former Soviet Union (between 6.8% and 22.3% in nine countries, including Moldovia and Azerbaijan) and four in China (7% in two provinces in China) [15, 16], A trend analysis of the 2008 report shows that between 1994 and 2007 the prevalence of MDR-TB in new cases (initial resistance) increased substantially in South Korea and two Russian Oblasts, Tomsk and Orel. By contrast, the prevalence remained stable in Estonia and Latvia, both of which have high rates of initial MDR-TB. The prevalence of MDR-TB in all TB cases decreased in Hong Kong and the United States [14].

Of 37 countries and territories that reported representative data on XDR-TB, five countries, all from the former Soviet Union, each reported 25 or more cases of XDR-TB, with MDR-TB prevalence ranging from 6.6% to 23.7% [15, 16], data from Eastern Mediterranean countries showed that the prevalence of initial MDR-TB was higher than previously estimated, with the exception of Morocco and Lebanon, with rates of respectively 0.5% and 1.1%. Initial MDR-TB rates in Jordan and Yemen were respectively 5.4% and 2.9%. The Americas, Central Europe and Africa reported the lowest rates of initial MDR-TB, with the notable exceptions of Peru, Rwanda and Guatemala, which reported rates of respectively 5.3%, 3.9% and 3.0%. [15, 16]. Data on previously treated cases from the WHO/ Union 2008 report were available for 66 countries and two regions of China [15]. Drug susceptibility testing (DST) results were available for 12 977 patients. Resistance to at least one anti-tuberculosis drug ranged from 0% in three European countries to 85.9% in Tashkent, Uzbekistan. The highest proportions of MDR-TB were reported in Tashkent (60.0%) and Baku, Azerbaijan (55.8%). data from Gujarat State, India, providing the first reliable descriptions of previously treated cases in India, showed 17.2% MDR-TB in this group [15].

The 2008 WHO/Union report also included a global estimation of the MDR-TB problem [14]. Based on drug resistance data from 114 countries and two regions of China reporting to this project, combined with nine other epidemiological factors, the proportion of MDR-TB among new, previously treated and combined cases was estimated for countries with no survey

information available. The estimated proportion of MDR-TB for all countries was then applied to incident TB cases (also based on indirect estimates). It was calculated that 4 89 139 (95% confidence limits [95%CL] 455 093–614 215) cases emerged in 2006, and that the global proportion of MDR-TB among all cases was 4.8% (95%CL 4.6–6.0). India, China and the Russian Federation were estimated to have the highest number of MDR-TB cases: India and China have approximately 50% of the global burden and the Russian Federation a further 7%. Twenty seven countries accounted for 86% of the world's MDRTB burden [14].

Caminero et al, divided the world into four large regions according to the influence of the three factors i.e., past and present management of TB and transmission of MDR-TB.

1. Countries with an epidemic: high prevalence and incidence of MDR-TB.

2. Countries with high MDR-TB prevalence but low or decreasing incidence.

3. Countries with low prevalence and incidence of MDR-TB and

4. Countries with low prevalence but an increasing incidence of MDR-TB.

The fourth edition of WHO *Guidelines for surveillance of drug resistance in tuberculosis* is an updated version of earlier editions published in 1994, 1997 and 2003. These guidelines incorporate the 2007 WHO *Interim recommendations for the surveillance of drug resistance in tuberculosis* and the conclusions of an Expert Committee Meeting on Anti-Tuberculosis Drug Resistance Surveys held in Geneva in September 2008. In addition experience gained from 15 years of the Global Project on Anti-Tuberculosis Drug Resistance Surveillance is also included [7].

Given below are some of the updates and clarifications in surveillance methodology that have been incorporated into the 4th edition:

1. At a minimum, surveillance should evaluate susceptibility to the following drugs:

a. Isoniazid and rifampicin;

b. If resistance is detected to rifampicin, then susceptibility to the fluoroquinolones and second- line injectable agents most often used in the setting have be tested. Testing for susceptibility to the first-line drug ethambutol should also be considered.

2. Statistical and epidemiological methodology is a fundamental aspect of designing surveys that sample patients, and appropriate technical assistance should be received in the early stages of planning. In particular, for surveys that use cluster-based sampling methods, results should be adjusted to correct for biases introduced by these sampling techniques. Missing values should also be accounted for, e.g. using multiple imputation techniques when possible.

3. MDR-TB management is a component of the Stop TB Strategy and WHO Member States have committed themselves to achieve universal access to diagnosis and treatment by 2015. Therefore, all drug resistance surveillance activities should be linked to patient treatment and care. Planning a comprehensive treatment programme for patients identified during a survey as having drug-resistant TB should run in parallel to planning the survey itself.

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3.2. MDR-TB and immigration

Gilad et al [9] assessed the incidence of TB in Southern Israel in the period between 1992 and 1997, and studied the prevalence of resistance to anti-TB drugs and its distribution among the various subpopulations inhabiting that region, with the intention of tailoring the empirical anti-TB treatment guidelines to those subpopulations. This study described the unique epidemiology of drug-resistant TB in Southern Israel, a region inhabited by both native and immigrant populations. Significant differences in age, gender, and resistance rates were found among the four distinct subpopulations inhabiting the Negev region. They attributed the observed differences to immigration from countries of high prevalence of drug-resistant TB. According to an earlier 10-year survey (1978- 1987) of TB in the Negev, Ethiopian immigrants and Bedouin Arabs comprised 76% of TB cases and 33% of them were extrapulmonary TB [17]. However the study of Gilad et al [9], recorded only 20% of isolates as extrapulmonary, and Ethiopian immigrants and Bedouin Arabs comprised only 40% of the cases. These differences demonstrate how dynamic this disease might be, tremendously influenced by immigration, and demonstrate the importance of continued surveillance in such a setup [9].

A worldwide survey of drug resistance rates by the WHO [18] demonstrated high rates of resistance among isolates in the former Soviet Union, with the highest rates detected in Latvia. The Resistance rates observed rom the study for the immigrants from the former Soviet Union (IFSU) were much higher than those encountered in the Russian republic for every drug or drug combination [9]. These rates were very similar to those found in Latvia, and were even higher overall (50% and 41.5%, respectively). In this current era, import of infectious diseases across international borders occurs much readily [9]. The studies done in Germany and Canada also had reported an increased incidence of multidrug resistance due to the immigration [19-21].

These studies have shown the impact of immigration on the incidence and distribution of drug-resistant TB in a particular country and the importance of continuous surveillance and immediate therapeutic decisions to prevent the dissemination of such resistant strains to their general populations [9].

3.3. HIV and MDR-TB

The epidemiological impact of HIV on the epidemic of drug-resistant TB is not known and may depend on several factors. HIV-positive TB cases are more likely to be smear negative. In addition, delayed diagnosis of drug resistance and unavailability of treatment (particularly in previous years) have led to high death rates in people living with HIV. Both of these factors (smear negativity and short duration of disease due to mortality) may suggest a lower rate of general transmission. However, HIV-positive cases progress more rapidly to disease and in settings where MDR-TB is prevalent (either in the general population or in the local population such as a hospital or a district), this may lead to rapid development of a pool of drug-resistant TB patients or an outbreak [7].

According to Cohn et al, 1997 [13], though the association of MDR-TB with AIDS has been well documented during outbreaks [22-24], the role of HIV infection as a risk factor for the development of drug-resistant TB in other settings was not clear [25]. In Kenya, Malawi, Tanzania, COte d'Ivoire, and France, drug resistance was not associated with HIV infection [26-30]. In contrast, in a survey of eight metropolitan areas of the United States, HIV infection was associated with resistance to antituberculous drugs, both within and outside the New York City area [31]. The acquired MDR-TB also occurs in largely immunocompetent hosts, which was seen in India, Korea, Nepal, and Bolivia [32-35].

The studies by Borrell and Gagneux [36] pointed out that, from a scientific point of view, the actual evidence for primary transmission of MDR -TB in HIV-negative individuals that has been confirmed by molecular methods is very limited, and that more studies including molecular data are needed to know the true extent of primary MDR-TB & XDR –TB in a general population.

3.4. Inadequate treatment and development of MDR and XDR-TB

Multidrug-resistant tuberculosis (MDR-TB) is a major challenge for TB control worldwide. Inadequate treatment of MDR-TB inevitably results in high mortality and the development of XDR-TB [37]. The study of Jeon et al, 2011 [38], shows how inadequate treatment has contributed to the high prevalence of MDR and XDR-TB in Korea. According to Jeon et al, the three TB referral hospitals in the public sector are responsible for the management of MDR-TB in the public sector of Korea. This study showed poor outcome for patients with MDR-TB at the 3 TB hospitals in Korea: low treatment success rate (37.1%), high default rate (37.1%), and high all-cause mortality rate (31.2 %) during the 3-4 yr after treatment initiation. Since the National Tuberculosis Program (NTP) of Korea has focused on new cases, there have been limited nationwide data about the incidence and prevalence of MDR-TB and its treatment outcomes. Treatment success rate of their study was the lowest ever reported among MDR-TB cohorts in Korea [38].

4. Molecular epidemiology of MDR-TB

4.1. Molecular epidemiology

Many different definitions of molecular epidemiology have been published and all mention the use of molecular tools, but not all explicitly mention epidemiology. Molecular epidemiology is not just molecular taxonomy, phylogeny, or population genetics but the application of these techniques to epidemiologic problems [39]. Epidemiology attempts to identify factors that determine disease distribution in time and place, as well as factors that determine disease transmission, manifestation, and progression. Further, epidemiology is always motivated by an opportunity or possibility for intervention and prevention [39]. What distinguishes molecular epidemiology is both the “molecular,” the use of the techniques of molecular biology to characterize nucleic acid- or amino acid-based content, and the “epidemiology,” the study of the distribution and determinants of disease occurrence in human populations [39].

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Molecular epidemiology makes use of the genetic diversity within strains of infectious organisms to track the transmission of these organisms in human populations and to evaluate the host and parasite -specific risk factors for disease spread.

Therefore molecular epidemiologic techniques can be incorporated into almost any epidemiologic assessment to improve exposure and outcome measures

4.2. Molecular epidemiology of TB

The molecular epidemiologic approach to studying tuberculosis epidemiology has identified several new observations that could not have been obtained by conventional epidemiologic or laboratory approaches [39]. Mycobacterial strain typing by means of molecular methods has become an important instrument for tuberculosis surveillance, control and prevention [40]. Among DNA fingerprinting methods which restriction fragment length polymorphism (RFLP) typing is the most common method used has permitted novel investigations of the epidemiology and pathogenesis of tuberculosis. The use of IS*6110*, an insertion sequence which is present in *Mycobacterium tuberculosis*, is generally considered to be the gold standard for tuberculosis molecular epidemiology studies [41], but other molecular typing techniques could be used as adjuncts in selected circumstances [42].

Spoligotyping is a technique based on the polymorphism of the direct repeat (DR) locus present in *M. tuberculosis* DNA. The DR sequences are composed of multiple 36bp copies, interspersed by short non repetitive sequences [43]. The direct-repeat locus in *M. tuberculosis* contains 10 to 50 copies of a 36-bp direct repeat, which are separated from one another by spacers that have different sequences. However, the spacer sequences between any two specific direct repeats are conserved among strains. Because strains differ in terms of the presence or absence of specific spacers, the pattern of spacers in a strain can be used for genotyping (spacer oligonucleotide typing, or "spoligotyping"). Spoligotyping has two advantages over IS*6110*-based genotyping. As small amounts of DNA are required, it can be performed on clinical samples or on strains of *M. tuberculosis* shortly after their inoculation into liquid culture. In addition the results of spoligotyping, which are expressed as positive or negative for each spacer, can be expressed in a digital format. However, spoligotyping has less power to discriminate among *M. tuberculosis* strains than does IS*6110*-based genotyping.

Mycobacterial interspersed repeat units (MIRU) genotyping categorizes the number and size of the repeats in each of 12 independent MIRUs, with the use of a polymerase-chain-reaction (PCR) assay, followed by gel electrophoresis to categorize the number and size of repeats in 12 independent loci, each of which has a unique repeated sequence. Two to eight alleles are at each of the 12 loci, yielding approximately 20 million possible combinations of alleles. The discriminatory power of MIRU genotyping is almost as great as that of IS*6110*-based genotyping. Unlike IS*6110*-based genotyping, MIRU analysis can be automated and can thus be used to evaluate large numbers of strains, yielding intrinsically digital results that can be easily catalogued on a computer data base.

The PGRS, the DR and the GTG repeated sequences have mainly been used for sub typing strains for which differentiation by IS*6110* finger printing appeared insufficient. This

is useful when *M. tuberculosis* strains contain no or lesser than six copies of IS*6110.* According to a recorded study in Sri Lanka, 68% of the isolates had less than five copies which were similar to that of other countries in the Asian region, such as India, Malaysia, Oman and Hong Kong [44].

The study by Ghebremichael et al [45] determined the transmission pattern of TB strains in Sweden. By MIRU-VNTR 31 (45%) of the 69 patients with Beijing strains were found in altogether 7 clusters (2–11 per cluster), yielding 45 different patterns. Thus the MIRU-VNTR typing, with fewer and larger clusters, was less discriminatory than IS*6110* RFLP. The two strains where a possible epidemiological linkage was established differed in one allele and thus did not cluster in MIRU-VNTR. All strains that clustered by MIRU-VNTR were identical also by RD deletions, mutT gene polymorphism and Rv3135 gene analysis, but not by spoligotyping and IS1547. Four of the IS*6110* RFLP clusters contained isolates that differed by MIRU-VNTR. The combination of MIRUVNTR with RFLP resulted in the disappearance of two clusters, and a reduction of the number of isolates in two clusters, compared to the clustering observed with IS*6110* RFLP clustering alone. In this study they found that patients with DR Beijing strains have been diagnosed for more than a decade in Sweden. The majority of the patients were foreign born, and their country of origin reflects areas where the Beijing genotype is prevalent [45].

4.3. Molecular epidemiology of MDR-TB

A study by Calver et al [46], investigated an outbreak of tuberculosis using a molecular epidemiologic approach and clinical and epidemiologic data to identify inadequacies in the implemented DOTS-plus strategy that lead to the emergence of pre–XDR TB and XDR TB in South Africa. They genotyped the drug-resistant *M. tuberculosis* isolates using molecular techniques including insertion sequence (IS) *6110* RFLP, spoligotyping and MIRU typing (12-loci format). Genotyping results indicated an on-going transmission of drug-resistant TB, and contact tracing among case-patients in the largest cluster demonstrated multiple possible points of contact. Phylogenetic analysis demonstrated stepwise evolution of drug resistance, despite stringent treatment adherence. These findings suggested that existing TB control measures in South Africa were inadequate to control the spread of drug-resistant TB in their HIV co-infected population. Diagnosis delay and inappropriate therapy facilitated disease transmission and drug resistance.

Hsu et al, 2010 [47], investigated the transmission and predominant genotypes of MDR- TB in Eastern Taiwan using both spoligotyping and MIRU-VNTR. Of the tested MDR isolates of 73 (94%) Spoligotyping, identified the Beijing strain as the predominant genotype (n = 48, 66%), followed by Haarlem H3 (n = 15, 21%), T1 (n = 3, 4%) and East-African Indian 2 MANILLA (n = 1, 1%). Six (8%) isolates did not match any spoligotype in the SpolDB4 database. Using MIRU-VNTR typing, they observed a unique pattern in 27 isolates, and 46 had clustered pattern strains (10 clusters). According to them by MIRU-VNTR they observed an isolate in cluster 9, however from spoligotyping, it had a unique pattern and therefore they did not considered it as a clustered pattern strain. By considering both spoliotyping and MIRU-VNTR into account, 28 (38.4%) isolates were judged to have a unique pattern and 45 (61.6%) were clustered pattern

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strains (classifying into 10 clusters). Assuming that there was one source case in each cluster and the rest in the cluster were due to transmission, Hsu et al, concluded that 47.9% ([45 − 10]/ 73) of the patients had MDR-TB due to recent transmission [47].

To better understand the epidemiology of MDRTB, the New York City Tuberculosis Control Program began DNA genotyping of MDRTB strains from new cases in 1995 [48]. The objectives of the study were to provide descriptive molecular epidemiology of MDRTB cases in the city during 1995–1997 and to identify predominant MDR strains present during the three years, as well as the extent and risk factors for clustering among the tested cases. Genotyping results were available for 234 patients; 153 (65.4%) were clustered, 126 (82.3%) of them in eight clusters of >4 patients. Epidemiologic links were identified for 30 (12.8%) patients; most had been exposed to patients diagnosed before the study period. From the analysis, the largest cluster observed was from the "W" strain (59 patients) representing almost 25% of the 241 MDRTB patients during the 3 years. This strain caused a well-documented multi-institutional outbreak in New York City from 1990 through 1993 [49-53]. Strain "W1", which was isolated in seven patients, is a variant of the W strain. It had an additional IS*6110* copy and was a part of the W strain outbreak [52, 53]. Forty percent (12 of 30) of the epidemiologic links in this cohort were to patients with these two strains. According to Munsiff et al [48] these strains were likely transmitted in the early 1990s when MDRTB outbreaks and tuberculosis transmission were widespread in New York.

To analyze the molecular epidemiology of *M. tuberculosis* strains at a hospital in Buenos Aires, Argentina, and mutations related to MDR and XDR-TB, Gonzalo et al [54], conducted a prospective case –control study. Spoligotyping identified predominance of the Haarlem family among the MDR TB cases (family responsible for the 1990s [55] outbreak) as well as the LAM and T families. A similar strain family distribution was reported for the French Departments of the Americas [56] and Turkey [57]. The Beijing family was seldom encountered in these areas, which is in line with recent observations in 7 countries in South America, including Argentina [58]. According to them [54] the MDR TB Haarlem2 strain appears to be more successful than other circulating MDR- TB strains and also than its susceptible counterpart (of 25 Haarlem2 strains, 20 were MDR TB).

By genotyping all isolates and combining with the mutational results, Perdigão et al [59] were able to assess the isolates' genetic relatedness and determine possible transmission events. According to their study strains belonging to family Lisboa, characterized several years ago, were responsible for the majority of the MDR-TB. Even more alarming was the high prevalence of extensive drug-resistant tuberculosis (XDR-TB) among the MDR-TB isolates, which was found to be 53%.

4.4. Transmission of MDR-TB and XDR-TB

Mathematical models predict that the future of the multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis (TB) epidemic will depend to a large extent on the transmission efficiency or relative fitness of drug-resistant *Mycobacterium tuberculosis* compared to drug-susceptible strains. Molecular epidemiological studies comparing the spread of drug-

resistant to that of drug-susceptible strains have yielded conflicting results: MDR strains can be up to 10 times more or 10 times less transmissible than pan-susceptible strains [36].

Experimental work performed with model organisms has highlighted a level of complexity in the biology of bacterial drug resistance that is generally not considered during standard epidemiological studies of TB transmission. However, much more work is needed to understand the detailed molecular mechanisms and evolutionary forces that drive drug resistance in this pathogen. Such increased knowledge will allow for better epidemiological predictions and assist in the development of new tools and strategies to fight drug resistant TB [36].

In infectious disease epidemiology, the relevant measure that reflects the reproductive fitness of a pathogen is the number of secondary cases generated; this measure is also known as the basic reproductive rate, R_0 [60]. In addition to the absolute number of secondary cases (i.e., absolute fitness), an often more useful measure is that of 'relative fitness', where the success of a particular pathogen variant is compared to the success of another. For example, the fitness of a drug-resistant bacterial strain can be expressed relative to the fitness of a drug-susceptible strain. In addition to epidemiological measures of relative fitness, differences in relative fitness can be measured experimentally [36].

The results of experimental studies performed with strains resistant to INH, SM or RMP suggested that, in clinical settings, there was a strong selection pressure for drug resistance-conferring mutations that cause minimal fitness defects [61]. Although these findings support the notion that virulence and competitive fitness assays can be predictive of the epidemiology of drug-resistant TB, they do not capture the overall complexity of the life cycle of *M. tuberculosis* [36]. Although several mechanisms of compensatory evolution have been described in other bacteria [62] little work has been done on this topic in *M. tuberculosis.*

Various molecular tools have been developed to genotype *M. tuberculosis* strains [63]. These tools have been applied to molecular epidemiological investigation of TB transmission for many years. According to the standard concept, patient isolates sharing a particular genotype or DNA 'fingerprint' can be considered epidemiologically linked and represent cases of active TB transmission (i.e., they are clustered TB cases), whereas strains with distinct or 'unique' DNA patterns are thought to reflect reactivation of latent infections. They compared molecular epidemiological fitness estimates from two previous reviews and more recent studies [60, 64]. Overall, the relative fitness estimates for MDR-TB vary dramatically, ranging from an almost 10-fold increased fitness compared to fully drug-susceptible strains found in a study from Russia [65] to about 10-fold lower fitness in Mexico [66] other studies have reported that MDR strains do not cause any secondary cases at all [67]. The reasons for this high variability in relative fitness of MDR strains have likely to do with the differences in study design and setting, differences in sample size and different methodologies and also to the variation in the quality of the TB control programmes [36]. According to Borrell and Gagneux, in addition to methodological, socio-economic and environmental factors, the variation in MDR fitness also reflects biological heterogeneity. Current epidemiological evidence for transmission of MDR- and XDR-TB, particularly compared to pan-susceptible TB, is very inconclusive. This can be partially explained by the fact that *M. tuberculosis* is more genetically diverse than is often

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appreciated [68] and because drug-resistant strains can exhibit heterogeneous fitness compared to drug-susceptible strains [36].

5. Conclusion

An understanding of the epidemiology of multidrug resistant tuberculosis (MDR-TB) and the extensively drug-resistant tuberculosis (XDR-TB) is critical for effective control of the global burden of tuberculosis (TB). For a comprehensive study on epidemiology of multidrug resistant tuberculosis (MDR-TB), please refer the reviews in the reference list.

6. Future studies

Future Studies on Epidemiology

In all epidemiological studies it is essential to have a clear definition of a case of the disease being investigated by delineating the symptoms, signs or other characteristics indicating that a person has the disease. A clear definition of an exposed person is also necessary. This definition must include all the characteristics that identify a person as being exposed to the factor in question. In the absence of clear definitions of disease and exposure, it is very difficult to interpret the data from an epidemiological study.

Future Studies on Transmission of TB

Future epidemiological studies on the transmission of drug-resistant TB should incorporate more comprehensive strain data, including specific drug resistance-conferring mutations and information on the strain genetic background. These variables, as well as their interaction, could play an important role in the transmission success of particular drug-resistant variants.

Future Studies on HIV/TB

The investigators who conduct the studies on HIV/ TB need to consider other possible risk factors for drug resistance such as demographics; prior therapy, socioeconomic status, and quality of TB control programs, etc.

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Chapter 12

Management of Drug-Resistant TB

Zakaria Hmama

Additional information is available at the end of the chapter

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1. Introduction

Although major progress has been made to reduce global incidence of drug-susceptible tuberculosis (TB), the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB over the past decade presents an unprecedented public health challenge to which countries of concern are responding far too slowly. Indeed, a recent WHO TB surveillance report indicates the highest global level of drug-resistance ever recorded, which affected disproportionately developing countries with an estimated 440,000 MDR-TB cases worldwide resulting in 150,000 deaths in 2009 [1]. Even more troubling is being the recent emergence of new strains of totally drug-resistant *M. tuberculosis* (Mtb), currently occurring in densely populated cities such as Teheran (Iran) [2] and Mumbai (India) [3]. Given that an untreated TB patient can infect up to 15 contacts in a year in overcrowded areas [4], it is highly likely that totally drug-resistant TB will continues spreading and one would worry that TB will again become an incurable disease.

While part of the increase in drug resistance can be attributed to difficulty in treating patients who are double infected with HIV, which represent about 13% of total TB cases [5], detailed field studies revealed that the emergence drug-resistant TB is clearly a direct consequence of misdiagnosis and mismanagement of drug susceptible TB, which result in only a fraction of TB patients getting correct diagnosis and appropriate therapy ([6,7] and Fig.1). In other words, "Resistance is man-made, caused by exposure to the wrong treatment, the wrong regimen, the wrong treatment duration" says TB expert Giovanni Miglio [8]. Therefore, a comprehensive approach to ensure rapid detection, proper treatment and public health measures needs to be applied globally to cure TB patients and prevent further transmission of the disease. This chapter discusses various challenges facing the management of drug resistant TB and presents the efforts of WHO and its partners for the development of strategies and guidelines for optimal TB control.

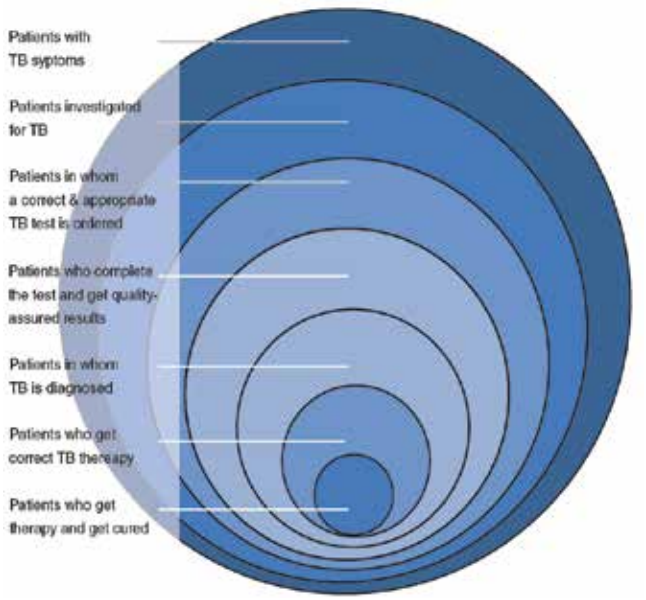

Figure 1. Misdiagnosis and mismanagement can result in only fraction of TB patients getting correct diagnosis, appropriate therapy, and positive outcomes. Reprinted from Ref. 9 with permission from Dr. Madhukar Pai.

2. Standard treatment for drug-susceptible TB

Symptoms associated with active TB are generally defined as loss of weight and energy, poor appetite, fever, a productive cough, and night sweats. Although highly suggestive of TB, such symptoms might easily be assigned to another disease. Therefore accurate diagnosis is important before initiating drug therapy. The current standard laboratory test consists on the analysis of 3 sputum specimens for acid-fast bacilli smears and culture, with nucleic acid amplification performed on at least 1 specimen [10].

In 1994, the WHO introduced the DOTS (Directly Observed Treatment, Short-course) as a major plan to control TB globally [11]. The DOTS strategy focuses on five main points of action: 1) government commitment to control TB, 2) diagnosis based on sputum-smear microscopy tests done on patients with TB symptoms, 3) direct observation short-course chemotherapy treatments, 4) a continuous supply of drugs, and 5) standardized reporting and recording of cases and treatment outcomes [11]. The standard short course (SSC) treatment recommended by WHO [12] consists of 2 months of intensive phase of daily oral administration of isoniazid (INH), rifampicin (RMP), pyrazinamide (PZA) and ethambutol (EMB) followed by 4 months continuous phase of daily INH and RMP alone.

INH is only active against growing tubercle bacilli [13]. RMP is active against both growing and stationary phase bacilli with low metabolic activity and is associated with high sterilizing activity *in vivo* [14]. PZA plays a unique role in shortening TB treatment from the previous 12

months to 6 months because it kills the persistent Mtb population in the lung [15]. EMB is active against growing Mtb but has no effect on dormant bacilli. The combination of drugs acting at different stages of the Mtb life cycle during SSC therapy has been successful in TB treatment in most endemic countries when patients adhere to a fairly strict daily regimen. SSC therapy causes minor or no side effects and is affordable, costing less than $40 for a full course of treatment. Side effects, if they occur, are manageable and usually do not result in the interruption of the treatment.

Aproximately 90% of people infected with Mtb develop an efficient immune response that successfully contains the infection but unfortunately without killing all the bacteria. Surviving bacteria persist in the lung as non-replicative (i.e. dormant) organisms [16]. In this stage of latent TB infection (LTBI), people do not exhibit TB symptoms and cannot pass the infection on to other individuals. However, in weakened immune system conditions (old age, HIV infection or therapeutic immunosupression), dormant bacteria revert into dividing organisms leading to TB reactivation [16].

LTBI is highly suspected in individuals previously exposed to those with known active TB, which would include people in hospitals, homeless shelters and prisons, or people having recently traveled to countries where TB is highly endemic. The stage of clinical latency is of surpassing importance for TB control as most cases of active TB arise from the vast reservoir of the latently infected population [17]. In fact, it estimated that the infection reactivates and cause active TB in approximately 5 to 10% of latently infected persons [18].

Purified protein derivative (PPD) skin test (also known as the Mantoux test) is the major diagnostic tool used to identify LTBI patients. A positive skin reaction to the PPD test reflects a local cellular immune response, which is interpreted as recent or remote exposure to the TB bacterium. However, despite its usefulness and simplicity, the PPD test have a low predictive value since false-positive reactions can occur as a result of previous BCG vaccination or sensitization to environmental mycobacteria [19,20,21]. In fact, the skin test uses a crude mix of Mtb antigens shared among many mycobacterial species. As a replacement for the PPD test, new interferon-gamma release assays (IGRAs) have been recently developed and shown to be more accurate for LTB diagnosis [22]. IGRAs measure *ex-vivo* production of IFN-gamma by circulating T cells in whole blood in response to more specific Mtb antigens such as ESAT6, CFP10 and TB7.7.

Although LTBI is symptom-free and non contagious, many countries have adopted its treatment in order to reduce the risk of infection progression to active TB and the spread of the disease to the general population. Six to 9 month treatment with INH alone was proven to be effective and safe [10]. Unfortunately, if LTBI results from exposure to a person with MDR- or TB XDR-TB, preventive treatment options are very limited or may not be possible. In both active and latent TB cases, it is crucial that health care providers make every effort to ensure that infected persons complete the entire course of treatment. They must explain clearly the benefit of the treatment and also possible side effects (or drug interactions). Additionally, They should identify potential barriers to the course of treatment, which will help to establish an efficient plan to ensure adherence.

Understanding the mechanisms of TB latency is crucial to development of better control strategies. Infection with Mtb occurs initially in alveolar macrophage, in which the bacteria replicate and induce cytokines that initiate the inflammatory response in the lungs, leading ultimately to the formation of granuloma [23]. Granuloma is defined as an immune structure consisting of connective tissue, lymphocytes and activated macrophages, which has a central necrotic core containing extracellular bacteria. Within the granuloma the bacterium is exposed to multiple stresses that include, among others, hypoxic, nutrient limiting, oxidative, nitrosative and acidic conditions [24,25], which trigger a genetic program controlled by the transcription factor DosR [25]. The later regulates the development of a quiescent physiological state, which maintains viability of non-dividing bacteria for extended periods of time. The granuloma contains the infection and prevents its spread to other organs [26]. However, dormant bacteria are capable of reactivation controlled by Rpf (resuscitation promoting factor) genes, which is associated with reversal of the non-replicating state into a metabolically active growing and dividing bacteria [27]. Thus, life-long immunity is not gained by a first episode of active TB disease and the disease may develop again at a later stage, either through relapse with the same strain or reinfection with a new strain.

Deciphering the molecular basis of dormancy and reactivation is therefore necessary for developing more efficient TB therapies. Adjuncts of agents that would block transitions between active growth, dormancy, and resuscitation or kill effectively dormant bacteria can significantly enhance the efficacy of current treatments for latent infection. Such agents would also shorten the treatment duration of active TB.

3. Molecular basis of Mtb resistance to SSC drugs

The frequency of spontaneous mutations that confer resistance to an individual TB drugs *in vitro* are well known and vary from 1 in 10^5 (EMB) to 1 in 10^{10} (RMP) [28].

Resistance to INH: INH is a drug precursor that is activated by Mtb catalase-peroxidase enzyme (KatG) to generate a range of highly reactive species [29]. Active INH targets essentially enoyl-acyl carrier protein reductase (InhA enzyme), which is involved in mycolic acid synthesis [29]. Resistance to INH occurs more frequently than for most anti-TB drugs, at a frequency of 1 in 10^6 bacilli *in vitro* [13]. Clinical isolates of INH-resistant Mtb often lose catalase and peroxidase activities due to KatG S315T mutation [30]. Resistance to INH can also occur through mutations in the promoter region of *inhA*, causing overexpression of InhA, or by mutations at the InhA active site, lowering InhA affinity for INH [31]. *katG* mutation can be associated with *inhA* mutations, leading to higher levels of INH resistance [32].

Resistance to RMP: RMP interferes with RNA synthesis by binding to the β subunit of mycobacterial RNA polymerase, which is encoded by *rpo*B. Mtb resistance to RMP occurs at a frequency of 10^{-7} to 10^{-8} as a result of mutations in *rpo*B. Mutations at positions 531, 526 and 516 in *rpo*B are among the most frequent (96%) in RMP-resistant strains [33].

Resistance to PZA: PZA requires conversion to its active form, pyrazinoic acid (POA), by the pyrazinamidase/nicotinamidase enzyme encoded by Mtb *pncA*, which then permeates

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through the membrane, disrupts bacterial membrane potential and affects membrane transport [34]. PZA resistance is linked to defective pyrazinamidase/nicotinamidase activity, which results from mutations that might occur at different regions (3-17, 61-85 and 132-142) of *pncA* [34]. While most PZA-resistant strains (72–97%) have *pncA* mutations, some do not have *pncA* mutations but rather express defective pyrazinamidase/nicotinamidase activity [13], which suggests possible mutations in a putative *pncA* regulatory gene, yet to be identified.

Resistance to EMB: Arabinosyl transferase, encoded by *embB*, an enzyme involved in the synthesis of cell wall arabinogalactan, has been proposed as the target of EMB in Mtb [35]. Mutation to EMB resistance occurs at a frequency of 10^{-5} [13]. The *embB* codon 306 mutation account for only 68% EMB resistant strains [36], suggesting that there may be other mechanisms of EMB resistance. Therefore, further studies are needed to identify potential new mechanisms of EMB resistance.

Because the mutations described above are unlinked, the probability of developing bacillary resistance to 4 drugs used simultaneously is unlikely. Clinical drug-resistant TB is definitely the result of genetic mutation amplification through mismanagement of the TB disease. This includes intermittent therapy due to irregular drug supply, inappropriate drug prescriptions and most importantly poor patient adherence to treatment [37]. Sequential accumulation of mutations in different genes involved in individual drug resistance results in the emergence of multiple drug resistance.

4. Diagnosis of multidrug resistant tuberculosis

Conventional culturing of the etiologic agent combined with drug susceptibility testing (DST) is the 'gold standard' for diagnosing drug resistant TB in order to initiate adequate treatment. However, this approach is rarely used because it requires 3 to 4 months to produce results. Indeed, only 7% of all MDR-TB cases are detected globally [1]. Hence, the deficiency in tools for rapid DST is associated with inadequate treatment regimens, which tragically increase transmission and spread of drug resistant TB, especially in HIV-infected individuals [38]. This alarming situation stimulated the development of a great number of rapid culture- and molecular-based methods that are currently being evaluated in TB diagnosis laboratories. The Nitrate Reductase Assay (NRA) is based on detection of nitrate reduction into nitrite by Mtb organisms capable of growth in the presence of the test antibiotic [39]. Whereas the Microscopic Observation of Drug Susceptibility (MODS) uses inverted microscope to detect the formation of cord-like structure by Mtb isolates resistant to the test drug [40]. The commercial Mycobacterium Growth Indicator Tube 960 (MGIT 960) is a drug-containing culture system based on the fluorescence detection of resistant bacteria [41]. The Genotype MTBDR*plus* is a molecular line-probe assay that detects simultaneously mutations in the rpoB gene that confers resistance to RMP as well as mutations in the katG gene and the inhA promoter, which are associated with resistance to INH [42]. The Alamar blue and resazurin assays are liquid-based colorimetric tests [43]; a color change in wells containing drug-exposed bacteria reflects resistance. The MTT assay relays on the ability of drug-resistant (viable) bacteria to cleave the tetrazolium

rings of MTT, which produces a violet-purple color [44]. Many of these assays gave excellent detection of MDR-TB, within a significantly shorter time frame when compared to conventional culturing methods (Table 1).

The effective implementation of these rapid diagnostic tests for TB and drug resistance will increase the proportion of patients promptly placed on appropriate therapy, and therefore will improve substantially management and control of TB disease globally. However a major limitation to the use of these rapid tests is their affordability and the availability of equipped laboratories in resource-constrained countries, which unfortunately tend to have the highest burden of MDR-TB cases. Thus, global initiatives are needed to make new diagnostics accessible to low-income countries.

	MTBDRplus	MODS	NRA	AB	Resazurin	MTT	MGIT 960	LJPM
Average time to results, days	2	7	7	8	8	8	9	30
Results within 8 days, %	100	90	77	87	87	74	42	-
Results within 10 days, %	100	100	100	100	97	87	81	-

MODS = microscopic observation drug susceptibility; NRA = nitrate reductase assay; AB =Alamar Bbue ; MTT = 3-[4,5-dimethyl- thiazol-2-yl)-2,5-diphenyltetrazolium bromide; MGIT = Mycobacterium Growth Indicator Tube; LJPM = Löwenstein-Jensen proportion method.

Table 1. Time to results and percentage of results obtained within 8 and 10 days. Reprinted from Ref. 45 with permission of the International Union Against Tuberculosis and Lung Disease. Copyright © The Union.

5. Treatment of drug-resistant TB

The emergence of MDR- and XDR-TB has shattered the initial optimism that DOTS based programmes would progressively eliminate TB. MDR TB is defined as resistance to at least the two most potent first-line TB drugs—ie, INH and RMP [46,47]. XDR TB strains are resistant to INH or RMP, any fluoroquinolone, and at least one of three second-line injectable drugs—ie, capreomycin, kanamycin, and amikacin [46,47]. In order to control the spread of drug resistant TB, the WHO extended the DOTS programme in 1998 to include the treatment of MDR-TB (called "DOTS-Plus") [48]. Implementation of DOTS-Plus requires the capacity to perform drug-susceptibility testing and the availability of second-line agents, in addition to all the requirements for DOTS. Clinical pilot experiences from the past few years showed that high cure rates of drug resistant TB are achieved in settings where DOTS-Plus has been established [49-51].

Resistance to INH is the most common form of TB drug resistance reported, either in isolation or in combination with other drugs [13]. INH monoresistant TB is relatively easy to treat with SCC treatment. Up to 98% cure and less than 5% relapse can be achieved when all four drugs

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INH, RMP, PZA and EMB are used during the 6-month treatment period [52]. RMP-resistant TB often carries a much more ominous prognosis, as the outcome of SCC treatment is poor in terms of both disease status at the end of the treatment and relapse [13]. Moreover, RMP monoresistance in Mtb is rare and usually reflects resistance to INH as well, i.e., MDR-TB [53]. In fact, SCC cures less than 60% of MDR-TB, with a recurrence rate of about 28% among patient with apparent success [38,54].

The current recommendation for individualized treatment regimens is a combination of at least four drugs to which the Mtb isolate is likely to be susceptible [55]. Drugs are chosen with a stepwise selection process through 5 groups of TB drugs (Table 2) on the basis of efficacy and safety [55]. More than 5 drugs can be used if the sensitivity to a given drug is unclear or if the regimen contains few bactericidal drugs. The duration of the intensive phase of treatment (when an injectable drug is given) should be at least 6 months (or 4 months after culture conversion). The continuation phase (without the injectable drug) should last until 18 months after culture conversion [55].

Although the effectiveness and feasibility of MDR-TB management in resource-limited settings have been demonstrated, less than 2% of all estimated MDR-TB patients currently receive appropriate treatment [5]. Thus, the growing MDR-TB epidemic globally requires moving beyond the pilot project stage in order to scale up DOTS-plus based TB management as a routine component of national TB control programmes. However, there are potential difficulties with implementing DOTS-Plus in low-income countries as it can absorb a large part of resources dedicated to existing DOTS programmes, and subsequently decrease the overall standard of care [56]. Note that the emergence of drug resistant TB in these countries is actually the result of limited resources to implement the simple DOTS programme.

A major barrier to the management of drug resistant TB in low-income countries is the prohibitive price of second-line drugs. Therefore in an attempt to address this issue, in 2000, the WHO and its partners established the Green Light Committee (GLC) initiative to facilitate access to quality-assured second-line TB drugs at reduced prices [57,58]. Evaluation of the first GLC-endorsed pilot projects of MDR-TB management in five resource-limited countries showed treatment success rates of 59%–83% [59]. During 2012, the number of patients with MDR-TB approved for treatment by the GLC Committee was only 42,033 with 13,000 actually starting treatment. It is clear that these numbers remain small compared to the estimated annual incidence (440,000 cases) of MDR-TB [1]. Therefore, substantial funding through public-private partnerships is desperately needed to scale up the availability of second line drugs.

Other than the price of second-line drugs, frequent adverse events and the long duration of the regimen further compromise adherence to TB treatment, even in the most advanced industrialized countries. These drawbacks have resulted in resurgence in research efforts during last decade to develop new TB drugs. In recent years, a number of new drug candidates with novel modes of action and excellent activity against Mtb have entered clinical trials [60]. OPC-67683 (nitro-dihydro-imidazooxazole) and diarylquinoline TMC207 are the most promising of these new drugs since both are highly active against drug-resistant and susceptible Mtb strains and possess excellent sterilizing activity [61]. These and other drugs under

TB drug group	Daily dose
Group one: first-line oral TB drugs (use all possible drugs)	
Isoniazid	5 mg/kg
Rifampicin	10 mg/kg
Ethambutol	15–25 mg/kg
Pyrazinamide	30 mg/kg
Group two: fluoroquinolones (use only one, because they share genetic targets)	
Ofloxacin	15 mg/kg
Levofloxacin	15 mg/kg
Moxifloxacin	7.5–10 mg/kg
Group three: injectable TB drugs (use only one, because they share very similar genetic targets)	
Streptomycin	15 mg/kg
Kanamycin	15 mg/kg
Amikacin	15 mg/kg
Capreomycin	15 mg/kg
Group four: less-effective second-line TB drugs (use all possible drugs if necessary)	
Ethionamide/Prothionamide	15 mg/kg
Cycloserine/Terizidone	15 mg/kg
P-aminosalicylic acid (acid salt)	150 mg/kg
Group five: less-effective drugs or drugs on which clinical data are sparse (use all necessary drugs if there are less ily than four from the other groups)	
Clofazimine	100 mg
Amoxicillin with clavulanate (every 12 h)	875/125 mg
Linezolid	600 mg
Imipenem (every 6 h)	500–1000 mg
Clarithromycin (every 12 h)	500 mg
High-dose isoniazid	10–15 mg/kg
Thioacetazone	150 mg

Table 2. Categories of TB drugs. Reprinted from Ref. 55 with permission of the International Union Against Tuberculosis and Lung Disease. Copyright © The Union.

development give hope that a safe and effective TB regimen of shorter duration will be available within the next few years.

6. Adverse drug reactions to second line TB drugs

The treatment of MDR-TB is a challenging issue due to the adverse events associated with long-term exposure (18 to 24 months) to second line drugs, all in great contrast to the short treatment period of drug sensitive TB. Adverse events significantly influence treatment

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outcome and patient compliance, leading to acquisition of more resistance and spread of drug-resistant strains. Initial evidence of the prevalence of adverse events associated with the use of second-line drugs was deducted from observation of patients enrolled in five DOTS-Plus sites: Estonia, Latvia, Peru, the Philippines and the Russian Federation. The data collected from these sites showed that among 818 patients enrolled on MDR-TB 30% required removal of suspected drugs from the regimen due to adverse events [62] and Table 3.

Adverse events can be distinguished as major or minor and may not be consistently found among all patients treated for MDR-TB [39]. The major adverse events associated with second line drugs include auditory toxicity (ototoxicity) and neurologic side effects [63].

Ototoxicity causes damage to the outer hair cells in the cochlea and vestibular labyrinth leading to permanent hearing loss. Ototoxic hearing loss is common in patients treated with aminoglycosides (Streptomycin, Kanamycin and Amikacin). A prospective cohort study of the incidence of ototoxicity in MDR-TB individuals (with normal hearing) showed that 57% of aminoglycoside-treated patients developed high-frequency of hearing loss [64]. The same study showed that HIV-positive patients (70%) were more likely to develop hearing loss than HIV-negative patients (42%). Susceptibility to hearing loss increases further in patients bearing mutations in mitochondrial genes [65]. Numerous mutations linked to susceptibility to ototoxicity have been identified in the mitochondrial MT-RNR1 gene that encodes the human 12S rRNA ribosomal subunit. In particular, the A1555G mutation causes increased binding of aminoglycosides to the 12S rRNA ribosomal subunit [66], which results in the disruption of mitochondrial protein synthesis and death of the cell. In this regard, a recent study in South Africa detected A1555G mutation in a significant proportion of the population (0.9% of Black and 1.1% of Afrikaner), indicative of high proportion of individuals genetically predisposed to developing aminoglycoside-induced hearing loss. It is unfortunate that the widespread and poorly controlled use of aminoglycosides will lead to many individuals suffering from permanent deafness. Auditory monitoring should be an integral part of the care programme of MDR-TB patients, particularly in countries where aminoglycosides are still commonly used. In addition, identification of patients who are genetically predisposed will significantly reduce the risk of developing ototoxicity.

Patients with neurologic side effects (depression, psychosis and suicidal tendencies) have less favorable outcome and increased risk of death. Cycloserine is the most significant TB drug associated with central nervous system (CNS) toxicity. Cycloserine is used as second line drug in TB treatment based of its structural analogy to D-alanine. Cycloserine competitively inhibits two necessary enzymes (alanine racemase and alanine ligase) that incorporate alanine into an alanyl-alanine dipeptide, an essential component of the mycobacterial cell wall [67]. Early studies revealed that neurological and psychiatric manifestations are present in as many as 33% of patients treated with cycloserine [68]. The principal side effects associated with cycloserine therapy are convulsions, exacerbations of bipolar states and multiple neurological symptoms including excitation, dizziness, headaches, insomnia and anxiety [69]. Cycloserine-mediated neurologic side effects are exacerbated even more when used in combination with isoniazid [70]. These variable psychotropic responses are related to cycloserine action as an

Adverse event*	Suspected agent(s)†	Affected *n* (%)
Nausea/vomiting	PAS, TM, FQ	268 (32.8)
Diarrhoea	PAS, TM	173 (21.1)
Arthralgia	FQ, TM, CS, AG	134 (16.4)
Dizziness/vertigo	CS, CM, AG, FQ	117 (14.3)
Hearing disturbances	CM, TM, AG	98 (12.0)
Headache	CS, FQ	96 (11.7)
Sleep disturbances	CS, FQ	95 (11.6)
Electrolyte disturbances	CM, TM	94 (11.5)
Abdominal pain	PAS, TM	88 (10.8)
Anorexia	PAS, TM	75 (9.2)
Gastritis	TM, PAS	70 (8.6)
Peripheral neuropathy	TM, AG, CS	65 (7.9)
Depression	CS	51 (6.2)
Tinnitus	CM, CS, AG	42 (5.1)
Allergic reaction	FQ	42 (5.1)
Rash	FQ, PAS	38 (4.6)
Visual disturbances	CS, TM	36 (4.4)
Seizures	CS	33 (4.0)
Hypothyroidism	TM, PAS	29 (3.5)
Psychosis	CS	28 (3.4)
Hepatitis	TM	18 (2.2)
Renal failure/nephrotoxicity	AG, CM	9 (1.2)

Table 3. Frequency of adverse events and suspected agents among 818 patients receiving MDR-TB treatment.PAS: para-aminosalicyclic acid; TM: thioamides; FQ: fluoroquinolones; CS: cycloserine; AG: aminoglycosides; CM: capreomycin.. Reprinted from Ref. 62 with permission of the International Union Against Tuberculosis and Lung Disease. Copyright © The Union.

agonist of the neuronal NMDA (*N*-methyl-D-aspartate) receptor for glutamate [71], which is a major excitatory neurotransmitter in the mammalian CNS [72]. The most dramatic effect of cycloserine reported so far is the suicide of 2 patients during the postoperative antibiotic treatment course following pulmonary resection [73]. Because of its neurological toxicities, cycloserine was prevented very early from being part of first line TB drugs but was recently reintroduced as one of the cornerstones of treatment for MDR- and XDR-TB [46]. Although co-administration of pyridoxine (vitamin B6) with cycloserine can reduce partially the neurological side effects, the later should be prescribed after psychiatric evaluation for patients with apparent convulsions and agitation [55]. Some clinicians favor terizidone (two cycloserine molecules combined) as they found the side effects associated with it are less severe and more manageable [55]. However, given the little evidence demonstrating safety and efficacy of terizidone, it should be used with caution in TB patients intolerant to cycloserine.

Although adverse events associated with second-line drugs are a major obstacle in the management of MDR-TB, compared with first line treatment, DOTS-Plus programmes have achieved cure rates of greater than 70% even in resource-poor settings [74,75]. In general, the

main adverse effects of anti-TB drugs occur during the first two to three weeks of treatment. If they are recognized in time and managed properly, high rates of treatment completion and cure can be achieved. Proper monitoring should include patient education, clinical examination and appropriate laboratory tests. Special training for staff on the various adverse events associated with second line drugs is essential for successful management. In particular, staff should consider altering dosages when appropriate, supplementary drugs to treat adverse events and replacement of drugs when toxicity cannot be managed.

7. Management approaches for the contacts of MDR TB patients

MDR-TB and XDR-TB cases are currently on the increase and it is expected that the number of their contacts will also increase, especially in densely populated area. Therefore, identification and proper management of these contacts are major components of drug resistant TB containment. In this regard, WHO recommend the identification of all close contacts of MDR-TB cases through contact tracing and their evaluation for TB infection.

A contact is defined as an individual who has a risk of acquiring TB because it has been exposed to Mtb by sharing air space with a person with infectious TB (the source case). The index case (a person with suspected or confirmed TB disease) is defined as the initial case of TB for a contact investigation [76]. He is not necessarily identical with the source case [76]. Many guidance documents focus on the source case and not the index case, as it is the source case who will have exposed the contacts, not necessarily the index case. Close contacts are those people sharing common habitation rooms with the source case. This can also include individuals with evidence of prolonged and frequent exposure to a source case in the workplace, school, prison, hospital ward, or social settings [77]. Contact tracing is defined as the systematic finding of contacts of patients with infectious TB disease [77]. The tracing helps identifying individuals who are particularly at high risk, such as individuals with HIV infection, young children and elderly.

The management of contacts of drug-resistant TB patients, in term of preventive chemoprophylaxis, remains a complex issue with a significant ethical dimension. In case of drug-susceptible TB, the provision of preventive INH therapy to suspect LTBI individuals is effective at reducing the risk of developing disease among infected contacts [10]. In theory, such a preventive approach should also work for LTBI individuals exposed to MDR and XDR Mtb strains. Unfortunately, health care providers cannot predict with certainty the susceptibility pattern of a contact's isolate from the source case's isolate. Indeed, many divergent drug susceptibility test profiles in source-contact pairs have been reported [78,79], due either to infection of the contact by another source case or to infection before the source case acquires resistance. Such a scenario likely occurs in high-burden TB areas where different drug resistant strains may circulate in homes, schools, and work places. Therefore, the lack of effective drugs with acceptable adverse-event profile in an otherwise healthy individual is a prominent barrier to the treatment of drug resistant TB contacts. Indeed, if, to some extent, the occurrence of toxicity is accepted by MDR-TB patients (since the alternative is high risk of death), convincing healthy contacts to cope with adverse-events during preventive therapy in is fundamentally different.

Given the lack of clear evidence in support of preventive therapy, the WHO does not recommend universal use of second-line drugs for chemoprophylaxis in MDR-TB contacts. Current guidance for the management of drug resistant TB contacts are largely based on expert opinions, which do not reject nor support provision of preventive therapy with the currently available drugs. In this context a guidance document presenting the most up-to-date evidence and expert opinion regarding the management of contacts of MDR- and XDR-TB patients has been recently proposed (March 2012) by the European Centre for Disease Prevention and Control (ECDC) [76]. Box 1 summarizes key recommendations provided by ECDC document.

Which factors should be evaluated to decide whether to provide preventive therapy to MDR TB contacts considered to have LTBI?

When evaluating an MDR TB contact and deciding between the two options (to provide preventive therapy and/or careful clinical observation and information), an overall individual risk assessment should be conducted, taking into consideration the following: the MDR TB contact's risk for progression to TB disease; the drug susceptibility pattern of the source case of infection; and the contact's risk for adverse drug events if initiating preventive therapy.

Are there any specific risk groups to whom special attention should be paid?

Children below the age of five years and immunocompromised persons in close contact with MDR TB patients and considered to have LTBI are at particular risk of progressing to TB disease. These risk groups might benefit from preventive therapy. The preventive therapy may be interrupted if, based on further examination, infection is found to be unlikely.

Persons over five years of age in close contact with MDR TB patients and considered to have LTBI could also be considered for preventive therapy if the individual risk assessment indicates this course of action.

If the decision is made to put an individual on preventive therapy, the selection of the drugs should be based on:

the drug susceptibility pattern of the source case's likely infecting strain;

local patterns of drug resistance;

the potential adverse events in individual patients, taking into account age and other risk factors;

the selection of single or multiple drugs and the duration of treatment will depend on the availability of drugs with bactericidal activity for the particular infecting strain; alternatively, the decision can follow national guidelines.

Which arrangements should be in place if preventive therapy is considered?

If preventive therapy is considered by the expert physician or other healthcare provider, national legislation should ensure that the treatment costs for the patient are covered.

If preventive therapy is considered to be relevant for a particular individual, careful clinical monitoring and follow-up is essential for the detection of drug-adverse events and signs of TB disease if the preventive therapy is not effective.

Specific opinions for XDR TB contacts

As the currently available treatment options are very limited for XDR TB, it is likely that the risks of preventive therapy outweigh the benefits for contacts of XDR TB patients. Thus, the option to inform and observe the contacts will be preferable, given the currently available drugs and evidence.

How should health authorities conduct follow-ups for MDR TB and XDR TB contacts suspected to have LTBI?

All MDR TB and XDR TB contacts considered to have LTBI who, after a comprehensive individual risk assessment, are not given preventive therapy should be followed-up by careful clinical observation.

Follow-ups should be performed according to existing national guidelines.

All persons in contact with MDR TB or XDR TB (after exclusion of TB disease) should be informed about the risks and symptoms, carefully observed, and provided with easy access to a specialized TB clinic in case of symptoms between

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assessments. No specific time period for follow-up or periodicity of clinical assessments is recommended, but regular systematic, clinical observation is essential for the early detection of TB disease.
Individuals repeatedly in contact with infectious MDR TB or XDR TB cases (e.g. healthcare workers) should be re-examined periodically.

Box 1. ECDC expert opinions regarding preventive therapy of MDR- and XDR-TB contacts.

Studies conducted so far on the benefits and adverse events of preventive therapy are not conclusive in term of optimal treatment and duration for preventive treatment of MDR-TB contacts [80]. Therefore, well-designed randomized clinical trials for preventive therapy are urgently needed in settings where MDR-TB therapy and a strong national programme infrastructure are already in place. Further research is also needed to define the most effective contact-tracing procedures for contacts and the most effective follow-up procedures in healthcare workers constantly exposed to drug resistant TB. In addition, specific management approaches need to be established for children below the age of five years, children with HIV infection, immunocompromised individuals, pregnant women, and the elderly. Finally, whether MDR Mtb strains are more or less infectious and/or transmissible than drug-susceptible strains need to be clarified.

8. Compliance issues from patients and health care providers

The treatment for MDR-TB is long and complex and relies on a handful of antibiotics with uncertain efficacy. The WHO has launched an 8-point plan to ensure optimal management of XDR-TB patients with currently available drugs (Box 2). However, guidelines do not always translate easily into real world practice. In addition to directly observed treatment, what support can be offered to convince a patient to continue painful treatment? And how should patients who have exhausted all treatment options with existing second-line drugs be cared for?

1. Strengthen quality of basic TB and HIV/AIDS control
2. Scale up programmatic management of MDR-TB and XDR-TB
3. Strengthen laboratory services
4. Expand MDR-TB and XDR-TB surveillance
5. Develop and implement infection control measures
6. Strengthen advocacy, communication and social mobilization
7. Pursue resource mobilization at all levels
8. Promote research and development of new tools

Box 2. WHO 8-point plan

In many cases, MDR-TB treatment results in poor compliance with subsequent development of further drug resistance (i.e. XDR-TB), which leaves infected patients, namely HIV positive individuals, virtually untreatable using currently available drugs. The WHO defines a

defaulter as being off drugs for more than 8 weeks after completing at least one month of treatment [81]. It is an operational definition to guide physicians in the decision of using a retreatment or second line regimen if the patient comes back to the health facility after defaulting. However it is imperative that health providers understand predictive factors for treatment default so that they can implement additional measures to target the population at risk. In this context, a recent review (2010) assessed TB treatment compliance and the factors predictive for poor adherence based on the analysis of 4 studies performed in Sub-Saharan Africa in the last 10 years [82]. The review revealed a high proportion of patients defaulting, which varied between 11.3% and 29.6%. Defaulting appears to be associated with many factors such as distance from the hospital, not being on the first course of TB medications, lack of repeated smears, drug-associated side effects, transportation difficulties, absence of family support and poor knowledge about TB disease and its treatment. Thus it is unfortunate that health care institutions continue to blame vulnerable and powerless patients who are unable, for this multitude of reasons, to comply with the treatment. Since distance from health care centers is a major factor, national programmes should at least consider making drugs more widely available, by either providing TB treatment in smaller health centers, or organizing mobile TB clinics, especially in rural areas.

It is time to admit that TB disease is not a 'patient problem' by default but rather a social and community responsibility that requires close cooperation and collaboration at all levels of the health care system. Forcing a patient to continue an ineffective, toxic regimen that results in uncertain outcome also raises an ethical issue yet to be resolved. Ereqat and colleagues reported a recent case of a MDR-TB patient who withdrew from treatment after 2 years while still sputum-positive [83]. Due to persistent efforts to force compliance, the patient disappeared carrying with him the potential to infect all people with whom he has contact. The authors suggest consulting with legal practitioners about the legality of enforced treatment and how patients who refuse or interrupt treatment can be managed to protect them and their potential contacts. It is obvious that in the absence of alternative treatment, this approach might end up with a response to TB without medication (i.e incarceration). For this type of recalcitrant patients, other TB specialists [84,85] propose directing efforts towards exploring possible regimens with better chances of cure and securing an appropriate living environment. Indeed, the threat of incarceration will just encourage patients to disappear and propagate the disease. Providing supportive accommodation with access to counseling and palliative care, when required, should reduce the risk of transmission to others [84]. Overall, until newer drugs become available, management that balances the risk of disease spread with individual human rights is likely to be more humane and less costly to health services compared with involuntary detention [85]. In this context, Upshur and Colleagues propose a list of additional considerations to the management of drug resistant TB as moral correlates to the current WHO 8-point plan (Box 3).

A paramount issue in TB management is that in many countries with limited resources most of the healthcare is provided by the private sector where the number of qualified medical personnel to prevent and treat drug resistant TB remains very limited. In this regard, the World Medical Association (WMA, http://www.wma.net) revealed that many doctors are no longer being taught to diagnose and treat TB. Thus, private physicians make frequent errors in dealing

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1. Adherence research
2. Building the evidence-base for infection control practices
3. Supporting communities
4. Enhancing public health response while addressing the social determinants of health
5. Embracing palliative care
6. Advocacy for research

Box 3. Additional considerations to the WHO 8-point plan

with TB cases. They prescribe too few drugs or the wrong drugs, give inadequate doses of drugs, or prescribe an inadequate duration of treatment [86]. The standardized method of determining cure is based on bacteriologic laboratory testing for the growth of Mtb on culture media. However many health care providers rely on clinical observation to determine treatment outcome [87], either because of shortages in equipment and adequate infrastructure or because they trust their own observation above test results. Such mismanagement is a major cause of acquired drug resistance and treatment failure. On the other hand, on many occasions lung cancer was misdiagnosed and treated as sputum negative TB, a medical error due to high TB prevalence and radiological similarities [88].

It is therefore important that healthcare personnel at the forefront in the fight against TB acquire appropriate and state-of-the art training on TB management. In this regards, the WMA launched in its website a new online refresher course for care providers in many languages. The course provides basic clinical care information for TB including the latest diagnostics, treatment and information about multidrug-resistant TB. It also provides information on how to ensure patient adherence and infection control and includes many aspects of TB care and management. Dr. Julia Seyer, medical adviser at the WMA, said: 'When we started an online MDR-TB training course in 2006, we discovered that many physicians were missing the most basic knowledge about normal TB'.

In summary, both the lack of patient adherence to treatment and deficiencies in programme managements are compromising the effectiveness of MDR TB treatment and the interaction of these two issues raises further the barrier to achieving efficient TB control. From the various opinions on the issue of non-compliance it can be concluded that:

- Addressing therapy-related adverse events should contribute positively in improving patient's compliance. Therefore, potential adverse effects must be carefully evaluated when designing the therapy plan. Alternative plans should be discussed with the patients to minimize the possibility of therapeutic barriers.
- Healthcare system quality is significantly related to compliance. Long waiting times and unhappy experiences during clinic visits are frequent complains from TB patients. A healthcare system that considers patient satisfaction would enhance patient adherence to TB treatment.
- Compliance is also affected by the characteristics of TB disease. While non-adherence is not a major issue when treating short duration infections, this is not the case for TB, a chronic

disease by definition. Therefore, special effort should be made to explain the nature of the disease with a particular focus on the asymptomatic stage of TB.

- Healthcare expenditures are a very important factor that affects compliance. TB patients often feel that the cost of long-term treatment would be a financial burden, which definitely threatens therapy compliance. Therefore, health care personnel should discuss the patient's resources and help identifying sources that might provide financial assistance to low-income patients.

Overall providing care centered on patient needs and expectations is a key component for the success of TB control programmes.

9. Programmatic management of drug resistant TB

Policies, strategies, protocols and guidelines for TB management are well explained and articulated on the paper. However, their implementation in resource-limited sittings remains challenging due to weak case finding strategies, unclear patient tracing mechanisms (especially defaulters), a lack of MDR-TB rapid diagnostic tools, absence of childhood TB case finding approaches and inadequate patient support services. Furthermore, specialized drug resistant TB services are limited to locations that often exclude many patients living in remote areas from receiving adequate health care. Recording systems, mainly paper-based, are time consuming for health care providers, and therefore reduces time for quality care. Addressing these many challenges requires collaboration from different components of national TB control programmes. These components include case detection, treatment, prevention, surveillance, and adequate monitoring/evaluation of the programme's performance. Such objectives are the backbone of DOTS-Plus management programme introduced by the WHO in 1998 [48]. Many DOTS-plus pilot projects provided evidence base for this strategy in the management of drug resistant TB [59,89,90]. Based on the success of pilot projects, the WHO issued in 2006 guidelines for what is now called programmatic management of drug-resistant TB (PMDT), which were recently updated with extra focus on the detection and treatment of drug-resistant TB in resources limited settings [91]. Priority topics identified in the new WHO document are:

- Case-finding through use of rapid molecular tests; investigation of contacts and other high-risk groups;
- Regimens for MDR-TB and their duration in HIV-positive and HIV-negative patients;
- Monitoring during treatment;
- Models of care.

PMDT is currently highly supported by funding for MDR-TB treatment, which has dramatically increased in the past few years. Multi-billion dollar funds are now available through governments and donors, including the Global Fund to Fight AIDS, Tuberculosis and Malaria, UNITAID and the GLC committee [90]. Such fund was essential to demonstrate the feasibility and effectiveness of PMDT in many resource-limited settings, where it is needed most [92].

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The current status of MDR-TB epidemic requires urgent moving of PMDT beyond this pilot project stage in order to respond to the call of Stop TB for the treatment of 1.6 million MDR-TB patients in the near future [93].

Although the last WHO document on TB control provides comprehensive guidelines for good PMDT, The WHO recognizes that many crucial management issues remain to be addressed. Thus, during the development of the recent PMDT document, a review published in 2008 [92] revealed some important gaps in knowledge that need to be addressed in order to optimize PMDT:

- Lack of high quality evidence from randomized controlled trials for optimizing treatment regimens in patients with MDR-TB, including the best combination of drugs and treatment duration;
- Very limited information about treatment and management of pediatric MDR-TB;
- Identification of the most effective chemoprophylaxis for contacts of MDR-TB cases;
- The therapy for symptomatic relief from adverse reactions linked to second-line TB drugs.

It is also important to note that social stigma and discrimination are still major obstacles for access to TB care services in many countries [94,95]. Similarly, financial issues and geographical accessibility is also a barrier for the continuation of treatment [96,97]. Misconceptions about TB are highly prevalent, which discourages seeking help in time or encourage those with TB to seek help from traditional healers [98]. Therefore national TB programmes must also include specific strategies to combat these issues in order to optimize the implementation of good PMDT.

Diversity in the epidemiology of MDR-TB poses a challenge for its management in various settings [99]. Ideally, TB management approaches need to be adapted to each particular setting. However it is possible to build a minimal package that could be adapted to specific countries wishing to implement proper TB management approaches. Accordingly, in 2003 a Stop TB Working Group on DOTS-Plus for MDR-TB identified key research questions to be answered in order to scale up the management of all forms of drug-resistant TB and to maximize its public health impact [99]. The working group felt that evidence is needed to address the following questions:

- How can regimens be selected (either at the programme or at the individual patient level) based on standardized and reproducible DST that adequately reflects *in vivo* responsiveness to treatment?
- How can setting specific treatment strategies be optimized with respect to effectiveness, complexity (dosing, eligibility, duration, and monitoring of outcome and side effects), safety, adherence, and affordability?
- What is the minimum infrastructure needed to scale-up PMDT, in terms of:
  - laboratory and treatment provision
  - efficient and equitable patient selection
  - prevention of transmission to other patients and health care workers

- How should infected contacts of DR-TB patients be managed?

Ensuring that these research questions are addressed is the responsibility of all parties involved in the management of MDR-TB. If adequately addressed, they will generate a solid evidence-base to support existing WHO guidelines.

The integration of PMDT into existing TB control programmes beyond the limited pilot project phase has become a critical emergency in order to respond to the increasing spread of MDR/XDR-TB worldwide. However, analysis of current WHO strategies by many experts in the field of TB management [100] suggest that successful PMDT will still require:

- New and improved tools for drug resistance testing;
- Clinical trials to test the efficacy and effectiveness of simplified and shorter second-line treatment regimens as well as of candidate second-line drugs;
- New and improved strategies for identifying patients with drug-resistant disease, promoting treatment adherence, and improving infection control;
- Better epidemiological data to explain geographic variations in occurrence of drug resistance and to identify the greatest contributors to development of drug resistance in specific settings;
- Clinical trials to test the efficacy and effectiveness of new regimens for prophylactic treatment of contacts of patients with DR-TB.

10. Patient centered care approach

Acquiring adequate care for TB is becoming increasingly complex and costly when patients are infected with drug resistant Mtb. This problem is further amplified when patient access to health care centers is limited and adequate patient-clinician relationships are absent. To address this issue Stop TB included patient centered approach as one of its important underlying principles. It insists on respect for patients right as individuals and as partners in TB care and control. Therefore what are usually characterized as 'patient problems' need to be reconfigured into solidarity with patients and programmatic challenges. Patient-centeredness can be traced back to the adoption of the right to health as part of the International Human right declaration in 1948 [101], but it is only in the 1990s, as result of the community reaction to the AIDS epidemic, that its importance has been perceived. Since then, the rise in prevalence of all forms of TB in HIV individuals has become one of the major stimulating factors for implementation of patient-centered approaches in TB care.

Promoting patient-centered case management involves assessing each TB patient's needs and identifying a treatment plan that ensures the completion of therapy. Policies and guidelines for patient-centered approaches are currently widely distributed. Unfortunately, their application in the field is progressing very slowly. Applying a patient-centered approach takes time and requires effort at many levels. It is a new way of thinking, teaching, providing care,

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prevention and communication [102]. Therefore, many countries will continue to experience substantial difficulties in treating drug resistant TB patients at high risk of defaulting.

A systematic review of factors that contribute to non-adherence [103] indicate that many social and economic barriers prevent patients from successfully completing their treatment. A wide range of interacting factors impact on the patient behavior, which is subject to changes during the course of treatment. According to Doctor Without Borders [104], one of the major challenges faced by drug resistant TB patients is the long and arduous treatment period, which can involve large numbers of tablets each day, as well as injectables, both expensive and not always easily available or accessible. Low-income patients living in remote area often struggle to reach specialized TB clinics, in particular when the harsh winter weather affect severely the country's road network and compromises the transportation system. Therefore, in the absence of efficient patient-centered approaches, it is almost impossible to convince patients to continue this forceful effort every day for up to two years.

In the Russian Federation, the proportion of MDR-TB patients defaulting from treatment has increased from 12% in 2001 to almost 30% in 2004 [105], despite the expansion of the DOTS programme to include the treatment of drug resistant TB in 2000. This failure to control drug resistant TB was mainly attributed to the absence of patient-centered strategies adapted to many risk factors for non-adherence such as poverty, unemployment, homelessness, alcoholism, drug abuse and mental illness, to name a few [105]. In response to the alarming proportion of defaulters, a novel patient-centered TB treatment delivery programme (Sputnik) was introduced in Tomsk City [106]. Sputnik care providers accompanied patients through treatment by remaining responsible for patients from the time of enrollment in the programme until the end of treatment (Box 4). The programme paid a particular attention to care giving. In addition to clinical preparation, nurses received a comprehensive training on how to care for patients facing a myriad of biosocial challenges. Indeed, a review of the emotional support that nurses provide to patients living with MTR-TB [107] concluded that nursing of TB patients could be improved with an integrated approach where the nurses are responsible for treating not only the patient, but also ambient factors that affect health, such as family and community.

A high nurse-to patient ratio (2:15),
More staff time per patient to facilitate bonding and defaulter searching
Provision of portable phones to nurses, which increases flexibility
Easier access to specialists
Expanded social and psychological support, which included clothing and assistance with procuring documentation required to access social services

Box 4. Sputnik programme package

The application of the Sputnik programme led to a mean adherence of 79.0% and a cure rate of 71.1%, indicating that adapted patient-centered approaches contributed significantly to improving TB patient adherence.

In 1998, the Centers for Disease Control and Prevention (CDC) and the Institute of Medicine of the National Academy of Sciences conducted a study to determine if TB eradication in the United State was feasible. The resulting report "*Ending Neglect: The Elimination of Tuberculosis in the United States*" concluded that TB elimination would require "aggressive and decisive action beyond what was in effect." One of the top objectives of the new CDC plan is to ensure that patient-centered case management and monitoring of treatment outcomes are the standard of care for all TB patients [108]. In particular, the CDC guidelines recommend all patients with active TB must be tested for HIV infection and that all patients double infected with Mtb and HIV infection must be appropriately and adequately treated. To ensure adherence to treatment, the CDC recommend the inclusion of multiple enablers (e.g., transportation vouchers and housing for the homeless) and incentives that will motivate the patient (e.g., food coupons), and other treatment enhancers such as alternative treatment delivery sites, and strategies to overcome social and cultural barriers.

Addressing social and economic barriers definitely increases patient access to adequate TB care. However, health education would also strengthen patient-centered approaches. Understanding an illness and how it affects ones life, as well as available treatment options, are necessary for a patient and community to take an active role in TB management. With input from community health professionals from several countries, a literacy tool kit "Within Our Reach: A TB Literacy Toolkit" was developed in 2009 for health educators, outreach workers, counselors, and supervisors who provide services to TB patients [109] and (Box 5).

Increasing knowledge about TB, the link between TB and HIV, TB treatment, and TB transmission
Raising awareness that TB is a serious but treatable disease
Giving patients confidence that they can complete TB treatment and be cured
Educating caregivers and families about how to support TB patients
Reducing stigma attached to TB and HIV

Box 5. Supporting objectives of the TB Literacy Toolkit

The tools are designed to educate TB and HIV patients, their caregivers, and their communities about TB and what it takes to complete a full course of TB treatment. The kit developers suggest that individual sessions should be conducted with flipcharts between the health provider and patient, and videos should be played in a waiting area or during community education events.

Patient-centeredness has become a central approach towards realizing universal access for all patients to efficient TB care. However scaling up this approach is progressing slowly in high TB-burden countries and is mainly challenged by the socio-economic determinants of knowledge and attitudes about TB among health care providers and the general population. Therefore optimal patient-centeredness approach requires collaborative efforts between all organizations serving TB patients to ensure that health care providers, policy makers, community leaders and the public are knowledgeable about TB disease.

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11. Drug resistant TB infection (tansmission) in the community and hospitals

TB is a highly contagious disease, acquired mainly through inhalation of airborne aerosols. Infection can occur by inhaling as few as 5-10 living bacteria. People with active TB infection spread the bacterium not only by coughing and sneezing but also by spitting, speaking, singing or laughing. The infectiousness of a TB patient is directly related to the number of droplet nuclei carrying Mtb that are expelled into the air. These droplets rapidly evaporated to form tiny particle nuclei, which could remain airborne for several days [110]. Given this mode of propagation, a person with active TB can spread the germs to up to 15 people in a year, if left untreated [4]. Therefore the process of TB spread also needs to be controlled in order to successfully combat the TB epidemic. In this regards, Nardell recommend the term "transmission control of TB" instead of "infection control of TB", since a third of the world's population is already infected with TB, a situation that appear to be stationary [111].

Because MDR strains carry mutations in major metabolic activities, in particular INH resistant strains lacking catalase activity [112], some researchers have suggested that they may be less virulent and less transmissible [113]. Contrasting with this hypothesis, the epidemic that occurred in New York City in the 1990s [114,115], which affected mainly HIV-infected persons, proved that MDR strains are highly virulent and transmissible. Current data on MDR TB prevalence in Africa, Eastern Europe and Asia [116] provides further evidence of this phenomenon. Drug resistant TB can be transmitted in virtually any setting but healthcare settings, correctional institutions and homeless shelters have an increased risk of transmission. The level of drug resistance TB in hospital settings varies according to local TB prevalence. For instance an university hospitals in Paris (France), reported MDR rates of respectively 1.2% among TB cases [117], while in university hospitals in Manila (Philippine), this figure was an alarming 53.5% [118].

It is generally thought that the emergence of drug-resistant TB (usually termed acquired) occurs in settings where patients fail to adhere to proper treatment regimens or receive inadequate treatment. It is difficult to assign the current magnitude of the epidemic to acquired resistance alone. Another mechanism for the perpetuation of resistance, which has largely been neglected in the development of TB control programmes, is the direct transmission of drug-resistant strains (called primary or transmitted resistance) [119]. In the 2006 XDR-TB outbreak in KwaZulu-Natal (South Africa), 52 of 53 people who contracted the disease (all of them HIV infected) died within weeks [120]. This outbreak received international attention because 85 percent of infected patients had genetically similar XDR strain, indicating that resistance was likely transmitted rather than acquired. Consistent with these findings, a study conducted in Tomsk (Siberia) –a setting where HIV infection is not widespread and effective TB treatment is available– to identify factors leading to increases in MDR-TB cases [121], revealed that a patient was six times more likely to develop MDR-TB if hospitalized for drug-susceptible TB than if not hospitalized. These results strongly suggest that nosocomial transmission of TB rather than resistance (acquired predominantly by nonadherence) is increasingly responsible for the rising MDR TB case rates in Russia and probably in many other places.

Worthy of note is that nosocomial transmission of TB is a risk not only to inpatients but also health care workers (HCWs). In fact, early studies revealed transmission of MDR-TB from patient to patient and from patient to HCWs [122]. A systematic review by Joshi and colleagues [123] demonstrated that TB is a significant occupational problem among HCWs in many low- and middle-income countries and that most health care facilities in these sittings lack resources to prevent nosocomial transmission of TB. HIV-infected HCWs have a particularly high risk of TB, which may be fatal if the disease is caused by a drug-resistant strain [124]. Indeed, dramatic nosocomial outbreaks of MDR TB occurred the late 1980s, largely in HIV infected HCWs, and caused many deaths [110]. This situation increased the concern of HCWs about the safety of working in institutions with a large numbers of admissions for active TB. Indeed, it is estimated that 1% to 10% of HCWs are infected annually in hospitals with more than 200 admissions per year for TB [110]. The risk of TB transmission to HCWs is particularly high in certain areas of the hospital, such as emergency rooms and units that admit patients with active TB [125].

A review of several reports of TB outbreaks with transmission to HCWs in industrialized countries revealed that many factors contribute to nosocomial transmission, such as delayed diagnosis, poor ventilation with positive pressure in isolation rooms, aerosolization of bacilli through mechanical ventilation, bronchoscopy and dressing change [110]. There was also strong evidence that technicians involved in cough-inducing procedures, histologic preparations and autopsies are at high risk, even in institutions caring for few patients with TB [126]. These outbreaks revealed many deficiencies in the knowledge of TB and its transmission as well as strategies used to control the disease. Therefore, various health authorities have implemented effective control programmes based on the early detection of TB and the prompt isolation and treatment of persons with TB in addition to introducing strong measures to prevent nosocomial transmissions of TB. For instance, the US Centers for Disease Control (CDC) [127] recommended the following levels of controls: **1. Administrative controls,** which reduce risk of exposure. **2. Environmental controls,** which prevent spread and reduce concentration of droplet nuclei. **3. Respiratory-protection controls,** which further reduce risk of exposure in special areas and circumstances. (Box 6).

Implementation of a full hierarchy of these measures lead to a significant reduction in nosocomial transmission of TB in the United States [128]. Whereas the extent of the epidemics in low and middle income countries is still attributed, in large part, to ineffective transmission control strategies. In these countries, double infection with TB and HIV has further accelerated the transmission of drug resistant TB and increased the spread of HIV. Such a dramatic situation blocks the efforts of both the Stop TB Partnership and anti-retroviral therapy programmes. It is regrettable that many health care institutions continue to house HIV positive individuals with patients who have drug-resistant TB, thus leading to nosocomial transmission with subsequent community transmission. In this regards, health authorities in Haiti implemented an effective community-based transmission control programme with a baseline triage and separation strategy [111]. Patients are admitted to either the general medical ward, a TB pavilion, or very basic isolation rooms based on smear results and HIV status (Fig. 2).

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Administrative Controls
• Assign responsibility for TB infection control • Conduct TB risk assessment • Develop and institute a written TB infection-control plan • Ensure the timely availability of recommended laboratory processing, testing, and reporting of results • Implement effective work practices for the management of patients with suspected or confirmed TB disease • Ensure proper cleaning and sterilization or disinfection of potentially contaminated equipment • Train and educate health-care workers • Test and evaluate health-care workers for TB infection and disease • Apply epidemiology-based prevention principles • Use posters and signs demonstrating and advising respiratory hygiene and cough etiquette • Coordinate efforts with the local or state health department.
Environmental Controls
Reduce concentration of infectious droplet nuclei through the following technologies: • Ventilation technologies (Natural ventilation and Mechanical ventilation) • High efficiency particulate air filtration • Ultraviolet germicidal irradiation
Respiratory Protection Controls
• Implement a respiratory-protection programme • Train health-care workers on respiratory protection • Educate patients on respiratory hygiene and the importance of covering their cough • Test HCWs for mask fit and functionality

Box 6. TB Infection-Control Programme: Level of Controls

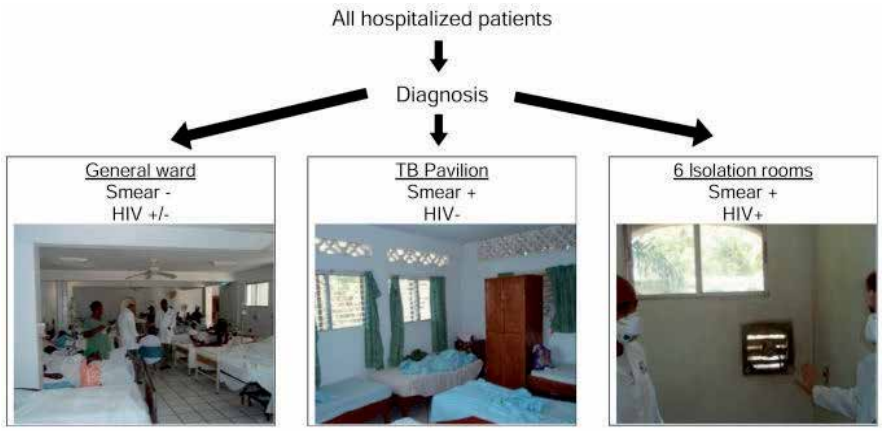

Figure 2. Community-based TB treatment triage strategy in Haiti. The general medical ward has natural ventilation and UV air disinfection. The TB ward has natural ventilation with fenestrated brick and more UV fixtures to disinfect the air than the general ward has. The six isolation rooms are off a common corridor, and each has a large exhaust fan built into the wall that draws air into the room from the corridor, as well as a UV fixture. Reprinted from Ref. 111 with permission of the International Union Against Tuberculosis and Lung Disease. Copyright © The Union

Although this simple baseline strategy is not sufficient, Nardell considers that its implementing in other resource-poor settings would contribute significantly to reduce the burden of TB epidemic [111].

12. Conclusion

Founded in 2001, the goal of The Stop TB Partnership for 2015 was to reduce the global burden of TB disease (deaths and prevalence) by 50% relative to 1990 levels. The reality of the global incidence of all forms of TB in 2012 indicates that this timetable is unrealistic. Despite billions of dollars already spent on TB control programs, less than 2% of drug resistant TB cases currently receive appropriate treatment in high burden countries allowing the disease to spread faster than the implementation of adequate management programs. This dramatic situation severely attenuates global efforts to control TB.

One of the major challenges now is to develop innovative approaches to expand the detection and treatment of drug resistant cases globally. To achieve this goal, substantial funding and development of extensive human resources is needed. Among the response priorities, the following are of paramount importance: 1) developing tools for rapid detection of drug resistance; 2) clinical trials to test simplified, safe and shorter second-line treatment regimens; 3) developing approaches to enhance treatment adherence; 4) clinical trials to test the efficacy of new prophylactic treatment regimens for contacts of patients; and finally 5) developing safe and more efficient second-line drugs.

However, even with increased detection and treatment of drug resistant TB, focusing on the care of TB and neglecting living conditions in low-income countries has little chance of completely reversing the burden of TB. The severity of the TB epidemic in the Western world during the 19th century was largely due to the low living standards prevalent among the poor during the industrial revolution. As living standards improved, TB mortality began to decrease long before any vaccinations or specific therapy was introduced. Reminiscent of the 19th century situation is the persistence of TB in poor and marginalized populations in most modern cities of the world. As the British historian Thomas Mc-Keown said in 1976, "the overall health of the population is less related to medical advances than to standards of living and nutrition" [101]. Thus the control and eventual eradication of the TB epidemic will need support and cooperation from multiple levels within the medical and scientific community, as well as all levels of government worldwide in order to address living standards and develop better drugs and therapeutic tools for the clinic.

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Chapter 13

Drug-Resistant Tuberculosis – Diagnosis, Treatment, Management and Control: The Experience in Thailand

Attapon Cheepsattayakorn

Additional information is available at the end of the chapter

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1. Introduction

Multidrug-resistant tuberculosis (MDR-TB) is defined as TB bacilli revealing resistance to at least isoniazid and rifampicin whereas extensively drug-resistant tuberculosis (XDR-TB) is TB bacilli that develops resistance to at least isoniazid and rifampicin as well as to any quinolone drug and at least one of the second-line anti-TB injectable drug : kanamycin, amikacin, or capreomycin. Report from the World Health Organization (WHO)/International Union Against Tuberculosis and Lung Disease (IUATLD) Global Project on Drug Resistance Surveillance revealed that the prevalence of the primary multidrug-resistant tuberculosis during 1996-1999 ranged between 0-14.1 % [1]. In 1994, the first WHO-IUATLD global anti-TB drug resistance surveillance was carried out in 35 countries and subsequently, the second, third and fourth surveillances occurred in 1996-1999, 1999-2002 and 2002-2007, respectively. The emergence of clinically significant MDR-TB was in the early 1990s. Reports of Primary Drug-Resistance Surveillance in Thailand during 1997-1998, 2001-2002, and 2005-2006 were 2.02 % [2], 0.93 % [3], and 1.65 % [4], respectively while the secondary drug-resistance in 2005-2006 revealed 34.54 % [4]. However, number of patients with MDR-TB demonstrated in 2008 Annual Report of the Bureau of Tuberculosis, Thailand were only 294 while the WHO' s estimated number of patients were 2,774 [5]. The prevalence of primary plus secondary MDR-TB among prisoners in Thailand in 2002-2003 was 5.3 % while the prevalence of primary MDR-TB was 5.9 % [6]. Report from the 10th Zonal Tuberculosis and Chest Disease Centre, Chiang Mai, Thailand, 10th office of Disease prevention and Control, Department of Disease Control, Ministry of Public Health, Thailand in 2011 revealed only 88 patients who registered with MDR-TB at hospitals in northern Thailand [7] while only 16.3 %, 18.6 % and 18.6 % of 43 patients with laboratory confirmation were cure, completeness of treatment, and dead, respectively in 2009 report [8]. Findings from the Bureau of Tuberculosis of Thailand ' s 2007-2009 Research

Project on Anti-tuberculosis Drug Resistance Surveillance in Thailand (Situation of Multidrug-Resistant Tuberculosis in Thailand : Fiscal Year 2007-2009) that studied in 126 hospitals countrywide showed 877 patients with laboratory-confirmed MDR-TB and 64 patients with laboratory-confirmed XDR-TB while 21.5 % were dead and 12.74 % of these MDR/XDR-TB patients had human immunodeficiency virus (HIV) infection /acquired immunodeficiency syndrome (AIDS) compared to 21.57% of probable or presumptive MDR-TB patients co-infected with HIV/AIDS [9]. Only 18.2% of the studied data sources came from TB registered book for MDR-TB patients and most of them came from the hospital medical registry [9]. A previous study by Scano F *et al*. revealed 52 of the 53 patients with XDR-TB died [10]. The median survival time from collection of specimen to death of these patients was 16 days [10]. The prevalence of XDR-TB among all MDR-TB patient was as the following : 10.3% in Germany and 14.3% in Italy (1993-2004) ; 1.5% in Asia, 15.4% in Republic of Korea, 13.6% in Russia, 0.6% in Africa and Middle East, 6.5% in industrialized countries, and 6.6% overall worldwide (2000-2004); 12% in Hong Kong (2004); 10.9% in Iran (2006); 7.3% in India; and 4% in France (2006) [11]. The WHO notified that Thailand possibly underreported of MDR/XDR-TB prevalence due to delaying of transportation of the specimens for anti-tuberculosis drug susceptibility testing to the specialized centres and processes of unstandardized data collecting of the country [9]. More than 400,000 new MDR-TB cases globally occur each year while approximately half of a million cases occurred in 2007 and accounted for more than 5% of the annually global cases of TB disease. The emerging of drug-resistant TB is a global health problem, although emphasis has been placed on several " hotspots " (higher than 3% of its prevalence) worldwide because of lacking of good global data reported to the WHO. The emergence of MDR-TB and XDR-TB is a real health threat to achieve TB elimination.

2. Epidemiology

Development of anti-tuberculosis drug resistance can occur due to *Mycobacterium tuberculosis* genetic factor, previous anti-tuberculosis treatment-related factors and many other factors [11]. The mechanism of drug-resistance is shown in Figure 1. There is a constant rate of spontaneous mutation of 0.0033 mutations/deoxyribonucleic acid (DNA) replication that is unique for a diverse spectrum of prokaryotic organisms [12]. The average rates of spontaneous mutation for rifampicin, isoniazid, pyrazinamide, streptomycin, and ethambutol are 2.25×10^{-10}, 2.56×10^{-8}, 1×10^{-3}, 2.95×10^{-8}, and 1.0×10^{-7}, respectively [13]. A previous study in India revealed 3.4% and 25% of primary and acquired MDR-TB, respectively [14] which were higher than the primary MDR-TB and acquired MDR-TB prevalence previously surveyed in Thailand [2,3,4]. Liu CH *et al*. reported their study in China which presented 19.4% of MDR-TB, 1.3% of XDR-TB, 19.8% of poly-resistant TB, and 47.1% of any anti-tuberculosis drug resistance [15]. A surveillance data in 2007 from the WHO demonstrated 4.8% of MDR-TB cases among new TB cases worldwide [16] compared to the study from MDR-TB surveillance in Thailand between 2007-2009 revealed the MDR-TB prevalence of 0-0.21% (average 0.08%) which was highest in the central part of Thailand [9]. The prevalence among patients with previously TB treatment from the same surveillance project was between 1.58-58.72% (average 8.49%, higher

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than the percentage of hotspot of MDR-TB prevalence set by the WHO (3%, an indicator for implementation of DOTS (Directly Observed Treatment, Short Course)-Plus programmes) which was also found highest in the central part of Thailand while the northern part of Thailand was the second [9]. This Thailand's serious MDR-TB situation needs urgently management such as implementation of DOTS-Plus programmes. In 2006, Gandhi NR *et al*. firstly reported of XDR-TB co-infected with HIV/AIDS which had been studied in Kwazulu Natal, South Africa (KZN) [11]. XDR-TB epidemic in South Africa appears to be the primary mechanism through the acquisition of 63-75% of XDR-TB cases [11] whereas the strain of *Mycobacterium tuberculosis* infected among a large number of XDR-TB cases in KZN was F15/LAM4/KZN [11]. Generally, individual who is infected with *Mycobacterium tuberculosis* has approximately 5-10% lifetime risk of developing TB disease, but in an individual with HIV-infection/AIDS the risk is 5-15% a year [11]. This can contribute HIV-infection/AIDS to facilitate the control of outbreaks of MDR-TB and XDR-TB, although it has contributed to outbreaks of drug-resistant TB [17]. The patients with MDR-TB and HIV/AIDS co-infection will have exceedingly high mortality [16]. Gandhi NR *et al*. recently reported their study on risk factors for mortality among MDR/XDR-TB patients with HIV/AIDS co-infection which revealed that 80% of XDR-TB patients died whereas 63% of MDR-TB patients were dead following the diagnosis [18]. The CD4-T cell count less than 50 cells/mm^3 was the strongest independent factor for mortality among both patient groups [18]. History of TB treatment is the most significant predictor of development of MDR-TB [19]. High prevalence of HIV/AIDS co-infection and inadequate resources for case detection and management have contributed to the emergence of untreatable XDR-TB [20]. Unfortunately, the presence of XDR-TB in non—HIV-infected patients with MDR-TB is independent poor prognostic factors [11]. Prevalence of XDR-TB is globally accounted for approximately 5.4% of MDR-TB prevalence [21]. Drug-resistant TB and drug-resistant gram-negative bacterial infection and disease are associated with the most serious health problems in developing countries [22]. Estimated 81,000 patients with MDR-TB (18.4% of the estimated MDR-TB patients worldwide in 2011) live in the 53 countries of the WHO European Region [23]. This European MDR-TB problem contributed to launching of a new WHO Regional Office for Europe Action Plan to fight MDR-TB to contain the spread of drug-resistant TB in the region by the end of 2015 [23]. The new action plan set the targets to be achieved by the end of 2015, are : 1) decreasing 20% of the proportion of MDR-TB cases among previously treated patients 2) diagnosis of at least 85% of the estimated MDR-TB cases and 3) treating successfully at least 75% of notified MDR-TB cases [23]. If this plan is fully implemented and expected, by 2015, to successfully treat 127,000 MDR-TB cases, and to prevent the emergence of 250,000 new MDR-TB cases and 13,000 new XDR-TB cases [23]. This would interrupt the transmission of MDR-TB and save 120,000 lives in this region [23].

3. Systematic management of MDR/XDR-TB

Diagnostic and treatment consultation networks for MDR/XDR-TB which set by the 10th Zonal Tuberculosis and Chest Disease Centre, Chiang Mai, 10th Office of Disease Prevention and Control, Department of Disease Control, Ministry of Public Health, Thailand beyond the year

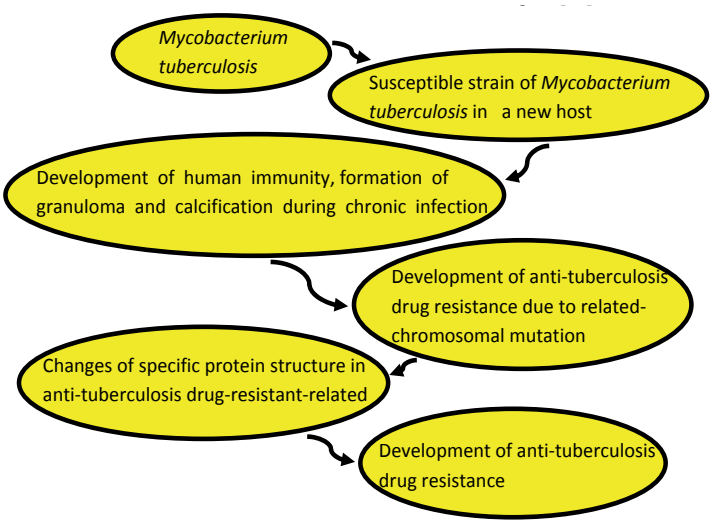

Figure 1. Mechanism of development of anti-tuberculosis drug resistance

2000 have been firstly initiated at tertiary care hospitals in northern Thailand. Data collecting on MDR/XDR-TB control has been tracked through the special project paper-based recording and reporting systems set by the 10th Zonal Tuberculosis and Chest Disease Centre which separated from the routine DOTS recording and reporting systems. Currently, a new computer programme has been developed by staff of this centre to efficiently record and report the MDR/XDR-TB data in the area of northern Thailand and attempt to gradually extend the use of this computer programme throughout the country.

4. Presumptive diagnosis of MDR-TB

The Thailand' s 2012 National Tuberculosis Management Guidelines [24] set the criteria for probable or suspected MDR-TB, are :

1. patients with history of TB retreatment, especially within 6 months after completeness of treatment or cure,
2. patients with interruption of 6 month-short course chemotherapy (2HRZE/4HR, H=isoniazid, R=rifampicin, Z=pyrazinamide, E=ethambutol) and having continuously positive-sputum smear examinations after treatment interruption,
3. patients with history of multiple TB treatments, irregular anti-TB drug taking, and persistently positive-sputum smear examinations,
4. patients having positive-sputum smear examinations at the end of second and fifth months of the 6-month short course chemotherapy (2HRZE/4HR),

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5. patients with evidence of HIV co-infection/AIDS before the 6-month short course chemotherapy starting,
6. general TB patients with history of MDR-TB patient exposure, including health or medical personnel with TB disease who have history of MDR-TB patient exposure, and
7. other high-risk patients of MDR-TB, such as general TB patient with huge lung cavity, diabetic patients, prisoners with TB disease, and TB patients who live in the cross-borderline areas.

5. Laboratory investigations for MDR-TB

Several plans have been announced for the WHO Stop TB Department to collaborate with the Foundation for Innovative New Diagnostics (FIND) to initiate and introduce rapid-culture technology and new rapid drug-resistant tests in the southern African countries and the world including the international standards for the second-line drug-susceptibility testing [11]. The Thailand' s 2012 National Tuberculosis Management Guidelines [24] set the laboratory investigations for MDR-TB which are direct acid-fast bacilli (AFB)-sputum smear examinations with sputum culture and drug susceptibility testing (DST). For reduction of the diagnosis time for unrecognized drug-resistant TB, new rapid diagnostic technologies for drug resistance from sputum smear or positive culture for smear-negative and extra-pulmonary TB must be prioritized [10]. All TB control programmes in moderate- and high-MDR TB prevalent settings should consider the promotion of culture and DST including implementation of use of algorithms for the diagnosis of pulmonary and extra-pulmonary TB [10]. Rapid DST is preferred to the conventional DST due to 1-2 days of resulting. Recently, the 2011 WHO Guidelines recommends " Xpert MTB/RIF® " and line-probe assay which are new molecular diagnostic technologies and can detect drug resistance to both isoniazid and rifampicin or only rifampicin [24], but their disadvantage is inability to detect the resistance to every drugs used in MDR-TB treatment for detecting probable XDR-TB, required expertise and expensive technologies/equipments which limit their wider uses [25]. The conventional DST takes 1-3 months of the results that take markedly longer than the new molecular methods and is labor-intensive [24]. Other alternative phenotypic methods based on the *Mycobacterium tuberculosis* metabolism such as CO2 (carbon dioxide) production, oxygen uptake, ATP (adenosine triphosphate) bioluminescence, etc. have been experimented and demonstrated promising in overcoming this obstacle of longer time resulting [25]. These methods also have impressive sensitivity and specificity compared to the conventional DST [25]. Currently, molecular line probe assays and automated liquid culture systems are recommended by the WHO as the gold standard for the first-line and second-line DSTs. Liquid culture DST has been demonstrated to have relatively good reliability and reproducibility for aminoglycosides, fluoroquinolones, and polypeptides for detecting XDR-TB [26]. However, liquid culture DST for other second-line drugs such as para-aminosalisylic acid, linezolid, clarithromycin, amoxicillin-clavulanate, cycloserine, clofazimine, terizidone, ethionamide, and prothionamide is not recommended by the WHO [26]. A recent study reported the use of spoligotyping and sequence 6110 restriction

fragment length polymorphism insertion analysis of genomic DNA which demonstrated that MDR-TB cases (74.0%) were more likely to be identified in clusters than anti-TB drug susceptible cases (33.6%) [27]. Xpert MTB/RIF assay recently has been introduced which meets the requirements of effective diagnosis of pulmonary TB as the following : allowing detection of both the *Mycobacterium tuberculosis* complex and resistance to the principal anti-TB drugs, especially rifampicin (RIF or R), availability on a global scale with standardized-easy use and robust diagnostic tools recently has been introduced [28]. This assay is a nucleic acid amplification test for detection of rifampicin resistance-associated mutations of the *rpoB* gene and *Mycobacterium tuberculosis* complex DNA in sputum [28]. It can be designed for use with other systems to automate and integrate sample processing, nucleic acid amplification, and detection of target sequences using reverse transcriptase polymerase chain reaction (PCR) and real-time PCR [28]. Between 2007-2009, the WHO has approved several drug-resistant TB diagnostic tests such as liquid culture (MGIT®, an automated liquid culture, developed by BD Diagnostic Systems, 2007) which has been used at the 10th Zonal Tuberculosis and Chest Disease Centre, Chiang Mai, Thailand, line-probe assays (INNO-Lipa®, line-probe assay that requires culture, developed by Innogenetics, 2008), noncommercial culture and drug susceptibility testing (Microscopic Observation Drug Susceptibility (MODS), developed by Academic Laboratories, 2009; Nitrate reductase assay, developed by Academic Laboratories, 2009; and Colorimetric drug susceptibility testing, developed by Academic Laboratories, 2009) [29]. GeneXpert MTB/RIF®, a new automated nucleic acid amplification technique which was developed by Cepheid, The Foundation for Innovative New Diagnostics (FIND) and University of Medicine and Dentistry of New Jersey (UMDNJ) was reviewed by the WHO in 2011 [29] and currently has been recommended to measure the *Mycobacterium tuberculosis* DNA and the rifampicin-resistance sequence worldwide. This new technique has been set in Thailand at least 6 sets including the one set at the 10th Zonal Tuberculosis and Chest Disease Centre, Chiang Mai, Thailand in collaboration with the Unites States Centres for Disease Control and Prevention (US-CDC) for reference laboratories in Thailand. GeneXpert test's sensitivity is moderate at 67.2% in AFB-smear negative cases at one-time smear staining of the specimens, and increases to 80% when is performed three times [28]. This test provides the results within two hours and requires minimal training of the laboratory workers [29]. The limitations of the test are requirement of a consistent source of electricity that will limit its use outside of the settings where a regular electric power supply can be guaranteed, its expensive cost of the instrument, and cost per test cartridge [29]. Mishra B *et al*. used the automated BACTEC 460 TB system in study the emergence of drug-resistant TB at an urban tertiary care hospital in South India which revealed that 37.2% were MDR-TB isolates whereas 42% of the pulmonary *Mycobacterium tuberculosis* isolates and 20.4% of extra-pulmonary isolates were MDR [30]. Phenotypic and genotypic detections of anti-TB drug resistance are described as the following [31- 49]:

1. Phenotypic detection

1.1 Slide DST

This method has less equiptment, suitable for decentralization, 93% rifampicin susceptibility at 88% predictive value of resistance.

1.2 Mycobacteria Growth Indicator Tube (MGIT) Systems

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This test has sensitivities of 100% for rifampicin, isoniazid, ethambutol and streptomycin, specificity of 100% for rifampicin, 97.7% for isoniazid, 98.0% for ethambutol and 89.8%% for streptomycin.

1.3 Microscopic observation broth-drug susceptibility assay (MODS)

This method has sensitivity of 72.7% and specificity of 99.7% for rifampicin, 72.6% and 97.9% for isoniazid, and 77.8% and 99.7% for MDR-TB.

1.4 Mycobacteriophage-based method

1.4.1 Commercial FASTPlaque assay (FASTPlaque TB test and FastPlaque TB-RIF™ (rifampicin DST) (Biotech Labs Ltd, Ipswich, UK))

This test has sensitivity and specificity of 31.2% and 94.9%, respectively in all anti-TB drug-susceptible and resistant TB patients, sensitivity and specificity of 33.3% and 93.9%, respectively in all anti-TB drug-susceptible and resistant TB patients with HIV-infection/AIDS.

1.4.2 Fluoromycobacteriophage assay (Figures 2, 3)

This method has 94% sensitivities for rifampicin and isoniazid and 98% sensitivity for streptomycin and specificities of 97% for isoniazid, 95% for rifampicin, and 98% for streptomycin (resazurin microplate technique), sensitivity of 94% for all three anti-TB drugs (rifampicin, isoniazid, and streptomycin) (*EGFP*-phage technique) and specificities of 93% for rifampicin, 90% for isoniazid and 95% for streptomycin (*EGFP*-phage technique).

1.4.3 Luciferase reporter phage assay

This test has 100% sensitivity and 89-100% specificity for culture isolates.

1.5 Nitrate reductase assay

This method has sensitivity and specificity of 100% and 100% for rifampicin, 93% and 100% for isoniazid, 76% and 100% for streptomycin, and 55% and 99% for ethambutol, respectively.

1.6 Microcolony method (Thin-layer agar (TLA) method)

This test has sensitivities and specificities of 100% for both rifampicin and ofloxacin, and sensitivity of 100% and specificity of 98.7% for kanamycin, 100% overall accuracy for rifampicin and isoniazid resistance.

1.7 Colorimetric redox indicator methods

This method has sensitivities of 100% for rifampicin, ofloxacin, kanamycin and capreomycin and 99.1% for isoniazid, specificity of 100% for rifampicin, isoniazid, ofloxacin and kanamycin, and 97.9% for capreomycin, overall accuracy of 98.4% for rifampicin, 96.6% for isoniazid, 96.7% for ofloxacin, 98.3% for kanamycin and 90% for capreomycin.

2. Genotypic detection (Nucleic Acid Amplification)

2.1 Line-probe assay (LPA)

2.1.1 INNO-LiPA Rif.TB® assay (Innogenetics, Ghent, Belgium)

This test has higher than 95% sensitivity and 100% specificity, sensitivity of 82.2% and specificity of 66.7% for MDR-TB detection.

2.1.2 GenoType® MTBDRplus kit (Hain Lifescience, Nehren, Germany)

This method has nearly 91% sensitivity for MDR-TB, possibly detects rifampicin and isoniazid resistance and confirms TB infection simultaneously.

2.1.3 Genotype MTBDRsl assay

This test has ethambutol, 89% sensitivity for ofloxacin, 87% sensitivity for capreomycin, 75% sensitivity for amikacin.

2.2 Real-time PCR

This technique has sensitivity of 89% and specificity of 99% (molecular beacons), overall sensitivity of 98-100% with 72% sensitivity in smear-negative specimens and specificity of 100% for Xpert MTB/RIF assay (Cepheid Xpert MTB/RIF®, Sunyvale, CA).

2.3 PCR sequencing

This technique has sensitivities of 96.7% for rifampicin-resistant isolates (*rpoB* gene), 64% for isoniazid-resistant isolates (*katG* gene) and 70% of ofloxacin-resistant isolates (*gyrA* gene) (PCR pyrosequencing).

2.4 DNA Microarrays (DNA biochip)

This technique has specificity of 97% and 95% for rifampicin, 91% and 60% for isoniazid, 96% and 67% for kanamycin, 93% and 73% for streptomycin, and 98% and 89% for ethambutol, respectively, simultaneous detection of multiple genetic sequences (oligonucleotide microarray).

Although genotypic methods have potentially fastest results, the massive cross-contamination is still the main risk. To prevent its, strict internal controls, special technique, and separation of working areas are required. The reproducibility of the results of the Xpert MTB/RIF assay under actual field conditions, the strength of the laboratories, and the manner and extent of its introduction are the impact of this new assay. The sources of error for genotypic methods are incomplete coverage of rifampicin-resistance gene-core region and mixtures (multiple mutations, wild-type strain/emerging mutant) and silent mutations. These sources of error may contribute to 1% false-resistant and 5% false-susceptible results [31].

Schematic representation of *phAE87* : *hsp60-EGFP* construction. Shuttle phasmid *phAE87* is a conditionally replicating derivative of phage TM4 in which the cosmid moiety is flanked by Pac I restriction sites. A plasmid derivative of *pYUB854* containing the *EGFP* gene (pMP14) was used to replace the cosmid in *phAE87* followed by lambda packaging and recovery in *E. coli*.

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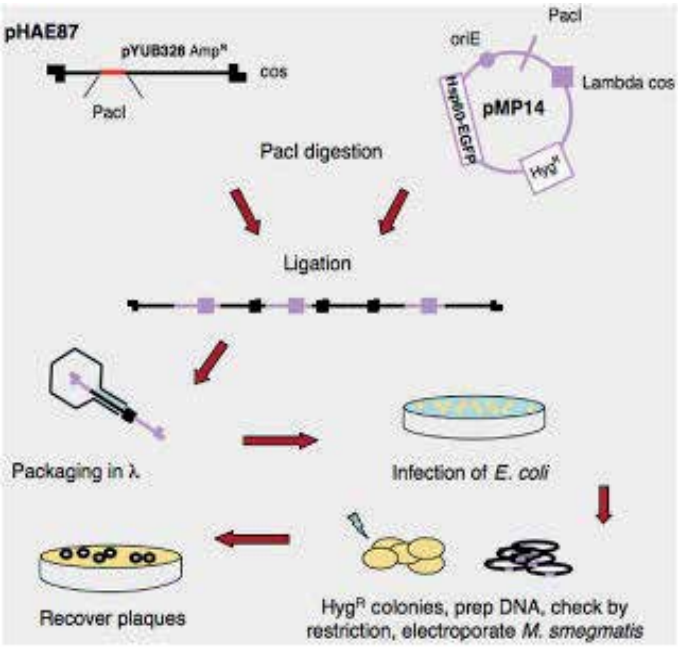
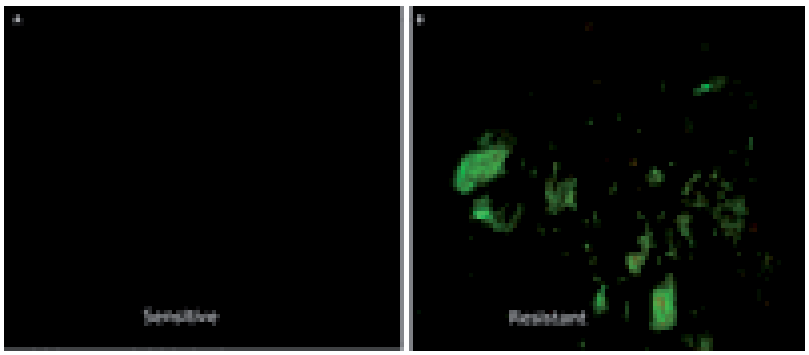


Source : Piuri M, Jacobs WR Jr, Hatfull GF. Fluoromycobacteriophages for rapid, specific, and sensitive antibiotic susceptibility testing of *Mycobacterium tuberculosis*. PLoS ONE 2009; 4(3) : e4870. doi: 10.1371/journal.pone.0004870

Figure 2. Fluoromycobacteriophages construction



Source : Rondo'n L, Piuri M, Jacobs WR Jr, Waard Jde, Hatfull GF, Takiff HE. Evaluation of fluoromycobacteriophages for detecting drug resistance in *Mycobacterium tuberculosis*. J Clin Microbiol 2011; 49 (5) : 1838-1842.

Figure 3. A, B). Two strains of *Mycobacterium tuberculosis* were incubated separately in 7H9-OAD with 2 µg of rifampicin/ml for 24 hours, infected with the *EGFP*-phage, killed with paraformaldehyde, and then fixed on microscope slides. The images, obtained with a fluorescence microscope, show a susceptible-to-rifampicin strain (A) and a resistant-to-rifampicin strain (B).

6. Radiological features in patients with MDR/XDR-TB

A recent study in South Korea showed 100% of lung nodules, 60% of lung consolidation, and 47% of lung cavities that were mainly located in the upper and middle lung zones in XDR-TB patients whereas less frequent lung nodules and ground-glass opacity lesions were found in XDR-TB patients compared to the patients with anti-TB drug-susceptible pulmonary TB [50]. More frequent multiple lung cavities, lung nodules, and bronchial dilatation were found in both MDR-TB and XDR-TB patients compared to the patients with anti-TB drug-susceptible pulmonary TB [50]. There was no different radiological findings between MDR-TB and XDR-TB patients [50]. Another recent study in South Korea revealed that micronodules and tree-in-bud appearance were found in 100% of the pulmonary XDR-TB patients whereas lung consolidations, lung cavities, bronchiectasis, lobar consolidations were found in 85%, 85%, 80%, and 70%, respectively [51]. This study showed a significantly larger extent of tree-in-bud appearance and lung consolidations compared to the MDR-TB patients [51]. In childhood patients, chest radiological features at the time of diagnosis demonstrates lobar opacification, intrathoracic lymphadenopathy, particular hilar lymph nodes, and airway narrowing [52]. Chest radiological features of three patients with MDR-TB who attended the 10th Zonal Tuberculosis and Chest Disease Centre, Chiang Mai, Thailand are shown in the Figure 4 (A,B,C) which demonstrated a single cavity at the upper lung zone in two patients and no lung cavity in another one. These three patients possibly attended the 10th Zonal Tuberculosis and Chest Disease Centre, Chiang Mai, Thailand at the earlier stages compared to the above study results.

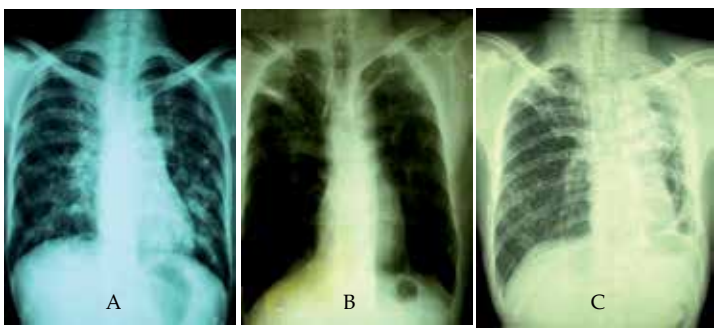


Figure 4. First-attendance chest radiological features of the three patients (A,B,C) with MDR-TB at the 10th Zonal Tuberculosis and Chest Disease Centre, Chiang Mai, Thailand A : Showing bilaterally diffuse lung infiltration with a cavity in the left upper lung zone, B : Showing fibrotic infiltration with surrounding new infiltration at the right upper lung zone with bilaterally diffuse emphysematous lung changes, C : Showing bilaterally diffuse infiltration with a cavity in the right upper lung zone with left pleural effusion.

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7. Regimens used in treating patients with drug-resistant tuberculosis

A recent study on MDR-TB treatment revealed that use of later generation quinolones (moxifloxacin, gatifloxacin, sparfloxacin, levofloxacin), ofloxacin or ethionamide/prothionamide, use of four or more likely effective drugs in the initial intensive phase, and three or more likely effective drugs in the continuation phase was associated with the treatment success compared to the treatment failure or relapse [53]. The duration of initial phase up to 7.1-8.5 months and the total duration of treatment up to 18.6-21.5 months increased the chances of treatment success [53]. In 2011, the WHO recommended the regimens containing a fluoroquinolone, pyrazinamide, ethionamide (or prothionamide), para-aminosalicylic acid (or cycloserine), and a second-line injectable drug with more than 20 months of treatment duration [54]. Five MDR-TB control projects with used DST results and previous treatment history were conducted among 1,047 MDR-TB patients in 5 resource-limited settings with well-established DOTS programmes (Manila, Estonia, Latvia, Lima, and Tomsk) in 1999 for Lima and Manila, 2000 for Tomsk and Latvia, and 2001 for Estonia [55]. At least 4 drug (ethambutol, pyrazinamide, cycloserine, clofazimine, para-aminosalicylic acid, ethionamide, or prothionamide, augmentin, clarithromycin or thiacetazone) including an injectable drug (kanamycin, amikacin, capreomycin, or streptomycin) and a fluoroquinolone (ofloxacin, ciprofloxacin, or levofloxacin) were administered for the duration of treatment (18-24 months) except for the injectable drug, which was administered for a specified interval after the patient' s specimens were culture-negative [55]. Monthly sputum-AFB smear and culture were monitored [55]. Every 6-months (Manila and Lima) and 3-months chest radiographs (Tomsk, Latvia, and Estonia) were performed [55]. The treatment outcomes among new and previously treated MDR-TB patients revealed 74.8% and 68.3% cured patients, 2.5% and 0.3% completed treatment patients, 4.2% and 7.0% failed treatment patients, 3.4% and 14.2% dead patients, and 77.3% and 66.6% treatment success rates (cure rate + completed treatment rate), respectively [55]. The results showed worsen outcomes among previously treated patients. Report from the 10th Zonal Tuberculosis and Chest Disease Centre, Chiang Mai, Thailand, in 2011 which had been collected from the data of laboratory-confirmed 254 MDR-TB patients (72.8% of all probable MDR-TB cases) among 349 totally suspected-MDR-TB cases with 15.8% of HIV co-infection in northern Thailand between 2005-2010 gradually increased from 62.2% of probable MDR-TB cases in 2005 to 78.3% in 2010 and revealed 3.2% treatment-denial patients, 75.2% treatment-registered patients, 30.2% died before starting the second-line drug treatment regimens (pyrazinamide, ethambutol, ofloxacin, para-aminosalicylic acid administered for 18-24 months and one injectable drug (kanamycin, or amikacin) administered 5 days per week for the initial 6-month phase), 25.4% default-treatment patients (continuous interruption of treatment more than 2 months), 54.8% treatment success rate, and 22.2% unavailable-data patients [7]. Among 19 cases with pre-treatment death, 10 cases (52.63%) demonstrated HIV co-infection. Extra-pulmonary cases accounted for 2.4% of the laboratory-confirmed cases which was lower than percentage of susceptible extra-pulmonary TB cases in the same area [56]. Four cases with laboratory-confirmed MDR-TB emerged as XDR-TB during treatment [7]. A previous study on outcomes of a daily supervised-MDR-TB treatment regimen which consisted of initial phase of 6-9 months with kanamycin, ofloxacin, cycloserine, ethionamide,

ethabutol, and pyrazinamide demonstrated that in cases of persistent culture positive at fourth month, the initial phase was extended for additional 3 months. Then ofloxacin, cycloserine, ethionamide, and ethambutol were continued for 18 months [57]. The results of the study revealed that 82% of cases demonstrated time to culture conversion at the second month or before. The culture conversion rates at third month and sixth month were 84% and 87%, respectively. The cure rate was 66%. At 24 months, 79% of the patients remained culture negative for more than 18 months. Adverse drug reactions were reported among 58% of cases and 2 failure cases emerged as XDR-TB during treatment [57]. A recent study on comparison between traditional hospital-based treatment-model of MDR-TB patient care and community-based model in rural areas of South Africa revealed that median times to starting the treatment and sputum smear conversion were shorter for community-based model (84 days versus 106.5 days and 59 days versus 92 days, respectively) [58]. Lack of sputum culture conversion at month 9 was a predictor of pulmonary MDR-TB treatment failure with 84% of sensitivity and 94% of specificity [59]. A recent study by Dheda K *et al*. demonstrated that the number of XDR-TB deaths was not significantly different compared between patients with and without HIV co-infection [60] whereas Well CD recently reported that lower cure rates and higher death rates were found in MDR-TB patients with HIV co-infection compared to the patients without HIV co-infection [61]. Survival of XDR-TB patients with HIV co-infection was associated with absence of biomarkers indicative of multiorgan dysfunction, less advanced stage of both diseases at time of diagnosis, and antiretrovirals provision [62]. Previous culture-proven MDR-TB, number of drug used in a MDR-TB treatment regimen, and treatment with moxifloxacin were independent predictors of death [60] while some previous reports showed that treatment success rates were poor (30-50%) in XDR-TB patients with HIV co-infection [63] and the MDR/XDR-TB prevalence was substantially precipitated by the HIV epidemic [64, 65]. In children with MDR-TB, the treatment guidelines are the same principles, using the same drugs as in adult patients with strict and prolonged supervision by expert pediatricians [66]. HIV co-infection are particular challenges and requires early starting of antiretroviral therapy with careful monitoring for drug-adverse side-effects [66]. Children with close contact with MDR-TB patients should be tested with tuberculin skin testing or interferon-gamma release assays, direct AFB-smear examinations, cultures, and DST and taking the chest radiological examinations [24]. Cases with close contact should be at least 2-year followed up [24]. If they are diagnosed MDR-TB, they must be treated with the empirical MDR-TB regimen [24]. Empirical MDR-TB treatment regimen is not recommended for MDR-TB chemoprophylaxis [24]. MDR-TB chemoprophylaxis for children with at least 2 second-line drugs for 6-12 months and reflecting the susceptibility profile of the source case' s isolate with daily supervision should be considered [52, 67]. The knowledge of mechanisms of the second-line drugs for children is necessary for ensuring the treatment adherence and long-term control of the disease.

A previous study on the second-line drug susceptibility among 40 MDR-TB strains in Turkey revealed mono-resistant to ethionamide 25%, amikacin 10.0%, kanamycin 2.5%, ofloxacin 2.5%, amikacin 0%, and clofazimine 0%, any resistant to ethionamide 37.5%, capreomycin 25.0%, kanamycin 5.0%, ofloxacin 5.0%, amikacin 5.0%, and clofazimine 2.5%, resistant to both ethionamide + capreomycin 5.0%, both capreomycin + clofazimine 2.5%, ofloxacin + ethionamide + capreomycin 2.5%, amikacin + ethionamide + capreomycin 2.5%, and kanamycin +

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amikacin + ethionamide + capreomycin 2.5% [68]. Currently, the data of the second-line drug resistance are not available [9].

The Thailand' s 2012 National Tuberculosis Management Guidelines [24] recommends the guidelines for both pulmonary and extra-pulmonary MDR-TB treatment as the following flow diagram :

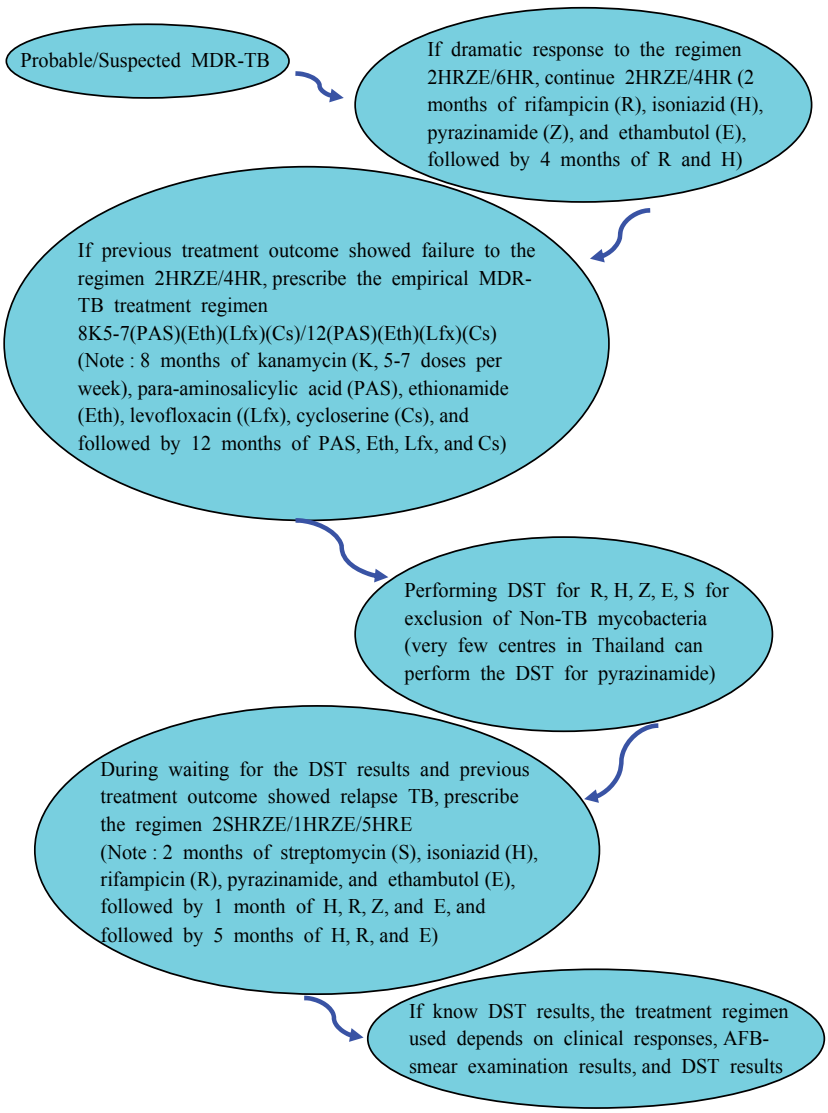

Figure 5. Flow Diagram of Management of Patient with Probable/Suspected MDR-TB

Currently, in Thailand, patients with persistent AFB-smear and/or culture positive after completeness of MDR-TB treatment will be prescribed isoniazid alone for lifelong while no

standardized treatment is recommended yet. Types of patients with MDR-TB, patient monitoring during MDR-TB treatment and assessment of sputum conversion, and classification of treatment outcomes are shown in the Table 1, 2, and 3, respectively [24].

Type	Description
New case	patient who does not take any anti-TB drugs before or take any anti-TB drugs less than 1 month duration and the DST results show resistance to at least rifampicin and isoniazid
Relapse case	patient who cured of MDR-TB in the past and present or pre-treatment of relapse-MDR-TB DST results reveal MDR-TB
After default MDR-TB	patient who continuously interrupts the TB treatment more than 2 months and the DST results before treatment interruption demonstrates MDR-TB
After failure of the first- TB treatment	patient' s AFB examinations show positive results at the end of the fifth month and the DST results at the end of the second or fifth month reveal MDR-TB
After failure of retreatment	patient who treated with the retreatment regimen (2 months of streptomycin, rifampicin, isoniazid, pyrazinamide, and ethambutol, followed by 1 month of rifampicin, isoniazid, pyrazinamide, and ethambutol , and then followed by 5 months of isoniazid, pyrazinamide, and ethambutol for relapse cases) and the DST results at the end of third or fifth month demonstrate MDR-TB
Transfer in	patient who is referred from one setting to the other setting for further diagnosis, treatment, and management, transferred setting must report the outcome of treatment to the referred setting (first setting) for discharge registration as " transfer in "
Other	other patients who cannot be registered as the above 6 patient-registration types including patients who treated with empirical MDR-TB treatment regimen at the non-NTP settings (not registered as the above 6 patient-registration types)

Table 1. Registration of the patients with MDR-TB [24]

Patient monitoring during MDR-TB treatment	Assessment of sputum conversion
Direct AFB-smear examinations and cultures will be performed at month : 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, , 14, 16, 18, and 20.	Results of 2 consecutively direct-AFB-smear negative and culture negative with 30-day intervals will indicate sputum conversion. Mostly, patients with sputum conversion will converse the positive to the negative results within the first 6-month of treatment duration.
Chest radiological examinations will be performed at month : 0, 3, 6, 12, and 18.	

Table 2. Patient monitoring during MDR-TB treatment and Assessment of sputum conversion [24]

8. Patient monitoring after completeness of the MDR-TB treatment [24]

For assessment of the MDR-TB relapse rate, direct AFB-smear examinations and cultures are performed and assessed every 3 months in the first 6-month completeness of treatment and

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then every 6 months for 18 months. Chest radiological examinations are preformed when indicated.

Cured	patient who treated with MDR-TB treatment regimen following the Thailand' s 2012 National Tuberculosis Control Management (or NTP) Guidelines with consecutively 5- negative results of the 30 day-interval AFB smear examinations and cultures of the last 12 months of treatment duration
Treatment completed	patient with completeness of the MDR-TB treatment following the Thailand' s 2012 NTP Guidelines but no consecutively AFB-smear examination and culture results during the last 12-month treatment duration
Died	patient with any cause of death during MDR-TB treatment following the Thailand' s 2012 NTP Guidelines
Failed	patient treated with MDR-TB regimen following the Thailand' s 2012 NTP Guidelines with 2 positive results of the 5 consecutive AFB-smear examinations and cultures during the last 12 months of treatment duration or patient with 1 positive-culture result of the last 3 consecutive cultures or patient with physician' s decision to stop the MDR-TB treatment due to clinical unresponsiveness or various adverse-drug reactions
Default	patient with continuous interruption of the MDR-TB treatment following the Thailand' s 2012 NTP Guidelines
Transfer out	partially MDR-TB treated patient with referring from (Transferring out) one setting to another setting with unknown treatment outcomes will be registered as " Transfer out "

Table 3. Classification of treatment outcomes [24]

9. MDR-TB management and treatment outcomes in Thailand between 2007-2009

The prevalence of laboratory-confirmed MDR-TB was 0.08%. Highest prevalence (0.21%) was found in the central part of Thailand. MDR-TB was mostly diagnosed and treated at the secondary care settings or general hospitals (31.5% and 31.14%), 24.3% and 25.08% of cases were diagnosed at the tertiary care settings and only 6.9% and 6.7% of the patients were diagnosed at the university hospitals, respectively [9]. The majority of the patients (63.82%) were registered as " after failure of the first-TB treatment " [9]. In Thailand, numbers of the secondary care settings or general hospitals are more than that of the tertiary care hospitals, this may reflex the above figures. Only 33.5% of cases were referred to the well-facilitated setting for directly observed treatment (DOT) [9]. Only 60.6% of MDR-TB cases were prescribed 4 oral second-line drugs and an injectable aminoglycoside drug [9] which recommended by the Thailand' s 2012 National Tuberculosis Management Guidelines while the rests were prescribed various treatment regimens [24]. Only 57.5% of cases had completed treatment adherence [9]. Low DOT implementation can contribute to high default rates, high treatment failure rates, high death rates, and low cured rates. There was 24.2 % of patients with com-

pleteness of treatment, 29.1% cured, 20.5% default, 2.2% treatment failure, and 21.5% died [9]. Treatment failure and treatment default rates were higher among new case compared to the patients with previous TB treatment whereas higher death rates were found among the patients with previous TB treatment. This could be due to inadequately strict- and intensive-health education provision to the new cases and more severe disease at the time of diagnosis among the patients with previous TB treatment. Only 27.5% of cases with completed treatment were followed up more than 2 months [9].

A recent study in South Korea revealed that the treatment regimen was individualized based on the history of anti-TB drugs taken by the patient and the most DST result [69]. Three to seven anti-TB drugs were self-administered except injectable drugs during hospitalization [69]. Injectable drugs were prescribed for 6-7 months [69]. The total treatment duration was at least 18 months after sputum culture conversion [69]. If the medical treatment was expected to fail or had failed in patients with localized lung cavities, or bilateral lesions and anticipated adequate postoperative lung function, surgical resection was considered [69]. The treatment outcomes showed that 37.1% of patients had treatment success, and 4.5% of them died of all causes during the 3-4 years after treatment initiation [69]. The independent predictors of all-cause mortality were age, history of MDR-TB treatment, XDR-TB, and prothionamide resistance [69]. Currently, there is no DOTS programme implementation in South Korea [69] while Thailand has been implemented several years ago, but the treatment outcomes were better than that of Thailand [9, 69]. These different results of both projects should be intensively investigated and explained. Kliiman K *et al*. recently demonstrated that predictors of poor treatment outcomes in MDR-TB were previous TB treatment, ofloxacin resistance, positive-AFB smear, and HIV-infection/AIDS [70].

The criteria for capacity of establishment of the specialized MDR-TB centre that recommended by the Thailand' s 2012 NTP guidelines [24] are as the following : 1.authorized persons' recognition of the MDR-TB threats 2.good laboratory networks and good patient-referral system 3.good DOT system, and 4.consistently continuous care for MDR-TB patients.

10. XDR-TB treatment

A previous study in Peru by Mitnick CD *et al*. demonstrated that 48 (7.4%) of 651 tested-isolate patients had XDR-TB [71]. The results showed various individualized regimens prescribed for 47 patients with XDR-TB as the following : 1) 14.9% of the patients included ethambutol, 2) 34.0% included pyrazinamide, 3) 38.3% included streptomycin, 4) 19.1% included amikacin, 5) 53.2% included capreomycin, 6) 17.0% included kanamycin, 7) 21.3% included ciprofloxacin, 8) 12.8% included ofloxacin, 9) 42.6% included levofloxacin, 10) 2.1% included spafloxacin, 11) 72.3% included moxifloxacin, 12) 100% included cycloserine, 13) 66.0% included ethionamide, 14) 95.7% included para-aminosalicylic acid, 15) 100% included amoxicillin-clavulanate, 16) 44.7% included clarithromycin, 17) 97.9% included clofazimine, 18) 17.0% included rifabutin, 19) number of drugs in regimen (number without documented resistance or prior exposure for more than 1 month) : 19.1) 5.3 +/- 1.3 agents among all available agents, 19.2) 3.2 +/- 1.2

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agents among 12 agents or classes for which routine DST was performed, 20) median duration of treatment with injectable agents : 15.4 months, and 21) median duration of treatment ranged 8.0- 24.9 months, median duration from treatment initiation to surgery : 11.6 months, and median duration of treatment for patients undergoing surgery : 31.2 months [70]. The median duration of follow-up was 19.4 months [71]. Treatment outcomes revealed that 60.4% of patients were cured or completed treatment [71]. This study is currently the up-to-date information of XDR-TB treatment. Positive AFB-smear, and urban residence could be predictors of poor treatment outcomes in XDR-TB [70]. For patients with mono/poly-resistant drug (s) TB, the recommended treatment regimens are shown in the Table 4 (13).

Mono/Poly-resistant Drug (s)	Regimen
Rifampicin mono-resistance	initial 2 months of isoniazid, pyrazinamide, and ethambutol, and followed by 10-16 months of isoniazid, ethambutol and a fluoroquinolone +/- initial 6 months of an injectable drug
Isoniazid mono-resistance	initial 2 months of rifampicin, pyrazinamide, and ethambutol, and followed by 4-7 months of rifampicin, and a fluoroquinolone (750-1,000 mg of levofloxacin or 400 mg of moxifloxacin substituted for isoniazid in the standard 6-month short-course regimen)
Rifampicin and pyrazinamide (+/- streptomycin) resistance	at least the initial 2-3 months of isoniazid, ethambutol, a fluoroquinolone, and an injectable drug (initial 6 months if extensive disease) for 18 months of total treatment duration
Rifampicin and ethambutol (+/- streptomycin) resistance	at least the initial 2-3 months of isoniazid, pyrazinamide, a fluoroquinolone, and an injectable drug (initial 6 months if extensive disease) for 18 months of total treatment duration
Isoniazid and pyrazinamide resistance	9-12 months of rifampicin, ethambutol,and a fluoroquinolon (longer if extensive disease)
Isoniazid and ethambutol resistance	9-12 months of rifampicin, pyrazinamide, and a fluoroquinolone (longer if extensive disease)
Isoniazid, pyrazinamide, and ethambutol (+/- streptomycin) resistance	at least the initial 2-3 months (6 months if extensive disease) of rifampicin, a fluoroquinolone, an oral second-line drug, an injectable drug for 18 months of total treatment duration

Table 4. Treatment of patients with mono-drug resistant and poly-drug resistant tuberculosis [13]

11. MDR/XDR-TB treatment-pipeline agents or compounds in clinical trials and related innovative researches

Currently, drugs in phase III clinical trials are moxifloxacin, gatifloxacin, and meropenem [72]. Heteronemin, nephalsterol, litosterol, and kahalalides are other interesting compounds which are in pre-clinical stage [72]. Okada M *et al*. conducted a study on granulysin and a new DNA

vaccine against MDR/XDR-TB and revealed that agglutinating virus of Japan/Heat-Shock-Protein65DNA+Interleukin-12-12DNA vaccine provided strong therapeutic efficacy in killing MDR/XDR-TB bacilli in mice and monkey models [73]. A recent experiment using MDR-TB monkey models which received normal and genetically altered Bacilli Calmette Gue'rin (BCG) vaccines demonstrated that these 2 groups of monkeys survived well compared to the control group [74]. Another study in XDR-TB mice model showed ability of interleukin-7 to kill the bacilli [74].

12. Totally drug-resistant tuberculosis

Totally drug-resistant tuberculosis (TDR-TB or XXDR-TB) was recently defined as TB bacilli which resist to all first-line and the 6 second-line drugs (para-aminosalicylic acid, fluoroquinolones, aminoglycosides, thiamines, polypeptides, and cycloserine) [75]. Meanwhile, a recent report from the US-CDC listed 7 challenges that should be addressed before new terminology of TDR-TB should be considered for adopting [76], following are the challenges:

1. The definition should not hinge on resistance to all drugs tested, because the number of drugs tested varies widely between laboratories.
2. In vitro testing data suggest cross-resistance among different drugs within a class of drugs or closely related classes of drugs (e.g., polypeptides and aminoglycosides) is not 100%.
3. Research and reference laboratories in many countries do not test for resistance to the third-line drugs (linezolid, thioridazine, other phenothiazines, monobactams (meropenem, imipenem), macrolides, metronidazole and other imidazoles, clofazimine, and amoxicillin/clavulanic acid).
4. DST for several anti-TB drugs is not sufficiently reliable or reproducible; retesting the same isolate provides a different result in many cases.
5. There are several new anti-TB agents in development pipeline that will be prototypes for new classes of antimycobacterial drugs or add new chemical entities to existing class.
6. Avoiding the unintended implication that patients with TDR-TB should not or cannot be treated.
7. Global laboratory capacity for DST of *Mycobacterium tuberculosis* isolates remains limited [75]. Two cases of TDR-TB with controversies of terminology and treatment occurred in 2003 in Italy and firstly reported in 2007 [77]. Currently, in Thailand, patients with all anti-TB drug-resistance will be prescribed isoniazid alone for lifelong whereas no standardized treatment is recommended yet. During 21-22 March 2012, the WHO had convened a technical consultation to discuss the feasibility and implications of a definition to cover more advanced patterns of TB resistance than XDR-TB [78]. The WHO concluded that reports of severe patterns of anti-TB drug resistance (worse than XDR-TB alone) are increasing whereas a new definition of anti-TB drug resistance beyond XDR-TB is not recommended [78]. This undefined resistance patterns contributed to technical difficulties

with DST of several anti-TB agents, the lack of standardized DST methods for several present and new investigational drugs, and insufficient evidence to link such DST results to patients' treatment outcomes [78]. No DST methods for group 5 and new investigational agents currently exist [78]. Molecular DST for the second-line drugs cannot yet replace phenotypic methods [78]. Collaboration between the national TB control programme, Ministries of Public Health, and the pharmaceutical companies will be required to resolve the limitations of treatment options by the compassionate use of new anti-TB agents [78]. The WHO will be the lead in ensuring that better patient data provide a more robust information for future policy decision [78].

13. Conclusion

As countries are purchasing and using second-line drugs, the likelihood of misuse and developing of TB-resistant strains increases. Currently, WHO and its partners have reached the phase of expanding MDR-TB control as a component of a comprehensive TB control programme. Launching in 2002, the Global Fund to Fight AIDS, Tuberculosis and Malaria (GFATM) expected that requests for second-line drugs for MDR-TB management should go through the Green Light Committee to prevent their misuse. The number of Green Light Committee-approved MDR-TB control programme is increasing rapidly as a result of main streaming of MDR-TB management into general TB control efforts. Expanding projects and accelerating evidence gathering are essential to further develop international policies. The TB-endemic countries themselves and the ability of the technical agencies, as well as the donor community are the factors of future success to expand MDR-TB control programmes.

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Section 4

Extra Pulmonary Tuberculosis

Chapter 14

Tuberculous Pleural Effusion

Wolfgang Frank

Additional information is available at the end of the chapter

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1. Introduction

Tuberculosis (TB) has traditionally been one of the major causes of pleural disease and until the earlier decades of the past century held as a principal paradigm of "pleuritis". Indeed in the presence of a distinctly exudative effusion and a compatible clinical presentation the widely used term "pleuritis exudativa" insinuated a tuberculous aetiology and has therefore been understood to be synonymous with "pleuritis exudativa tuberculosa". Whilst in the era of TB decline in the Western hemisphere the term "pleuritis exudativa" (which actually is a tautology!) has largely survived but should now describe exudative effusions in general, the full and precise term "pleuritis exudativa tuberculosa" is therefore suggested whenever the possibility of a tuberculous background is addressed. Otherwise the term "tuberculous pleurisy" or "tuberculous pleuritis" is used to describe this entity, in some countries also the term "specific pleurisy" is common. Apart from acute pleuritis exudative tuberculosa, TB of the pleura may however rarely present as a rather chronic disease state in terms of caseous pleurisy or specific (i. e. tuberculous) empyema, respectively. The following chapter reviews the different features and mechanisms of tuberculous pleural involvement as well as their diagnostic and therapeutic implications.

2. Epidemiology

In many regions of the world tuberculous effusion maintains its role as the leading inflammatory pleural disease. With the worldwide unabated HIV epidemic and related immune deficiency syndrome this state of affairs is likely to continue or being even aggravated within least in certain high risk populations. On a global scale the current significance of human immunodeficiency virus (HIV)-co-infection may be illustrated by WHO data, indicating at a TB-prevalence of 1/3 of the world´s population – similar to the past decade – a HIV-association

of approximately 13 % by the year 2009 [1, 2]. Conversely it is assumed, that 33 % to 50 % of HIV infected individuals are co-infected with M. tuberculosis [2]. The MTB/HIV-association however shows a huge intercontinental and regional variance, with the highest rate of HIV-pleural tuberculosis-coincidence being reported in Zimbabwe where 95 % of Patients with tuberculosis pleurisy were HIV positive [3]. In Burundi and Tansania a HIV-coinfection was found in 60 % of all cases of tuberculous pleurisy [4]. One of the lowest rates is reported from Spain with 10 % [5]. Am example of the impact of a high HIV-endemic environment on the incidence of tuberculous pleurisy is also given in a series from Ruanda, where TB accounted for as much as 86 % of all diagnosed pleural effusions [4]. Pleurisy incidence obviously and essentially parallels variability of global TB prevalence with an overwhelming share of 95 % occurring in developing countries. In TB-patients as a whole, pleural involvement varies between ~ 3-5 % in Western Europe and the USA vs. ~ 30 % in developing, HIV-high-prevalence-countries [6, 7, 8]. The differences clearly underline the modifying role of immunological determinants, stage and severity of the disease, general health status and nutritional factors. The effect of HIV on the occurrence of pleural involvement in a given TB-patient is illustrated by a study reporting a 38 % pleurisy incidence in AIDS-associated TB as compared with 20 % in matched HIV-negative TB patients [5]. On the basis of the presented data according to even conservative WHO estimates the TB-pleurisy incidence throughout the current decade is expected to remain grossly unchanged compared to the past decade, i. e. 18.2 – 62/100.000 in the developing countries vs. 0.42-0.77/100.000 in Western countries [6, 7, 10]. When the epidemiology of pleurisy (or pleural effusion in general) is analysed in terms of the magnitude of TB-contribution, a probably still valid estimate in Western countries is as low as 0.1 – 0.2 % and remains distinctly < 1 % even when referring to pleurisy in a strict sense (i. e. exudates) [11]. By comparison the previously reported percentages of 30-86 % in developing countries are – and remain – indeed dramatically different.

3. Pathophysiology and natural history

3.1. Immunological and microbiological factors

MTB may affect the pleura at different stages of pulmonary or systemic disease and by a number of different mechanisms. Thus pleural involvement occurs in primary, postprimary and reactivated TB alike and is basically believed to arise directly from contiguous macroscopic or microscopic lung lesions or else lymphogenic or hematogenic spread, but probably also via immunogenic mechanisms. Pleuritis exudativa tuberculosa is by far the most common clinical variety and has been classically interpreted as an early delayed-hypersensitivity-type phenomenon rather than direct organ involvement [12, 13]. Many clinical observations and experimental findings are in favour of this hypothesis such as:

- its frequent association with known primary infection and a typical 6-12 weeks latency,
- an often striking absence of significant pulmonary or systemic TB-lesions,
- an often culturally negative or paucibacillary effusion [14],

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- the sometimes abundant isolation of specifically purified protein derivative (PPD)- protein sensitized T-lymphocytes from pleural fluid [15] and
- more recently the inducible pleurisy in previously PPD-sensitized animals when exposed to intra-pleural mycobacterial protein.

Also the vigorous expression of inflammatory mediators interleukins (IL) like interferon (IFN) γ, IL-1 and IL-8 observed in this model (or conversely their suppression by antilymphocyte serum) support this view [16, 17].

On the other hand there is also strong evidence, that infectious invasion of the pleural space actually occurs at a substantial, albeit variable degree. At thoracoscopy, even with negative fluids studies, extensive inflammatory granuloma formation and fibrin deposits with unexpected abundant mycobacteria recovery are a common finding (see also section on invasive endoscopic-bioptic studies) [18]. The increasingly emerging evidence of a preferred association of TB-pleurisy with reactivated TB in Western populations clearly points to infectious as well as immunological mechanisms being interrelated and operative in a complex manner. Direct infectious invasion however clearly prevails in chronic tuberculous involvement of the pleura as in specific empyema.

According to present views and based on experimental evidence the sequence of immunological processes involved in TB-pleuritis appears to follow a three stage pattern of cellular reactions and granuloma formation as a topic variant of general interaction mechanisms between MTB and the human immune system. A schematic representation is given in figure 1 [19, 20].

Any trigger-mechanism that allows access of mycobacterial protein to the pleura will set off a rapid mesothelial cell initiated and IL-8 mediated polymorphonuclear neutrophil (PMN) influx within a few hours [21]. In addition macrophages and blood-borne monocytes determine this IL-1, IL-6 and tumor necrosis factor (TNF)-α-orchestrated *early stage* reaction.

Within roughly 3 days, in the following *intermediate stage* lymphocyte subpopulations, mainly of CD4+ helper cells but also a substantial CD8+ cytotoxic (natural killer cells) fraction dominate the scene resulting in a CD4+/CD8+-ratio of ~ 4.3 [22]. A minor contribution includes so-called T-cell receptor double negative (DN) $\alpha\beta$-T-cells and $\gamma\delta$-T-cells which appear to have regulatory functions. More recently in tuberculous pleural fluid another unique CD4+CD25+ T-cell-class could be demonstrated being specifically involved in the down-regulation of autoreactive IFN-γ-producing T-cells, thus preventing inflammatory overshoot [23]. IFN-γ a strong promoter of macrophage activation and granuloma formation (together with TNF-α) is the predominant interleukin in this stage. IFN-γ-producing cells have been phenotypically indentified as CDW29+ subpopulation and make up a substantial portion of the granuloma core structure [24].

The *late phase* is characterised by an equilibrated and sustained CD4+/CD8+ cell-based response with continued IFN-γ release and prolonged granuloma formation. Several modulating interleukins are involved in this process such as T-helper-cells (CD4+)-supporting IL-12 and counter-regulatory antiinflammarory cytokines like IL-10 and transforming growth factor (TGF-β).

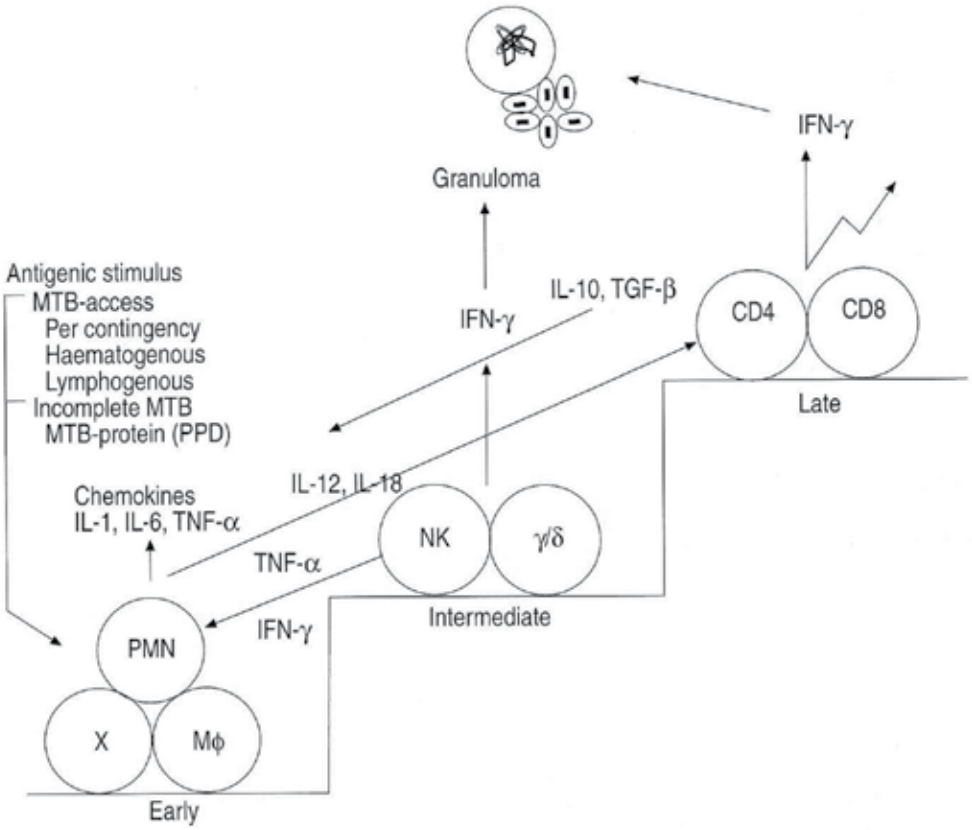

Figure 1. Mechanisms and immunogenesis of tuberculous pleurisy: the three stages of protective immune response. IFN: interferon; TNF: tumor necrosis factor; IL: interleukin; PMN: polymorphonuclear granulocyte; X: undefined cell; MΦ: macrophage, MTB: mycobacterium tuberculosis; PPD purified protein derivative

Results of HIV and AIDS research also emphasize the importance of T-cell response. Several working groups have shown, that the prevalence of tuberculous pleurisy in HIV-infected patients with TB is strikingly correlated with their CD4+ blood lymphocyte count. In one series pleurisy prevalence in individuals with a count of > 200 cells/ml was 27 % as compared to 10 % in those with a count of < 200 cells/ml [25]. The data support the view, that the clinical expression of exudative pleural effusion requires a largely intact cellular immune system and features pleurisy as a high activity response in a still immunocompetent individual. In epidemiologic terms one would conclude that pleural effusion should be more frequent in the still immunocompetent host than in patients with AIDS. In reality however in most HIV-high-prevalence countries like South Africa, Uganda and Zimbabwe the percentage of thoracic TB-patients with pleural effusion is reportedly higher in HIV+ patients [26]. As an explanation the situation is probably blurred by a variable and poorly defined immune status in HIV+ individuals.

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3.2. Other factors

The mechanisms of fluid accumulation and of abundant protein leakage to the pleura with often extensive fibrin deposits in tuberculous pleurisy have so far not been fully elucidated. In actual fact pleuritis exudativa tuberculosa generally presents with the highest protein levels commonly seen in exudative conditions. The intensity of inflammation and a proportionately increased vascular permeability would provide a satisfying explanation [25, 27] although at least in animal models, no such significantly altered vascular permeability could be demonstrated [12]. Current opinion holds that grossly impeded lymphatic protein clearance from the pleura due to altered parietal lymphatic channels is probably of tantamount importance.

Again the *entry mechanism* of mycobacteria to the pleura has remained unclear. It is usually assumed that the release of infectious material from a ruptured subpleural TB-lesion is the most common mechanism. While this is likely to occur in more or less extensive pulmonary TB, it would not explain the frequent association of tuberculous pleuritis with an – at least radiographically – unaffected lung. There are also no convincing data yet to quantify the contribution of a purported hematogenous or lymphogenous contribution. One might reasonably speculate that different patterns of pleural tuberculous involvement are operative which might correspond to the different clinical settings of primary, post-primary and reactivated TB.

Caseous tuberculous pleuritis or specific empyema is nowadays a rare condition which is believed to be the result of longstanding or chronic infection of the pleura, when either caseous material gains access to the pleura or chronic pleuritis develops on the background of impaired local defence such as pre-existing fibrous damage of the pleura or as a sequel and complication of artificial pneumothorax, oleothorax or other TB-specific surgery dating back to the pre-chemotherapy era. Correspondingly there is usually an extremely long history often with a remarkable paucity or even absence of symptoms. Penetration to deeper chest wall structures (specific abscess) and ultimately transcutaneous discharge (empyema necessitans) or creation of a specific bronchopleural fistula, as not infrequently seen in the pre-chemotherapeutic era, may complicate this condition [27]. Putrid discharge from a thoracic mass or putrid expectoration with or without haemoptysis may ultimately advert to the condition.

4. Clinical manifestations and natural course

Tuberculous pleurisy may occur as an acute, subacute or rather chronic disease. At times the course is also surprisingly oligosymtomatic. Therefore duration of symptoms or major illness prior to hospital admission and diagnosis varies considerably from < 1 week (31 %) to < 1months (62 %) or even longer (7 %) [28]. These data refer to the pre-HIV era and would not apply for HIV-seropositive patients and elderly populations, which both tend to have a particularly long symptomatic or else oligosymtomatic period. An infectious, i. e. febrile illness is nevertheless by far the most common clinical presentation. As a general rule, an acute febrile illness is the more likely to occur the younger and the more immunocompetent a given patient is. In developing, high-prevalence and high primary-TB-affected countries the age peak of incidence is in the mid

thirties, whereas in industrialized countries with a major contribution of reactivated TB it has shifted to about 50 yrs [29]. But still the age-related incidence peak of tuberculous pleuritis is distinctly lower than of parenchymal pulmonary TB which used to peak around 55 yrs [30]. Implicitly by the same statement in Western populations TB-pleurisy was historically more symptomatic than is currently the case. In a representative series from the 1960-1970s ~ 60 % of patients developed an acute illness mimicking bacterial (pleuro)-pneumonia with cough (70 %), chest pain (75 %) and low- to high-grade fever (86 %) as the most frequent symptoms [31, 32]. Other symptoms include those commonly occurring in various TB disease states such as weight loss, malaise and night sweat. Severe or even live threatening disease, defined as persistent high-grade fever > 38,3°C over > 2 weeks or respiratory distress has been in reported in a more recent series in only 7 % [31], whereas an oligosymptomatic or a febrile course is described in 14-33 % [32]. Tuberculous pleurisy usually involves one hemithorax only (90-95 %) and is of limited size (roughly up to one-half of the hemithorax volume). In a major series (n=254) effusions occupying more than 2/3 of a hemithorax were noted in only 18 % [33]. Rarely effusion will occupy the entire hemithorax and will almost never reveal compressive or displacing features [31]. Basically there are no specific clinical clues to tuberculous etiology in pleurisy unless some TB-contact is revealed or suspected. An HIV-related background may be suspected in a compatible clinical and history setting or when there is a long preclinical period, unusual additional symptoms like diarrhea and more hepato(spleno)-megaly or lymphadenopathy as might be attributed to the tuberculous condition. Untreated, lone pleuritis exudativa tuberculosa in the short term seems to be a self-limited inflammatory process in most instances, terminating in complete or incomplete resolution within weeks or month. Frequently observed otherwise unexplained diaphragmatic adhesions may be a late sequel of clinical silent or oligosymtomatic TB pleurisy. Importantly however progression or reactivation to active pleuropulmonary or extrapulmonary TB occurs in an important fraction. In one follow-up study the recurrence rate within 1 year was 5 %, where TB did not relapse earlier than 8 month after the onset of pleurisy. Within a 4-5 yr period however the rate was dramatically higher and in initially culture positive and culture negative subjects with 65 % and 60 % respectively roughly alike [27]. One major outcome determinant clearly is the presence and the extent of pulmonary involvement. At a similar therapeutic intensity in a very recent major clinical study from Taiwan, 51 (24,9 %) out of 205 hospitalised patients having been identified to have isolated (lone) pleuritis had a significantly better outcome, shorter hospital stay and less comorbidity than the patients with pleuropulmonary disease [34].

5. Diagnosis

5.1. Clinical findings

5.1.1. Signs at physical examination

Physical examination clearly will provide only non-specific signs of pleural effusion in general including dullness to percussion and the occasional demonstration of a pleural rub at auscultation ("snow-ball-crunching sign") in particular in the presence of chest pain. Signs of a trapped or loculated rather than free flowing fluid collection may suggest a tuberculous

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aetiology, but this observation holds also true for "plain" parapneumonic pleurisy. Usual signs of systemic infection, as mentioned above, that should be looked for, may alert to the possibility of a HIV-related background.

5.1.2. Imaging studies

Imaging techniques are engaged in the evaluation of tuberculous pleurisy following general diagnostic pathway recommendations for effusion. *Conventional chest radiography (CRX)* requires fluid amounts of at least 150 ml to become clearly detectable as blunting of the costodiaphragmatic angle in standard projections. Profuse effusion with opacification of an entire hemithorax would rather favour differential diagnoses like malignancy in the elderly and afebrile patient [35]. Free flowing effusion may be easily identified, but one should look specifically for signs of loculation, pleural thickening or adhesions and in profuse effusion for compressive signs interfering with the respiratory performance. Apart from pleural changes pulmonary infiltrates, nodules, lymphnodes and other suggestive signs of TB like encapsulated or cavitary lesions must be carefully looked for using routine *CT-imaging*. CT-based prevalence of lung perenchymal tuberculous lesions in mixed populations appears to be significantly higher than previously assessed based on conventional radiography. In one recent series from Korea comprising 106 patients with an age distribution from 16-89 yrs (mean 53) with 86% a remarkable high rate of parenchymal changes was found, presumed to represent active tuberculosis in 59 % [36]. Most of these lesions revealed features of reactivated rather than primary tuberculosis. *Sonography (Ultrasound, US)* using innovative technical achievements like high frequency (5-7.5 MHz) – US and convex or sector scanners allow extended exploration of the chest wall structures, the diaphragm and the anterior mediastinum up to a penetration depth of ~ 25 cm. Specific advantages of US are a more precise fluid volumetry than by CRX, precise localisation of septae, membranes and chambers as well as pleural thickening along with its particular versatility for bedside diagnosis. On demand guidance for interventions such as thoracentesis is a particular asset of US.. Examples are shown in figure 2, 3. *Magnetic-resonance imaging (MRI)* is a highly refined, not generally available technique, which will rarely be required but does have differential diagnostic merits in the analysis of critical borderline relationships i. e. distinguishing between inflammatory-infiltrative and malignant-destructive pleural processes via different T-weighted sequences [37]. Very recently a role of PET-CT has also been described. PET-imaging may indeed provide differently extensive focal and impressing laminar changes which however remain indistinguishable from malignant lesions [38].

5.2. Immunologic tests

5.2.1. Tuberculin skin reaction

The tuberculin skin reaction is traditionally considered an indispensable tool in the diagnosis of tuberculosis in general and likewise in tuberculous pleurisy although it is less reliable than in pulmonary TB. The rate of false negative reactions to PPD has been given as high 30 % of cases but even figures up to < 41 % have been reported [31, 32, 33], the variability possibly reflecting non-standardised test doses. Still however there remains an amazing false negative rate. There is no absolutely satisfying hypothesis to explain this paradoxon, let alone unequiv-

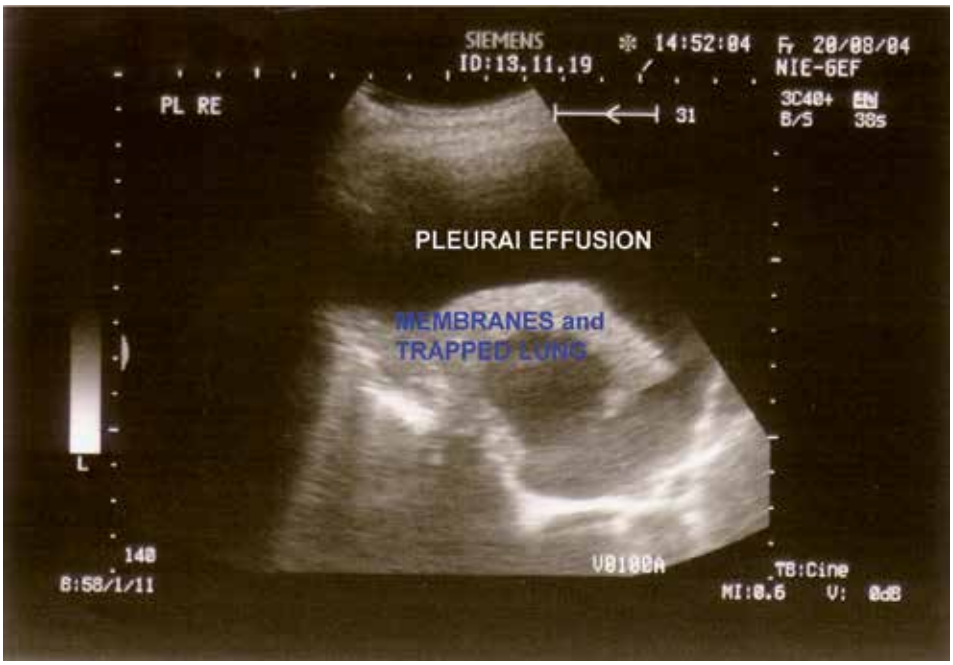

Figure 2. Ultrasound detection of inflammatory visceral membranes and consecutively trapped lung in pleuritis exudativa tuberculosa

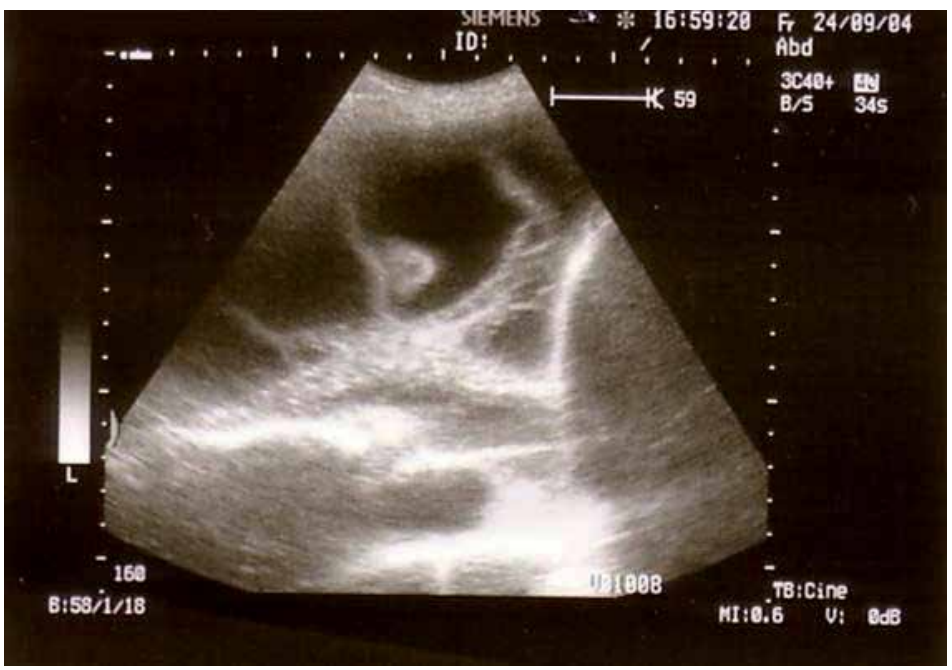

Figure 3. Ultrasound detection of multiple chambers in pleuritis exudativa tuberculosa

ocal experimental evidence. It appears a valid speculation to consider a local pooling of sensitised T-lymphocytes at the site of infection responsible. Animal experiments and clinical investigations have shown sequestration of PPD-sensitized lymphocytes to the pleural compartment actually to occur in the early phase of infection leading to their systemic depletion [39]. As a presumptive additional mechanism the presence of adhering suppressor cells to blood lymphocytes has been demonstrated in PPD-anergic patients [39]. While the explanatory evidence may remain scanty, it should be emphasised that in clinical practice the phenomenon appears to be transitory and restricted to the early phase of tuberculous infection. It might thus be associated with the pre-allergic phase of tuberculous infection, since conversion of skin reactivity has been subsequently observed within a 6-8 weeks delay [27]. As a reverse conclusion in the framework of discussed hypotheses the observation of a delayed PPD-conversion might be interpreted as a clue to primary infection to have occurred. Persisting anergy would then point to other immune-modulating factors like advanced age, certain drug interference or immune-compromising comorbidity.

5.2.2. Interferon-γ-release assays

In Europe commercially available Interferon-γ-release assays (IGRA) are the QuantiFERON-TB-Gold-Test and the T-Spot-TB-Test. Both use the MTB-RD1-region antigen sequences CFP10 and ESAT 6 and measure the specific lymphocyte-induced quantitative IFN-γ-response or the sensitized IFN-γ-producing lymphocyte response, respectively. There has been elaborated a body of clinical data in practical use highlighting both the assets and pitfalls of the investigation. In summary and in general there is distinct superiority to the PPD-skin-test with an overall sensitivity of ~ 85 % and a high specificity well > 90 % [40]. The concordance of the PPD-test and IFN-γ-release assays is in the order of 60-85 % [41]. However in the identification of active clinical tuberculosis blood-based IFN-γ-release assays also have revealed a considerable rate of false negative findings. In several studies including pulmonary a well as pleural tuberculosis, sensitivity was limited to 60-64 % [42, 43, 44]. There have also been a number of inconsistent and equivocal reports where the results obviously vary with different TB-prevalence settings (i. e. pretest probability). In a number of studies the variability of sensitivity ranges between 96 % in low prevalence settings down to 58 % in studies featuring high prevalence areas, also specificity setbacks are reported [45]. Blood-based IGRA´s therefore seem to share the limitations of PPD-testing. Since they cannot distinguish latent from active TB, in conclusion, the diagnostic value for identification of tuberculous pleurisy in high prevalence settings is very low and has even only limited value in industrialized countries.

5.3. Pleural fluid analysis

5.3.1. Biochemical parameters

When there is enough effusion to allow safe puncture and TB is suspected, *thoracentesis* is a mandatory diagnostic step. The effusion will be invariably and markedly exudative with a (unless in tuberculous empyema) clear, straw- to amber-coloured appearance and a mean protein content above 5.0 g/dl, in one series (n=83) it was 5.2 g/dl (range 3.5 – 7.0) [32]. Glucose and pH-values have traditionally believed to be characteristically low in TB. It appears however, that on the basis of more recent data, as also confirmed in the author´s own experi-

ence these values are not substantially different from exudates due to other aetiologies. SAHN [46] found pH-values < 7.29 and glucose values < 30 mg/dl in only 20% of patients and this has been confirmed by others [47]. Interestingly however, if low values actually occur, they appear to correlate with the pleural bacillary load and are to some extent predictive of cultural results. In one thoracoscopic study positive pleural fluid culture yield was 59 % when the glucose level was < 50 mg/dl but only 25 % when the glucose values were > 50 mg/dl ($p<0.005$) [18]. Lactic dehydrogenase (LDH) is a non-specific marker of pleural inflammation, which may be excessively elevated in tuberculous pleurisy, although with a mean value of 423 IU/ml (range 43 – 1.575) as reported in a representative series again does not discriminate TB from parapneumonic and not even from malignant effusion [32]. Adenosine deaminase (ADA) has been a promising and much hailed semispecific biochemical parameter. ADA is an inflammatory enzyme expressed predominantly by sensitized and activated T-lymphocytes. Isoenzymes (ADA2) in addition reflect to some extent monocyte/macrophage activation. Thus increased ADA-activity in general indicates various T cell/macrophage interactive inflammatory processes like granulomatous disease but also empyema and collagen vascular disease. It appears however particularly sensitive to TB. In a key study (n=129) in patients < 35 yrs a receiver operating characteristics (ROC) –derived cut-off level of 47 U/ml allowed distinction of tuberculous effusion from empyema, rheumatic and neoplastic disease with a 100 % sensitivity and 87.5 specificity. When empyema was eliminated, specificity and the positive predictive value even attained 100 % [48]. There are important limitations to the interpretation of these results and their clinical relevance:

- the data reflect the afore mentioned age group only, in more heterogenous groups both sensitivity and specificity have to be (down)-corrected to 95 % and 90 % respectively [22, 49, 50].
- the results strictly apply to high TB prevalence settings only and do not allow for different pre-test prohabilities [3].
- also immune suppression like in AIDS endemic areas may interfere with inflammatory ADA-release and invalidate diagnostic conclusions [3, 51].

Nevertheless, based on the most accepted cut-off level of 40 IU/l and provided its critical use in areas of at least intermediate TB-prevalence ADA determination must be regarded as a true diagnostic enrichment. An era of successful ADA-use has been recently summarized and confirmed by a large size metaanalysis (63 studies, 5297 tuberculous and non-tuberculous effusions) resulting in a sensitivity and specificity of 92 % and 90 % respectively [52].

5.3.2. Cytological analysis

Based on the immunological processes involved, a marked lymphocytosis is the predicted and characteristic feature of TB-pleurisy along with significantly increased total white cell counts as reflected in one representative study with a mean count of 2.309/mm^3 (range 30 – 24.009 mm^3) [32]. Usually 90 – 95 % of pleural fluid cells are T-lymphocytes, the remainder being B-lymphocytes and (mostly) activated mesothelial cells. Only exceptionally (in ~ 5 %) lymphocyte counts < 50 % may occur [27]. Thus when an 80 % lymphocyte reference line is chosen,

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pleuritis tuberculosa exudativa is by far the most frequent cause of pleural lymphocytosis [46]. Rarely, in particular in the early phase of inflammation fluid cytology may reveal neutrophil leucocyte (PMN) predominance. Expansion of the eosinophil compartment would be an extremely unusual finding. In the presence of significant numbers of eosinophils (> 5 %) differential diagnoses should be considered.

5.3.3. Microbiological studies

The microbiological yield from diagnostic (low volume) thoracentesis as far the smear is concerned is very low unless the whole effusion or large amounts are being centrifuged or the patient has a tuberculous empyema [14, 29]. In HIV positive individuals, particularly in those with CD4 cell counts < 200 x 10^6/l significantly higher yields are being reported amounting in one study to 37 % vs. 0 % in non HIV-patients [53]. In a comprehensive study on microbiologic smear findings in pleural fluid specimens in non-selected HIV negative out-patients, the positive acid fast smear yield (n=232) again was actually zero [54]. Cultures should be obtained both from the sputum and pleural fluid. The positive cultural yield from pleural fluid has been given in collective reviews with 10 – 35 %, being ~ 25 % in the mean [14, 30]. In one of the largest series (n=100] the sensitivity of pleural fluid culture was 28 % [18, 55]. The use of radiometric or non-radiometric liquid culture systems (BACTEC, MB/BacT, MGIT) will markedly accelerate results and possibly lead to an enhanced yield (~ 50 %), when bedside instead of laboratory inoculation is used [56]. The yield of sputum cultures in tuberculous effusion is expectedly largely dependent on the extent and nature of pulmonary involvement and may mount up to ~ 50 %. In the non-expectorating patient the use of induced sputum is advised [57]. The positive yield is also believed to be higher in HIV-infected patients [53, 57]. In the complete absence of pulmonary lesions according to most sources the sensitivity will be no more than 4-7 % [30]. Only exceptionally a surprisingly high figure of 31 % for induced sputum has been reported [57].

5.3.4. Immunological and molecular studies

Immunological studies of pleural fluid in TB-pleurisy focus on the measurement and analysis of chemokins and interleukins that are characteristically associated with the tuberculous immune response. TNFα and IFNγ revealed at a cut-off 140 pg/ml a sensitivity of 94 % and a specificity of 85 % [58,60]. Similarly as for ADA the major confounders were bacterial empyema and parapneumonic effusion respectively. Interestingly TNFα did not attain enough discriminatory power to separate TB from various inflammatory conditions and is no more considered a valid option in the diagnosis of TB. More recent meta analysis-derived collective data from 22 studies resulted in an overall sensitivity of 89 % at a 97 % specificity [61]. Thus at present IFNγ-determination in pleural fluid – contrasting to systemic IGRA-application – would appear a useful diagnostic test with a sensitivity and discriminatory power comparable to that of ADA-determination if one was to accept the significantly higher costs and disregard more powerful diagnostic options as provided by subsequently discussed invasive biopsy techniques.

Molecular mycobacterial identification methods employing a variety of *nucleic acid amplification techniques (NAAT)* have been applied in TB pleurisy with considerable enthusiasm and expectations ever since their first application in TB in 1989 [62]. The techniques that have been used include target amplification (polymerase chain reaction, PCR), strand displacement amplification (SDA), transcription mediated amplification (TMA), probe/primer amplification (ligand chain reaction, LCR) and Q-Beta replicase amplification mostly with the IS 6110, 16S recombinant ribonucleic acid (rRNA) and 65 XD target sequence [63-66]. So far published data, both biopsy- and pleural fluid-based have shown considerable variance of diagnostic yield, which ranged from 20-81 % as to sensitivity with an expectedly high specificity in the order of 98-100 % (table 1). When analyzing the sources of this high variance, apart from technical factors like contamination-related "carry over" or amplification inhibitors, the most important determinant appeared to be the number of bacilli in the pleura fluid or specimen sample [31]. Although theoretically requiring the presence of merely one microorganism to trigger amplification, similar to sputum anlysis, failed to detect pleural MTB in particular when the pleurisy was paucibacillar, correlating with cultural negativity. In addition to fluid samples numerous studies have evaluated the value of various nucleic acid extraction and amplifications techniques in formalin-fixed and paraffin-embedded pleural tissue specimen [67-71]. With the use of commercial kits of both DNA amplicons (ligand chain MTB assay, LCxMTB or AMPLICOR MTB) or RNA amplicons (amplified MTB direct test, AMTDT) according to the latest currently available sources, the sensitivity of each single technique did not exceed 63.2 % albeit at an expected 100 % specificity [71]. The so far largest meta-analysis including 40 studies and featuring commercial as well as in-house ("home-brew") tests, confirms a low and heterogeneous sensitivity (in the mean 62 %) and high specificity of 98 % [72]. Thus there is no convincing evidence, that generally and especially in the critical issue of paucibacillar (cultural negative) pleurisy, NAATs perform substantially better in tissue than in effusion specimens (table 1). Although in-house assays have been reported to be slightly superior [73], there remain significant sensitivity set backs both in liquid- and tissue-derived specimen. In summary NAATs may offer certain advantages like quick results within hours or added specificity. They may also improve sensitivity in combined and parallel use with conventional methods and multiple amplicors (diagnostic confirmation), but can certainly not replace or obviate the need for conventional tools in the diagnosis of TB pleurisy.

5.3.5. Invasive bioptic and endoscopic studies

Bioptic techniques in the evaluation of tuberculous effusions incorporate closed blind or imaging guided needle biopsy and medical (video)-thoracoscopy. Only exceptionally, if ever, surgical diagnostic efforts including video-assisted surgical thoracoscopy (VATS) would appear appropriate. Invasive techniques are indicated when clinical investigation and pleural fluid analysis provide only ambiguous or conflicting results and this is particularly true if relevant differential diagnoses like malignancy need to be reliably excluded. *Needle biopsy* may be considered a first step. There are no clear preferences as to the type of needle to be used, although in the author´s opinion the Tru-Cut or Raja-system may be preferable to the older Abrams- or Ramel-needle by providing a larger specimen along with easier handling. It is recommended, that at least six biopsies are obtained, since they will not regular-

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First Author	case-# TB / non-TB	Amplicor Kit	Sensitivity [%]			Specificity [%]
			overall	culture-positive	culture-negative	
effus.-based						
deWit	53/31	336 r.squ.	81	-	-	78
Lassence	14/10	IS 6110	60	100	50	100
		65 XD	20	66	8	100
Querol	21/86	IS 6110	81	100	60	98
tissue-based						
Salian	25/ 35	IS 6110	73	-	-	100
Marchetti	26/ 11	IS 6110	80-87	100	73-82	100
Gamboa	67/ 97	AMTDT	83	-	-	100
Palacios	18/168	LCxMTB	90.4	-	-	98.5
Ruiz-Manzano	57/ 17	AMTDT/ LCxMTB	80.7	-	-	100
				-	-	100
Pai (eff + tiss)	metaan	various	63.2	-	-	100

Table 1. Role of Nucleic-Acid-Amplification-Techniques (NAAT) in the Diagnosis of Tuberculous Pleuritis

ly contain a representative parietal pleural sample [74]. With this premise and the expected yield of at least two valid samples closed needle biopsy should be diagnostic in tuberculous pleurisy in ~ 60 % of cases, when histology and tissue-, as well as fluid-culture are being combined. In a major series (n=100 %) a 61 % positive yield was composed of 51 % biopsy yield and 28 % positive fluid culture (figure 4) [18, 55]. Distinctly higher yields have also been reported in the literature, leading in a collective review to an average sensitivity of 69 % (range 28-88 %) [75]. The difference and wide range is likely to be due to technical disparities and inclusion of data originating from largely different prevalence areas. In one study from a high prevalence area (South Africa) comprising 51 patients with undiagnosed pleurisy the positive closed needle yield in tuberculous pleurisy (histology+AFB-stain+culture) was 79%, when combined with pleural fluid ADA-determination and a lymphocyte/neutrophil ratio > 0.75 sensitivity increased to 93% at a specificity of 100% [76]. Thus with the parallel use of less invasive parameters needle biopsy approaches the diagnostic potency of more invasive techniques and would appear the second best diagnostic option in areas with limited medical logistics and resources.

Medical thoracoscopy as a *"window to the pleural space"* [77] is the gold standard procedure in the evaluation of exudative pleural effusion, hence also pleural pleurisy. The current and future role of thoracoscopy needs to be redefined for its diagnostic and interventional efficacy in the light of its close historical affiliation with TB. In fact tuberculosis was already a major focus of medical thoracoscopy or *"pleuroscopy"* as referred to and initiated by JACOBAEUS back in

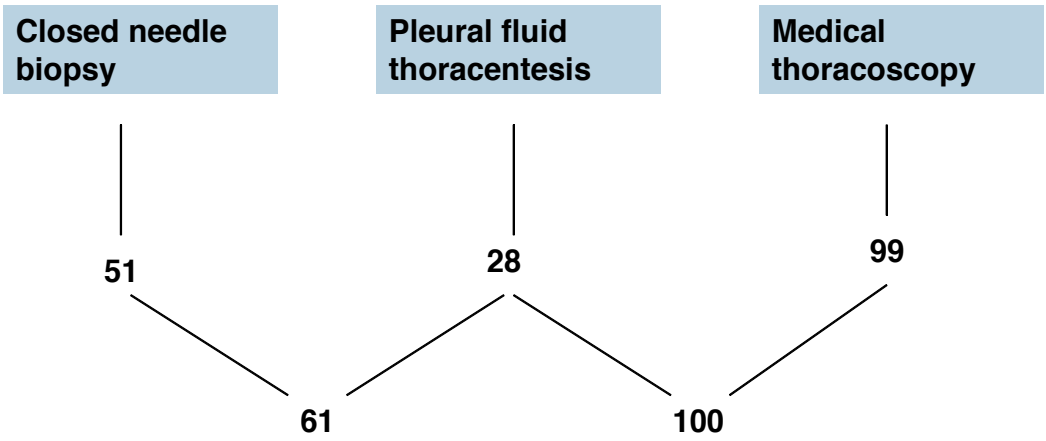

Figure 4. Single and cumulated yield (%) of various microbiological and bioptical investigations in tuberculous pleurisy

1910 [78]. Anticipating modern minimally invasive surgical techniques, now all included under the heading *video-assisted thoracic surgery (VATS),* his pioneering approach to thoracoscopy was basically interventional with the intention to optimize pneumolysis and to break strands for artificial pneumothorax induction in pulmonary TB *("Jacobaeus operation")*. However the ability to visualize major portions of the pleural surface, to intervene in the presence of membranes, adhesions and septae with the option of numerous dedicated biopsies also ensures optimum diagnostic results that are reflected in a yield of 94-99 % as confirmed in decades of clinical experience [18, 77-81]. At thoracoscopy tuberculous pleurisy usually appeals to the experienced investigator with characteristic and fairly diagnostic inflammatory patterns.

- One may present with abundant fibrinous membranes, septae, loculations and diffuse inflammatory thickening of the parietal and visceral pleura as the prevailing pattern. An example of this endoscopic pattern is shown in figure 5.

- A second characteristic feature is a more or less intensive seeding of the pleural surface with solid or caseous, sago-like nodules and only scanty fibrin deposits as shown in figure 6. Although usually fairly small, major nodules as also shown in figure 6 may easily be confused with malignant lesions.

- *Tuberculous empyema* as exemplified in figure 7 may be visually indistinguishable from non-specific bacterial empyema unless calcifications, irreversible lung trapping or suspect pulmonary lesions suggest a tuberculous origin.

Similarly to closed needle biopsy a sufficient number of biopsies – at least three- should be obtained to warrant optimum and representative results. This may often require mechanical debridement of membranes and septae to gain access to the inflamed pleura. When thoraco-

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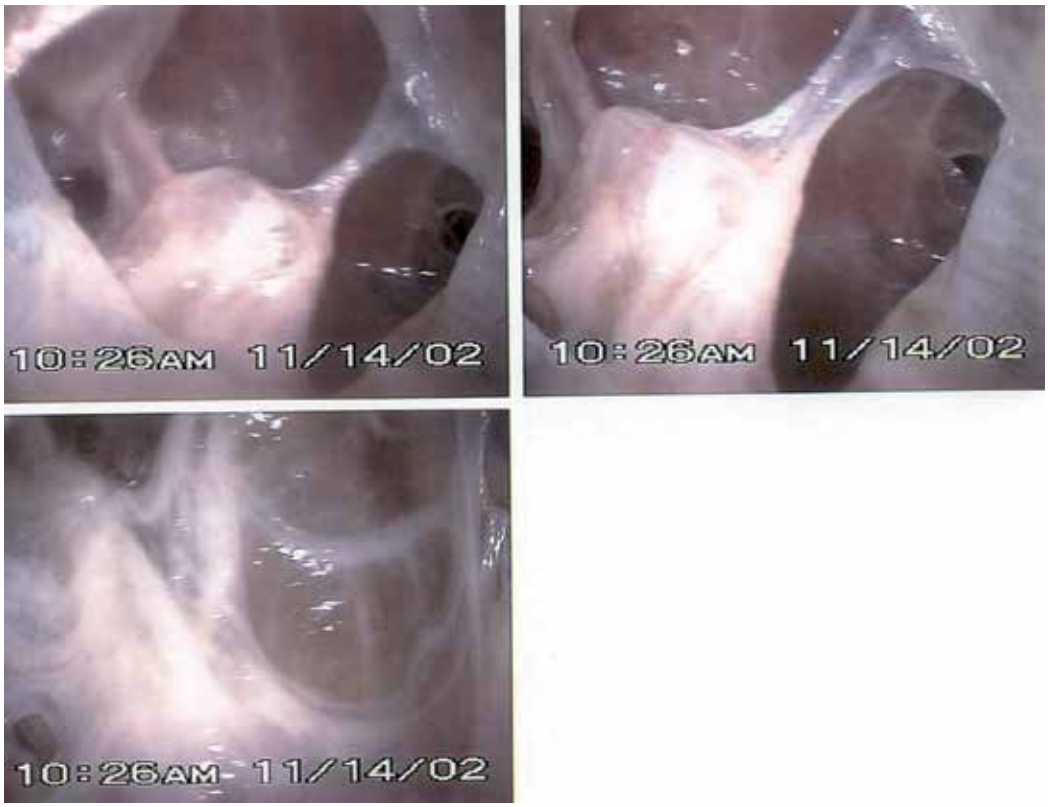


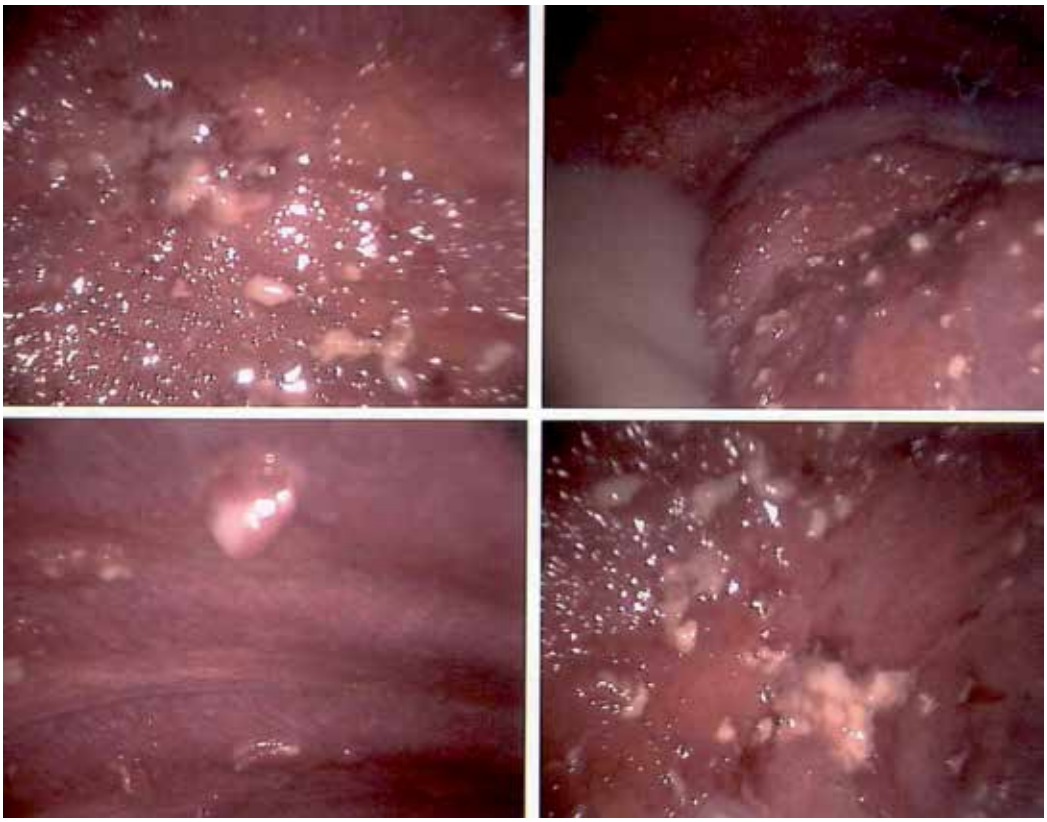
Male 48 years, pleuritis tuberculosa exsudativa

Figure 5. Typical thoracoscopic aspect of fibrin-type multi-loculated effusion including septae and chambers in tuberculous pleurisy

scopic results are combined with aforementioned techniques positive results may be augmented to virtually 100 % (fig. 2) [18].

Thoracoscopy also provides a number of additional advantages:

- With the reasonable diagnostic certainty of visual findings combined with an immediate histological yield of > 90 % it allows instant implementation of antituberculous chemotherapy
- The percentage of positive TB-cultures obtained from biopsies and fibrous membranes may be twice as high (78 %) as from needle biopsies and pleural fluid combined (39 %) [77]. This in turn provides superior opportunity for drug susceptibility testing.
- Complete removal and subsequent drainage of pleural fluid with pulmonary re-expansion provides instant relief to the patients and warrants better healing and outcome options (see section on therapy).



Male 24 years, pleuritis exsudativa tuberculosa

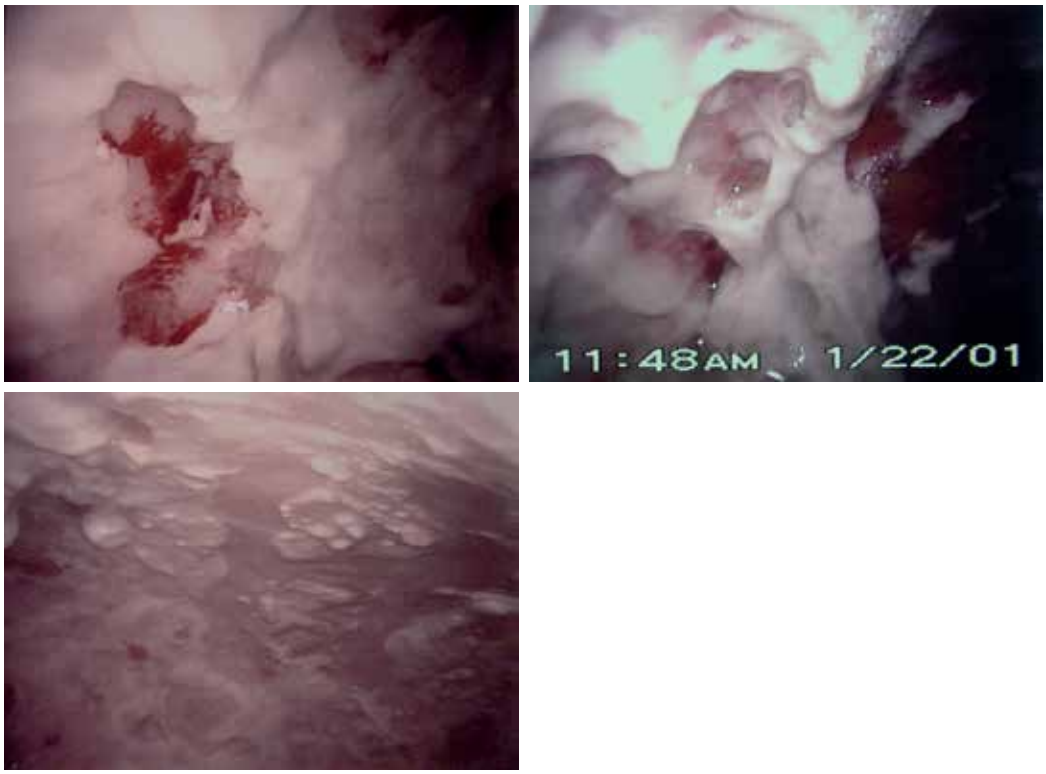
Figure 6. Typical thoracoscopic aspect of sago-type disseminated small and larger nodules both of the parietal and visceral pleura giving rise to confusion with malignancy

- In addition thoracoscopy may be easily expanded to an adjuvant therapeutic intervention by breaking adhesions and debridement of membranes as also discussed in the section on therapy.

In the overall assessment of biopsy techniques the experienced investigator will therefore bypass closed needle biopsy and prefer thoracoscopy. Closed needle biopsy however will remain the second best alternative if

- there is no logistic option for thoracoscopy or
- in the presence of clinical obstacles such as contraindications or mal-detachment of the lung due to adhesions or advanced obliteration of the pleural space.

In summary, for the diagnosis of tuberculous pleurisy it appears and remains a well-founded clinical policy to push for the recovery of biopsy specimens whenever possible and to combine these with less invasive test results to ensure optimum management of the condition.



Male patient, 48 yrs

Figure 7. Typical thoracoscopic aspect of tuberculous („specific") empyema showing putrid parietal coverings including bioptic lesion and circumscript coin-like pleural thickening

6. Therapy options

6.1. Systemic therapy

Basically systemic therapy of tuberculous pleurisy in the moderately ill patient neither differs in intensity nor duration from antituberculous chemotherapy of pulmonary and other organ tuberculosis in general. Current short term recommendations for non-complicated pulmonary and extrapulmonary organ tuberculosis call for a quadruple drug therapy in the 2-month acute phase in the combination of 5 mg/kg Isoniacid (INH), 10 mg/kg Rifampicin (RMP), 30-35 mg/kg Pyrazinamid (PZA) and 20-25 mg/kg Ethambutol (EMB) or 15 mg/kg Streptomycin (SM), where daily alternation of the SM and EMB component may be preferable [82]. In the second 4-month stabilizing phase a INH/RMP dual therapy is recommended. Until the 1990-years a triple therapy in the initial phase for Tb was considered safe enough in view of a low incidence of primary drug resistance. The quadruple therapy recommendation is therefore an amendment to a meanwhile globally changed drug susceptibility situation. Thus in an un-clarified clinical setting the extent of drug resistance expectation will modify treatment strategies. The

current policy in the case of tuberculous pleurisy therefore holds, that in lone and paucibacillar pleurisy (without lung parenchymal lesions) after immediate quadruple therapy a down-grading to a historical triple scheme is safe enough, provided full drug susceptibility is warranted. In the presence of lung parenchymal involvement the full standard scheme would however apply. The current average drug resistance probability is reflected in one major series (n=78) with a rate of 6.4 %, being probably representative for Middle Europe [55].

The addition of an oral or parenteral steroid regimen to antituberculous drug therapy has been discussed controversially. The rationale put forward for this approach focuses on

- the assumption of a shorter, attenuated clinical course in the severely ill patient,
- improved outcome by prevention of sequels in terms of pulmonary encasement and fibrothorax.

Three valid clinical studies employing a randomized, double-blind controlled design may be considered to have basically settled the issue [83, 84, 85]. These studies consistently showed, that a tapering steroid therapy for 4 and up to 12 weeks starting with 0.5, 0.75 and 1.0 mg/kg/day prednisone added to a standard antituberculous drug regimen, although mitigating and shortening the clinical course to a moderate extent in two studies, did not alter any of the outcome endpoints (clinical status, effusion resolution, pleural sequelae, lung volume and gas exchange). In conclusion from these data, steroids would generally not appear indicated in TB-pleurisy, a reasonable practice however would be a temporary use in the presence of a severe febrile and consumptive clinical course. Their long term use for the prevention of fibrotic sequels would appear obsolete.

6.2. Local therapy

Local therapy is an option, which is usually directly derived from a thoracoscopic approach to the management of the condition. First of all it needs to be emphasized that the (possibly complete) evacuation of pleural effusion is already an important topic treatment approach. While this can basically be achieved by non-endoscopic techniques like simple thoracentesis and small bore catheters as well, there is no doubt, that thoracoscopy will be disparately more effective due to the ability of visual guided optimum positioning and the use of large bore drains. In addition there is ample clinical evidence, that expert medical thoracoscopy can open intrapleural loculations and chambers, completely evacuate sequestrated effusion compartments and also to some extent produce effective debridement of membranes. Although no controlled study has so far proven the value of such efforts, from the view of the expert endoscopist it would appear a straightforward and convincing approach. Together with the early induction of antituberculous chemotherapy it might be responsible for the fact that in our institution over more than two decades of experience none of the patients needed decortication subsequently.

Another more recently discussed approach in topical therapy would be, by a rationale analogue to non-specific bacterial empyema, the use of fibrinolysis (streptokinase) which even need not necessarily be linked to an endoscopic protocol. There is so far only scanty experience [85]. However one fairly comprehensive study from Taiwan using a non-endoscopic pigtail

catheter technique and comparing a loculated streptokinase group (n=22) with a loculated normal saline irrigation group (n=22), reported significantly better outcome both in clinical terms of imaging and functional criteria [86]. Additional future evidence provided, this would seem an encouraging step towards further improvement in acute tuberculous pleurisy management.

Surgery in the era of antituberculous chemotherapy is only exceptionally required in the management of tuberculous involvement of the pleura. Remaining indications refer to rare instances of previously mentioned tuberculous empyema and in particular its complications. Specific issues in this context would be excessive membrane formation with trapped lung and significant long term pulmonary encasement due to fibrothorax. Due to the scarcity of pertinent cases and studies (at least in the western hemisphere) there are no generally accepted surgical guidelines for the management of these conditions. Surgical decisions must be created in an individual case-determined approach. A reasonable policy would appear to perform lone or combined empyemectomy/pleurectomy, also termed *early decortication* in clinically severe and functionally disabling conditions refractory to medical efforts. These indications may be amenable to video-assisted thoracic surgery (VATS)-based interventions. Formal thoracotomy would however be required if it comes to additional lung parenchymal resection or thoracoplasty in rare complicated cases e.g. with persisting pyopneumothorax with or without trapped lung due to a large, medically intractable broncho-pleural fistula.

A different issue is severe, chronically trapped lung due to fibrothorax. A reserved approach to surgical strategies is generally advised because unexpected long term remission of inflammatory peels is sometimes impressing. Although decortication has been performed as early as 6 weeks after the precipitating insult (empyema), the indication to *late decortication* is basically discussed in the context of definitely and irreversibly trapped lung (fibrothorax) i.e. when at least 6 months have elapsed. With a focus on repair of lung function and prevention of chest deformity most investigators agree that the indication requires a significant decrement of lung function (TLC<60% pred., reduction of perfusion>50%) and level of deformity in the absence of significant calcifications (*pleuritis calcarea*). Even then with extensive fibrotic fusion of both pleural sheaths not only will surgery be fraught with considerable technical problems but also the certainty and extent of functional improvement may not be predictable and warranted.

6.3. Sequels and prognosis

There are largely diverging data as to the prevalence of fibrothorax and permanent pleural thickening as the most important sequel of pleural TB. In one source based on standard radiographs in pleuritis exudative tuberculosa a percentage as high as 49 % has been given [32]. With the strict definition of fibrothorax as a pleural membrane of at least 5 mm thickness extending across major portions of the hemithorax and persisting > 8 weeks after initiation of therapy a figure of ~ 5 % is a more likely and widely accepted rate of this complication. The intensity of pleural inflammation expressed as interleukin levels and derangement of biochemical parameters is assumed to be to some extent predictive for this complication. In one study residual pleural thickening was indeed significantly correlated with the magnitude of the intitial change of inflammatory glucose-, pH- and TNF-α-levels [87].

Caseous tubercus pleurisy and specific empyema respectively is in its natural course and in prognostic terms an entirely different entity. These patients will invariably and typically develop an extensive calcified fibrothorax (pleuritis calcarea) with or without concomitant chest deformity. Also chronic non-specific lung disease (COPD) with or without bronchiectasis, late TB-exacerbations and internal or external fistulisation (specific empyema necessitans) may develop. Anecdotical occurrence of non-HODGKIN lymphomas arising from long term smouldering encasements has also been described.

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Chapter 15

Neurotuberculosis and HIV Infection

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Additional information is available at the end of the chapter

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1. Introduction

The incidence and mortality of tuberculosis (TB), the most common opportunistic infection in HIV patients has drastically increased with the emergence of the HIV pandemic

The HIV infection supported the re-emergence of TB as well as two major changes in the natural history of TB, namely it has increased the frequency of extrapulmonary TB and the mycobacterial multidrug resistance. The extrapulmonary TB involvement is present in up to 40% of the HIV cases and includes respiratory, digestive, lymphatic and neurologic localizations. Of these neurotuberculosis (NTB) is probably the most devastating extrapulmonary form of TB. The risk of acquiring NTB in HIV patients has been reported as 10 times higher than in non-HIV individuals and its related mortality exceeds 50%. The prognosis is further worsened by the HIV related progressive immunodeficiency which leads to the reactivation of opportunistic infections and the development of malignancies. The early diagnosis of NTB in HIV positive patients improves the short and long term prognosis of these patients and increases their life expectancy. Unfortunately the complexity of the clinical presentation and the variability of the bacteriological results accounts for significant difficulties in the diagnostic confirmation of NTB. Therefore treatment in these patients is often empirical. Moreover the antituberculous treatment is of long duration with serious adverse effects. Ensuing complications during treatment include the immune reconstitution inflammatory syndrome (IRIS) - a complication that is characteristic for HIV patients undergoing treatment for TB. Furthermore the multiple drug interactions between the antituberculous and antiretroviral treatment require close supervision of these patients.

This chapter summarizes the epidemiological, pathogenic, clinic and therapeutic challenges of NTB in HIV patients.

This chapter summarizes the epidemiological, pathogenic, clinic and therapeutic challenges of NTB in HIV patients.

2. Epidemiological data on the HIV/TB co-infection

TB is preventable and curable and its eradication was considered possible before the spread of the HIV pandemic. Since then the pathogenic mechanisms of HIV and TB have been closely entwined. Such is the complementary evolution of HIV and TB that the HIV/TB co-infection has been referred to as a ''syndemic'' by some authors [1]. The term ''syndemic'' reflects the similar social, epidemiological and pathological settings of both diseases. The close interrelation between HIV and tuberculosis overcomes by far the interactions between other community acquired infections. Thus epidemiological studies suggest that as many as 50% of the HIV patients develop mycobacterial infections. The rate of extrapulmonary TB could account for more than 50% of cases presenting with HIV and TB coinfection. In the pre-AIDS era the immunodeficiency status incriminated in the pathogenesis of extrapulmonary TB was induced by autoimmune diseases, aging, diabetes, alcoholism, malnutrition, malignancies or immunosuppressive chemotherapy. However the total amount of extrapulmonary TB in non-HIV immunosupressed patients did not exceed 15% of all TB cases. In addition meningitis and other forms of NTB represented less than 1% of all TB cases in non-HIV patients [2,3] but presently account for 10% of all TB cases in HIV patients [4]. Tuberculous meningitis (TBM) occurs in 5%-8% of the HIV patients [5,6] but tuberculomas and abscesses are also a common finding in late stages of AIDS [7]. Regarding the CNS infection with non-tuberculous mycobacteria one of the most important risk factors is the progressive immunodeficiency induced by HIV infection.

Co-infection with HIV not only increases the risk for central nervous system (CNS) TB [17] but also alters the clinical signs, delays the diagnosis and worsens the prognosis [8]. Thus the mortality of HIV patients with TBM is as high as 63% and nearly half of deaths occur in the first 21 days [9].

3. Pathogenic mechanisms of NTB

TB is a respiratory infection with a generally latent course. The immunodeficiency status favors the extrapulmonary dissemination of mycobacteria leading to inflammatory granulomas with diverse localisations. Some granulomas arise adjacent to the meninges or to the brain parenchyma and become the last station before the CNS invasion. Disruption of these granulomas into the subarachnoid space is followed by the cerebrospinal fluid (CSF) invasion with mycobacteria and meningeal infection.Release of mycobacteria from these granulomas is mainly associated with the severe depletion of macrophages and lymphocytes along with the imbalance of local cytokines. The CSF inflammatory reaction induced by mycobacteria antigens leads to a lymphocyte and fibrin-rich subarachnoid exudate which progressively envelops the blood vessels and cranial nerves. The expansion and intensity of this inflammatory exudate induces multiple complications including: the obliterative vasculitis followed by cerebral infarctions, the CSF obstruction and emerging hydrocephalus and the spinal extension of TB and chronic arachnoiditis. Some of the CNS granulomas could evolve as cerebral or spinal

masses further developing into tuberculomas or tuberculous abscesses [10,11,12]. In addition HIV patients characteristically present several TB cerebral lesions evolving simultaneously.

Below we enlisted the factors involved in the clinical progression and persistent CNS invasion with mycobacteria in HIV patients.

1. *The cellular immunosuppression in TB and HIV infection.*

The site of extrapulmonary mycobacterial infections and especially the CNS invasion depend on the efficacy of cell-mediated immunity. Both the HIV infection and TB trigger complex mechanisms which increase the cellular immunosuppresion.

On the other hand humoral immnity is increased but inefficient. The high titres of antimycobacterial antibodies are not protective and could instead result in numerous complications. The most important mechanism behind the cellular immunosuppression in the HIV-TB coinfection is the severe depletion of macrophage and lymphocyte cells.

Macrophage and lymphocyte cells. Macrophages play a crucial role in both HIV and mycobacterial infections. As phagocytes of the innate immunity they are considered the main cells involved in the immune response against mycobacteria.Infected macrophages recruit additional immune cells such as dendritic cells and T cell lymphocytes and release numerous chemokines and cytokines to form granulomas. The latter are specific stable inflammatory structures limiting the growth of mycobacteria. At the same time mycobacteria could develop inside macrophages from granulomas thus ensuring their persistence. In addition macrophages infected with Mycobacterium tuberculosis (M. tbc) augment the expression of the C-C chemokine receptor type 5, also known as CCR5, the most important HIV coreceptor [13]. Therefore infected macrophages perform a significant role in the protection and transport of mycobacteria and HIV to other tissues including the brain.

With the passing of time some of the macrophages infected with mycobacteria suffer apoptosis leading to a numeric decrease of the most important cells involved in the defence against mycobacteria invasion. Moreover HIV is directly responsible for the depletion of CD4+ T lymphocytes through its cytopathic effect and anti-gp120 antibodies. The depletion of CD4+ T lymphocytes raises the susceptibility to TB and most notably towards neurologic forms of TB [14]. In this respect the decreasing CD4+ T cell count was proven to vary inversely with the incidence of NTB. Most patients with HIV and NTB display a CD4+ T cell count below 200 cells/ mm^3 unlike patients with pulmonary TB who commonly present with a CD4+T cell count, between 250 and 550 cells/ mm^3. In conclusion in the late stages of infection the main pathogenic mechanisms of invasion with mycobacteria and HIV are closely interwined.

The Cytokine dysregulation. Both HIV and mycobacteria are intracellular pathogens. Their presence stimulates the release of cytokines by macrophages and Th1 cells which in turn regulate the cells involved in the immune response. The stability of the granuloma is usually ensured by a high number of CD4+ T and CD8+ lymphocytes along with a Th1 cytokine profile represented by IFN -γ and TNF-α. [15].TNF-α is a pro-inflammatory cytokine released at high levels by CD4+T cells and macrophages coinfected with mycobacteria and HIV. The role of TNF-α in the clinical outcome of the 2 diseases is contradictory. Regarding its role in the control of tuberculo-

sis a high level of TNF-α stimulates the apoptosis of infected macrophages and the cellular activation [16,17]. On the other hand the use of TNF-α neutralizing antibodies in inflammatory diseases has been associated with an increased risk of extrapulmonary TB including TBM [18].CD4-T-cell deficient mice [19] as well as mice able to neutralize endogenous TNf- α [20] or the gene for IFN-γ [21] are subjected to fatal TB. Nevertheless an in vitro experiment on human monocytes noted that higher levels of TNF-α could be associated with more virulent or faster growing mycobacterial strains [22].The contradictory effect of TNF-α was also observed in the HIV infection. Studies conducted by Lane and Osborn proved that TNF-α is a potent inhibitor over the primary HIV infection of the macrophages but enhances the HIV replication in latent HIV infections [23,24]. This finding could explain why mycobacteria infections which promote the synthesis of TNF-α could also augment HIV replication in chronic infected individuals. The level of TNF-α in the blood of patients infected with mycobacteria and HIV was documented to be 3 to 10 times higher than in non-HIV patients [25] showing a major imbalance in the release of this proinflammatory cytokine. TNF- α also plays a central role in the CNS localizations of mycobacteria. The excessive amount of TNF-α could accelerate the disruption of rich tuberculous foci adjacent to the CNS. Increased levels of TNF-α as well as IFN-γ were found in the CSF of patients with TBM at the disease onset [26] as well as several months after the acute episode [27] Experimental studies on rabbits proved that the excess of TNF- α acts as a persistent trigger of the inflammatory response and as a procoagulant factor associated with both the mycobacteria CNS invasion as well as cerebral vascular complications. [28]. The therapeutic use of TNF-α inhibitors in severe forms of TBM, tuberculoma and cerebral tuberculous abscesses was linked to a decreased inflammatory response and noticeable clinical recovery [29-31]. The major role of TNF-α in the progression of TBM was also proved in murine models by Tsenova as well [28,33]. Studies on HIV patients with TBM also emphasized the significance of increased levels of CSF TNF-α and of IFN-γ in advanced TBM stages [34].

In conclusion all these studies proved that important variations of the Th1 cytokine profile and especially of those involving the release of TNF-α represent one of the pathogenic mechanisms that aggravate the outcome of NTB in the HIV infection. Understanding these changes could be the first step towards the development of efficient complementary therapies in NTB to reduce the excessive inflammatory response. Thus TNF-α inhibition could be used as an antiinflammatory therapy in NTB with severe complications but should not be recommended in other forms of TB.

2. *The persistent activation of microglial cells.*

A significant role in the pathogenic mechanisms of CNS infections was assigned to the activation of microglial cells, the resident macrophages of the CNS. Microglial cells are involved in the local phagocytosis and play a central role in the pathogenesis of infections and inflammatory diseases [35]. These cells also represent the main target of both HIV and mycobacteria infection [36,37]. Thus the activation of microglial cells by mycobacteria induces the release of proinflammatory cytokines, some of which are able to add to the stability of cerebral granulomas. A moderate level of CXCL9 and CXCL10 chemokines released by microglial cells regulates the influx of inflammatory cells to the brain and interferes with the chemotaxis of monocytes/macrophages and T cells thus assisting the

formation of granulomas. However since microglial cells are the main source of cerebral TNF-α these could also induce an aggresive inflammatory response with severe meningeal inflammation, brain edema, protein accumulation, endarteritis and intracranial hypertension accounting for most of the complications described in NTB [28,38]. Therefore a balanced activation of microglial cells is critical against the CNS mycobacterial invasion. On the other hand the intracellular HIV replication in microglial cells leads to their activation, neuroinflammation and release of neurotoxins that cause AIDS associated neural dysfunctions. The complex role of the microglia in cerebral HIV/TB co-infection is explained by the rich number of HIV receptors and co-receptors expressed by these cells such as CD4, CCR5, CXCR4 as well as other receptors involved in the inflammatory response including IFN-γ, TNF-α,CD14 and MHC class I and II receptors [39].The CD14 receptor promotes the uptake of both HIV and nonopsonized M.tbc strains in microglial cells [40] while CD4 and CCR5/CXCR4 co-receptors interfere with HIV cell attachment. As a result microgial cells are the main target of HIV and mycobacteria once these enter the CNS. Therapies directed towards reducing the inflammatory response in the HIV/TB co-infection include the blockage of certain receptors (such as CD14), the use of CCR5 antagonists and TNF-α blockers (as thalidomide). Another alternative is dexametasone recommended in most forms of CNS TB. The clinical benefits of dexametazone were inspired by in vitro studies proving a potent inhibitory effect on the release of cytokines from microglia [39].

In conclusion simultaneous infection of the microglia with HIV and mycobacteria increases the meningeal inflammatory response, the fundamental pathogenic step in all forms of CNS TB. The synthesis of excessive inflammatory infiltrate is responsible for the clinical findings and possibly irreversible complications in NTB, such as hydrocephalus and vasculitis [41]. Moreover the excessive inflammatory response triggered in the HIV/TB co-infection could induce the immune reconstitution inflammatory syndrome – a complication that is specific for this patient category.

4. Pathogenesis of the immune reconstitution inflammatory syndrome

The Immune Reconstitution Inflammatory Syndrome (IRIS) is an uncommon inflammatory response encountered in those cases of severe immunosuppression in which the rapid administration of specific treatment abruptly restores the immune response. The HIV infection is the most frequent cause of immunodeficiency predisposing to IRIS. In addition TB is the most common opportunistic infection related to HIV-associated IRIS. The antiretroviral and antituberculous treatments rapidly restore the immune response. Such a rapid treatment response may sometimes lead to an aggressive lymphoproliferative reaction and massive release of proinflammatory cytokines. There are 2 clinical presentations of IRIS known as the paradoxical IRIS and unmasking IRIS. IRIS manifestations in HIV patients with NTB follow two possible scenarios:

a. A paradoxical reaction emerging in patients with NTB correctly diagnosed and appropriately treated in which HIV infection is subsequently detected and also treated but new severe neurological manifestations arise during treatment (paradoxical NeuroIRIS-TB).

b. An unmasking reaction appears in patients with HIV and latent unknown NTB in which the successful antiretroviral treatment unexpectedly induces neurological manifestations of TB (unmasked NeuroIRIS-TB)

The neurologic manifestation of IRIS-TB are rare (19% of the total cases) but with a mortality risk that is three times higher than other IRIS localisations [42]. The specific features related to NeuroIRIS-TB reside in the excessive CNS inflammatory reactions generated by the activation of microglia. The excessive inflammatory response is linked to the abundance of mycobacterial antigens and their high immunogenicity. Various studies have approached the immunologic mechanisms and risk factors for IRIS in HIV-TB patients.

The observations below on the pathogenesis of IRIS-TB were selected according to the potential clinical application.

- The release of multiple mycobacterial antigens in the first 2 months of antituberculous therapy and concurrent wide distribution of sequestered CD45RO memory lymphocytes in the bloodstream during HIV antiretroviral treatment are the principal mechanisms inducing an excessive inflammatory response. To avoid the overlap of these events the current WHO recommendations advocate an initial antituberculous treatment followed at a minimum interval of 2 weeks by the antiretroviral treatment in patients with a low level of Th CD4+ cells [43]
- The pathological overproduction of Th1 cytokines particularly IFN-γ was noticed in IRIS-TB/ HIV co-infection [44,45].Taking into account the experimentally increased levels of IFN-γ in IRIS the blood interferon-gamma (IFN-γ) release assays (IGRA) could be implemented to monitor IRIS evolution in the future. In addition the pathological overproduction of chemokines CXCL9 and CXCL10 induced by IFN-γ was observed in IRIS-TB/HIV co-infection [46]. The development of therapeutic strategies which could reduce the intracerebral level of these chemokines are essential to prevent and decrease ensuing granulomas thus protecting against IRIS.[47,48]
- The excessive release of IgG antibodies to PPD was observed in patients with IRIS-TB/HIV co-infection [45] Nonetheless the level of antibodies against the phenolic glycolipid antigen (PGL-TB1) was lower in IRIS hosts. The IgG anti PPD and especially the intrathecal synthesis of IgG/PPD could provide additional information on the humoral immune response in NeuroIRIS – TB [49].
- The restoration of a delayed type of hypersensitivity to mycobacterial antigens was reported in HIV patients with latent TB after starting the antiretroviral therapy [50,51]. All the same recent studies cast doubt on the tuberculin-specific Th1-responses in prompting IRIS [52]
- The profile of cytokines differs between the 2 types of IRIS as well as between TB infection and IRIS-TB. Hence certain cytokines (IFN-γ,TNF-α and IL-6) are more elevated in IRIS-TB than compared with patients presenting only TB. [53,54]. This finding could help distinguish

TB from IRIS-TB. Other studies have also investigated different profiles of immunological markers which could aid in the above distinction. Conradie et al. have identified a profile of makers including IL8, active NK cells, C reactive protein and lymphocyte count that is related to unmasking IRIS-TB. This profile could be further used in the differential diagnosis of the 2 manifestations or as a prediction of unmasking IRIS-TB [55].

5. Etiological data on the mycobacterial strains in HIV/TB co-infection

HIV patients are frequently infected by virulent strains of M.tbc. The virulence of a particular strain depends on the genetic composition of M.tbc. Thus the Beijing genotype of M.tbc mostly found in Asia is considered the most aggressive genotype and has been associated with CSF dissemination and multidrug resistance to antituberculous agents in HIV patients [56]. Infections with M. bovis are rare and occur mostly in HIV Hispanic patients. Despite the high environmental exposure to nontuberculous mycobacteria CNS involvement is rare even in AIDS patients and usually occurs at a CD4+ count under 10 cells/mm3. The pathogenic mechanisms behind the interactions established between the host and virulent mycobacteria are less documented. The infection with Mycobacterium avium complex (MAC) remains the most studied and most frequent nontuberculous mycobacteria accounting for the atypical tuberculous manifestations in the advanced stages of AIDS infection [57]. The Mycobacterium avium intracellulare (MAI) serotypes 4 and 8 are the most prevalent in AIDS patients [58].

Sporadic cases of NTB with other mycobacteria have also been recorded in AIDS patients following disseminated infection [59]. MAC is an ubiquitary environmental mycobacteria which colonizes the gastrointestinal and respiratory tract but is also able to invade the epithelial cells and the intestinal wall [60]. Virulent strains isolated from AIDS patients are able to penetrate the mucosal barriers and resist intracellular killing by macrophages resulting in a disseminated infection. Further studies on the interaction between M. avium and the HIV-infected cells confirmed the inhibition of several cytokines secreted by the Th_1 CD4+cells, natural killer cells and macrophages.These ultimately favour the intracellular survival of M. avium and even accelerate its growth rate [61,62]. The neurologic involvement due to MAC in advanced stages of AIDS generally presents as TBM following a disseminated infection with prolonged bacteremia [63-66]. The comparative aspects of the CNS invasions with M.tbc and nontuberculous mycobacteria in HIV hosts are presented in table 1

6. Clinical data on NTB in HIV patients

NTB is frequent in HIV patients compared with non-HIV patients. Reactivation of latent forms of TB is accelerated in HIV patients with a 10% annual risk of progression to active infection compared with 10-20% lifetime risk of developing TB in non-HIV patients. Literature data is contradictory as to the role of HIV on the clinical presentation or evolution of NTB. Although some studies found significant differences between HIV and non-HIV NTB [67-69] others

	M. tbc	Nontuberculous mycobacteria
Mycobacteria strain	M tbc, rarely M bovis	98% MAC, rarely other mycobacteria
Primary infection	Usually respiratory	Gastrointestinal or respiratory
Frequency	Moderate	Low/very low
CD4+ T cell count	< 200 cells/mm3	<10% cells/mm3 (usually)
Clinical forms	Meningitis, Tuberculoma, Abscess	Disseminated, Abscess
Diagnosis	Established diagnosis criteria	No standard diagnosis criteria
CSF mycobacteria detection	Essential to diagnosis confirmation	Not essential to diagnosis confirmation
Mycobacteria detection (other than CSF)	In blood	In faeces (frequently), in blood (if disseminated infections)
Prognosis	Reserved	Terminal infections (frequently)

Table 1. Comparative aspects of the CNS invasions with M. tbc and nontuberculous mycobacteria in HIV hosts

argued that the HIV co-infection does not influence the clinical evolution [70]. Nevertheless the differential diagnosis between NTB and numerous systemic and neurologic nontuberculous complications emerging in AIDS is difficult. Thus the clinical presentation of NTB in HIV patients could be influenced by numerous factors such as:

- various neurological manifestations caused by HIV itself;
- other opportunistic infections with CNS tropism, mainly toxoplasma, criptococcus, papilloma or herpes viruses infections;
- concurrent cerebral tumors : non-Hodkin cerebral lymphoma, Kaposi sarcoma;
- simultaneous evolution of various forms of NTB (meningitis, tuberculoma)- a characteristic finding in HIV patients;
- extra-neurological infections or malignancies related to HIV.

All these interfering factors could explain the variable descriptions of the clinical presentation, CSF manifestations or imaging aspects in the numerous studies on NTB in HIV patients.

NTB in HIV patients encompasses the following forms: TBM, disseminated TB of the nevrax, tuberculoma, and tuberculous abcess. En plaque tuberculoma, chronic spinal pahymeningitis and serous TBM are rare forms of TB not described in HIV patients.

6.1. Tuberculous meningitis in HIV patients

The real frequency of TBM in HIV patients is hard to assess as the various clinical presentations related to immunodepression could be confused with other neurologic manifestations. The epidemiological data on the subject is contradictory. Current statistics in areas with an increased prevalence of TB disclose M. tbc as the most frequent etiologic agent of meningitis in HIV patients [71]. Moreover TBM was recorded as the

initial presentation of AIDS in 42% of cases. A study performed in Kenya, a state with an increasing incidence of TB and HIV, revealed that 80% of the necropsies performed on HIV patients exhibited disseminated TB and 26% of these also displayed meningeal involvement [72].On the other hand the frequency of disseminated tuberculosis based on clinical and bacteriological criteria only did not exceed 14,5% of cases [73-74]. The conclusion arising from these studies is that the extent of the CNS invasion is highly variable and a large number of disseminated TB in AIDS probably remains undiagnosed.

Neurological presentation. TBM is the most frequent form of NTB in HIV patients. The neurological manifestations differ according to the degree of immunodeficiency.

- *TBM in the early stages of HIV immunodepression.* The onset of TBM is insidious. Fever and meningeal signs develop progressively (7-30 days) paralleling the changes in the cognitive status and mental state. Once the meningeal syndrome is established the evolution is rapid. The meningeal syndrome is intense and progressive. Under such circumstances the diagnosis could be aided by recognizing the paralysis of certain cranial nerves (mostly involving the sixth cranial nerve but also the second, third, fourth and eighth nerves) as well as the signs of hydrocephalus or cerebral edema (headache, convulsions, pyramidal or cerebellar signs). Encephalitic forms display an altered level of consciousness with progressive evolution to coma. In forms with major spinal involvement (TB spinal meningitis, spinal arachnoiditis) the inflammatory exudate surrounds the spinal cord and induces radicular compression. As a result radicular pains develop along with sings of transverse mielitis (paraplegia and urine retention).

- *TBM in advanced stage of HIV immunodepression*. In advanced stage of immunodepression the inflammatory exudate is decreased and the clinical presentation is atypical. Fever could be absent in these patients. The meningeal sings are discrete or missing [75]. Hydrocephalus is delayed. Tuberculous vasculopathy prompts frequent complications following thrombosis, or hemorrhagic infarcts. Focal lesions related to the vasculopathy are common. The cognitive dysfunction is severe [76] with a rapid evolution to profound coma [8].In this advanced stage of AIDS NTB rarely evolves as a solitary finding. Usually other infections or tumors are also associated with NTB and the wide spectrum of clinical manifestations implies various *neurological patterns with focal, perypheral or central nervous signs.*

CSF data. The aspect of the initial CSF could be suggestive disclosing lymphocytic pleocytosis, elevated proteins and low glucose levels. Nevertheless the etiologic confirmation is based on bacteriological criteria only. In patients with severe immunodeficiency the CSF white cell count is usually only slightly increased but could also be normal [67] The low number of lymphocytes in HIV could modify the differential count in the CSF to a predominant number of neutrophils [67] causing confusion with bacterial meningitis. Elevated proteins are a typical finding in TBM in non-HIV patients. However 43% of the HIV reported cases presented low or even normal protein values [5,8]. The most difficult cases are those in which the CSF is reported as normal, a common finding in patients with severe immunodeficiency. In the absence of a strong inflammatory response acid-fast bacilli smear retrieves positive results [67] in up to 67% of cases and the cultures are positive in 40 – 87,9% of cases [76,77].High rates of smear and culture

positivity facilitate the diagnosis in patients with an atypical clinical presentation and normal CSF exam.

Neuroradiological findings. The classic CT neuroradiological findings in TBM include basal meningeal enhancement, hydrocephalus, and infarctions in the supratentorial brain parenchyma and brainstem [78]. The concurrent finding of basal meningeal enhancement, tuberculoma or both on CT scans could disclose a sensitivity of 89% and 100% specificity for TBM in non-HIV patients [79]. In HIV patients contrast-enhanced MRI is generally considered superior to CT results [78]. Some MRI studies indicated that meningeal enhancement and cerebral infarctions were more common in HIV-infected individuals with TBM by comparison with non-HIV patients [5,70]. However the basal meningeal enhancement and hydrocephalus rarely occur in advanced stages of AIDS with reduced inflammatory response [76]. On the other hand cerebral infarctions and focal mass lesions are frequently encountered in late stages of AIDS [80-82]. In addition to the previous aspects imaging studies also disclose cerebral atrophy due to HIV infection. Tubeculomas also were reported in 15-24% of cases [5].

6.1.1. The diagnosis of TBM in HIV infected patients

The diagnosis is urgent and extensive including all tuberculous lesions, HIV status and other HIV associated lesions, bacteriological confirmation and neurological complications. It is based on clinical features, CSF analysis and MRI imaging. (table 2). A belated diagnosis increases the mortality, complications and the risk of relapse.

Clinical diagnostic criteria. Clinical features in HIV patients with TBM reflect the atypical inflammatory response and the extensive vasculopathy. The meningeal sings are inconstant and discrete especially in patients with severe immunodepression. The signs of encephalitis emerge from the onset and could be the first significant manifestation of the disease. The gravity of the altered level of consciousness parallels the increased mortality [8].Cerebral nerve paralysis is a common finding but could be also induced by other associated conditions such as HIV neurotoxicity, the cerebral reactivation of opportunistic infections (toxoplasma, JS virus, Herpes simplex virus) or cerebral malignancies (Non-Hodgkin lymphoma, Kaposi sarcoma). These patients particularly exhibit multiple extraneurologic manifestations. The presence of other active lesions like pulmonary TB or other extrameningeal sites of TB is highly suggestive for the CNS TB diagnosis [5,67,81]. Thus the presentation of HIV patients unlike non-HIV patients often includes peripheral, intrathoracic and intraabdominal adenopathies. The etiology of these adenopathies does not always imply a diagnosis of TB. The differential diagnosis for adenopathies should always include other lymphotropic opportunistic infections with neurologic manifestations (toxoplasma, CMV, syphilis). The tuberculous origin of adenopathies could be overestimated in the clinical diagnosis if the histological confirmation is not obtained. The histological examination is thus a prerequisite for a correct diagnosis of these adenopathies. Hepatosplenomegaly is commonly reported but could also occur as a result of other HIV associated infections (B or C hepatitis, CMV infections).To conclude no clinical criteria is highly suggestive for CNS TB in HIV patients. Moreover any neurologic or extraneurologic finding should prompt a thorough differential diagnosis that includes any other HIV related affections.

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Laboratory diagnostic criteria. The degree of immunodeficiency in HIV patients with NTB could be assessed using the CD4+T cell count. Most studies on TBM disclose a CD4+T cell count between 32-200 /mm^3 [5,81,82].Other findings including a lower hematocrit, peripheral low neutrophils, lower plasma sodium level [76] and moderate to severe anemia Hb < 8 gm/dl [69] were not constantly present in all studies and could be mostly related to the HIV infection than to TB. Moreover hyponatremia in patients with HIV-TB co-infection could arise due to the following: a) cerebral salt wasting syndrome observed in 65% of patients with numerous cerebral lesions, including patients with TBM [83]; b) the syndrome of inappropriate release of antidiuretic hormone secretion; c) hypothalamus pituitary-adrenal axis suppresion. Hyponatremia is a marker of the disease severity and the mortality in this patient group is significantly higher than that of patients with normal sodium levels (36,5% versus 19.7%) [84].

The CSF exam is decisive for the diagnosis. The specificity of the bacteriological diagnosis is 100% but its implication in the final diagnosis is quite low since the Ziehl-Neelsen stain is positive in less than 20% of cases and Lowenstein culture confirmation although positive in 73% of cases is tardy [85]. Methods of improving the sensibility of Ziehl-Neelsen stain have been described [86] but are less implemented. Tuberculin skin test and Interferon-gamma release assays if positive do not distinguish between latent TB and active disease. As well negative results should be evaluated with caution in severely immunodepressed patients. Several complementary diagnostic tools were explored in certain studies like specific antigens and antibodies detection, adenosine deaminase detection, PCR techniques, detection of tuberculostearic acid or IFN-γ levels in the CSF. However their use is limited due to discordant results or other inconveniences related to the cost, cross-reactivity, specificity or sensibility [87-90]. Recently the improvement of nucleic acid amplification assay techniques, particularly polymerase chain reaction (PCR) assay (especially nested PCR assay technique) increased the diagnostic sensitivity and specificity but its use in AIDS related CNS TB is still unconfirmed [91]. All in all the bacteriological confirmation is difficult and belated but remains the only diagnostic tool in AIDS related CNS TB.

Imaging diagnostic criteria. Imaging studies are required in the evaluation of neurological complications of TBM, in the treatment follow-up and differential diagnosis. Contrast enhanced MRI and Positron emission computed tomography – computed tomography (PET-CT) display the highest sensibility. Unfortunately most literature studies are based on the more inexpensive CT scans. No aspects are definitely characteristic to CNS TB in HIV patients. Atypical results showing the absence or minimal meningeal enhancement [8] or the absence of communicating hydrocephalus were reported on the CT scan in 69% of AIDS cases [5,8]. Nevertheless other studies found no significant radiological differences between HIV and non-HIV patients.

*In addition to the clinical, CSF and radiologic criteria, a medical history of TB and positive tuberculin skin test could help raise the diagnostic suspicion of a tuberculous infection.

Neurotuberculosis suspicion
Clinical investigations (assessing the risk of tuberculosis, neurological manifestations, other manifestations)
History of tuberculosis (TB antecedents, risk of exposure)
Physical examination disclosing:
1. Signs of menigeal irritation (suggesting meningitis or a meningeal reaction to localized cerebral lesions)
2. Neurologic examination (mental status, sensory and motor exam, focal signs, intracranial hypertension)
3. Other manifestations suggesting TB and nontuberculous lesions induced by HIV activity, opportunistic infections or malignancies like lymphadenopathy (given attention to lymphoma, syphilis, toxoplasmosis), pleural or pericardial effusion (given attention to Kaposi sarcoma), pulmonary lesion (given attention to pneumocystosis, Kaposi sarcoma, fungal pneumonia,CMV pneumonia, lymphocytic interstitial pneumonitis), skin lesions (given attention to Kaposi sarcoma, Moluscum contagiosum, fungal lesions, meningococcal purpura)
Laboratory data assessing the immune status, HIV activity, risk of opportunistic infections or malignancies
Complete blood count (pancytopenia suggests medullar invasion with mycobacteria but also invasive malignancies or drug toxicities)
Biochemical evaluation of liver and renal function; indicate associated co-morbidities; important for drug regimen recomandation,
Serum sodium level (hyponatremia is linked to disseminated mycobacteriosis and cerebral lesions/ it corelates with the mortality risk)
Immune status: CD4+ T cell count (CD4<200 cells/mm^3 is related to the risk of NTB and major HIV-related opportunistic infections; CD4< 50 cells/mm^3 is related to the risk of nontuberculous mycobacteriosis or to the risk of IRIS)
HIV viral status: blood/CSF RNA HIV viral load (if positive it point to the antiretroviral failure and needing to swich the regimen)
Serologic assays: serum specific antibodies IgG and IgM related to other HIV-opportunistic infections,mainly toxoplasma, CMV, syphilis.
Imaging studies: cerebral or spinal CT/MRI; (important in localized NTB and other cerebral opportunistic infections or malignancies
Eye fundus examination : shows choroid tubercles in disseminated tuberculosis
Neurotuberculosis confirmation
Lumbar puncture (if the MRI does not indicate mass lesions!): CSF analysis: cytochemistry, stains*, culture **, or complementary exams ***!
Other specimens analysis: sputum, pleural fluid, blood, urine, tissue specimens (lymph node, hepatic or cerebral biopsy): stains*, culture** other examination***

, human immunodeficiency virus; CSF, cerebrospinal fluid; TB, tuberculosis; NTB, neurotuberculosis; MRI, magnetic resonance imaging;CMV, citomegalvirus; * stains: Ziehl Neelsen (acid-fast bacilli), India ink (fungi), Gram smear (bacteria); ** culture on specific media: Lowestein or Bactec(mycobacteria), Sabourraud (fungi), blood agar (bacteria); *** PCR,polymerase chain reaction, detection of ADA activity, detection of antigens/ antibodies for toxoplasma, CMV, criptococcus, meningococcus, pneumococcus

Table 2. Neurotuberculosis diagnosis in HIV patients

6.1.2. The evolution of TBM in HIV patients

In the HIV-TB co-infection TBM is frequently associated with pulmonary TB or tuberculous lymphadenopathies. *The risk of a relapse* is considered 23%. The most important risk of relapse is the lack of adherence to the antituberculous and antiretroviral treatment. CSF blood glucose ratio and the presence of pulmonary TB could also be linked with the risk of relapse according to a study performed in Vietnam [92]. *The mortality rate* is high; the survival rate is difficult to evaluate taking into account the increased mortality of HIV patients due to other opportunistic infections or specific complications. Risk factors for death during hospitalization for TBM included: a) the CD4+ count lower than 50 cells/mm^3; b) the presence of advanced neurologic signs or hydrocephalus on admission; c) a diagnosis and treatment delay with more than 3 days [80];d) the absence of the antiretroviral treatment or failure of the highly active antiretroviral therapy (HAART) [93].TBM relapsing forms and multidrug resistant mycobacteria are linked to a high mortality rate. IRIS prognosis is generally good.

6.1.3. Conclusion

TBM comprises variable manifestations in HIV patients. Early stages of immunodepression in HIV patients usually set the same diagnostic difficulties as in non-HIV patients as a result of the variable clinical presentations and delayed bacteriological results. In the advanced stages of HIV the clinical presentation is atypical and the CSF cytochemical profile could be within normal parameters. Other concurrent lesions of active TB could ease the diagnosis. The differential diagnosis should always include other HIV-associated manifestations, other opportunistic infections or malignancies. The bacteriological exam is still the only tool able to confirm the diagnosis. The prognosis of TBM in HIV patients is shadowed by numerous diagnostic difficulties, increased risk of relapse and associated HIV pathology.

Below are NTB diagnosis criteria (table 2) and imaging aspects found in our clinical practice in patients with HIV and NTB: meningoencephalitis (figure 1), cerebral tuberculoma (figure 2) and cerebral tuberculoma in context of IRIS (figure 3)

6.2. CNS disseminated TB

CNS disseminated TB (CNS milliary TB, cerebrospinal granulia) is a form of cerebral milliary frequently associated with disseminated TB. It is rarely limited to the CNS. The diagnosis is usually based on findings at the necropsy or MRI results. *Constitutional symptoms develop progressively even in the absence of neurologic signs; mycobacteria could also be isolated in other pathological products than the CSF (most frequently from the blood). The eye fundus exam could disclose characteristic choroid tubercles*. A classical miliary pattern on chest radiograph frequently complements the aspects of cerebral miliary. Postconstrast MR brain images reveal intense nodular enhancing granulomas located at cortico-medulary junction and throughout the brain parenchyma. The differential diagnosis of cerebral military should include other opportunistic disseminated infections or secondary metastatic lesions. It is possible to underestimate this form of CNS TB as a result of the diagnostic difficulties and required expensive imaging studies.

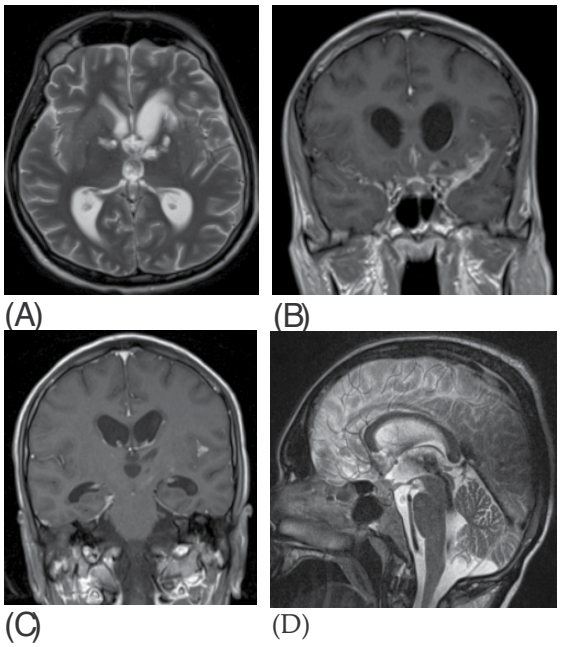

Figure 1. A-D. Cranio-cerebral MR: axial (A), coronal (B and C), and saggital (D) images showing tuberculous meningitis, cerebral thrombosis and hidrocephalus in a 23-year-old patient with AIDS. He had been receiving antiretroviral treatment for 3 months prior to the present hospitalization. He was admitted with milliary TB and meningoencephalitis associated with oral HCV infection, candidiosis and reactivated CMV infection. The clinical evolution was complicated by toxic hepatitis due to antituberculous treatment and cerebral thrombosis. On admission the CD4 count was 244/mm^3 and the RNA HIV load was 239 copies/ml. Contrast MRI before and after the administration of intravenous gadolinium and angioMRI(sag 3D PC phlebography) show: hyperintense lesions on FLAIR sequences and T2 weighted images, appearing hypointense on T1 with no contrast enhancement, located in the medial part of the lentiform nucleus and the head of the caudate nucleus; contrast filling of the basal cisterns extending to the sylvian fissure (more proeminent on the left side), the floor of the third ventricule and the infundibular area (involving the optic nerves, chiasm and optic tracts); asymmetric profound venous system with bilateral amputation of the superios talamostriate veins without the visualisation of the anterior left vein of the pellucid septum; enlargement of the ventricular system with no median shift or transependimar resorbtion. *Conclusions*: post ischemic sequelae, thrombosis of the profound venous system, basal meningeal contrast enhancement suggestive for meningitis and dilation of the ventricular system.

6.3. Intracranial mass lesions in HIV patients with CNS TB

6.3.1. Tuberculoma

CNS tuberculomas develop insidiously in the cerebral parenchyma following either the reactivation of local granulomas [94] or a paradoxical response to the antituberculous therapy (figure 2,3). The lesions could be solitary or multiple and their localisations are diverse. Cerebral localisations are more frequent than spinal ones. Data on HIV patients presenting tuberculomas is scarce [95,96]. The diagnosis is probably underestimated in low income countries taking into account the expensive CT/MRI importance in the confirmation. The clinical presentation is pseudotumoral with fever and headaches. The neurologic signs vary according to localisation and may be absent. HIV patients rarely present signs of intracranial hypertension or convulsions. On the other hand tuberculomas could be associated with other

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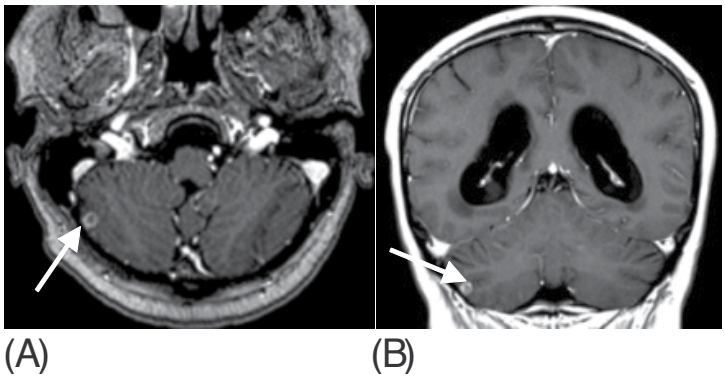

Figure 2. Cranio-cerebral *MR images* showing cerebelous tuberculoma in a 41 year-old patient with a 5 year history of HIV infection nonadherent to the antiretroviral treatment.The patient was admitted with a cerebellous tuberculoma and acute ischemic stroke.The laboratory data on admission disclosed a CD4 count of 145cells/mm^3 and RNA HIV load 240000 copies/ml.*Axial T1 weighted images shows (A)*: Focal enchancing triangular lesion in the anterolateral right-side of the pons of 5×9 mm with FLAIR hyperintensity, difussion restriction, no significant changes in the apparent diffusion coefficient (ADC) and no contrast enhancement (the aspect is suggestive for acute ischemia); a right focal cortico-subcortical cerebellous lesion with peripheral ring enhancement on T1 weighted images and mass effect (the aspect is compatible with a tuberculoma). *Coronal T1 weighted images shows (B)*: symmetrical enlargement of the ventricular system with no midline shift; transependimar circumferential resorbtion edema is present adjacent to the ventricular wall; no intraventricular obstruction or contrast enhancement. *Conclusions*: acute ischemic stroke in the anterolateral right side of the pons; focalinferolateral parenchymal lesion suggestive for a tuberculoma; significant hydrocephalus with no intraventricular obstruction.

manifestations of TB such as TBM, pulmonary TB or other signs suggestive for CNS TB such as tuberculous vasculitis. The CSF usually displays no changes or few cytochemical abnormal findings (low glucose, elevated proteins); the acid-fast bacilli smear and culture are frequently negative. The aspect on the CT suggestive for a tuberculoma presents as isodense or lightly hypodense lesions with annular contrast enhancement and the ''target sign'' as a result of central calcifications. Nevertheless these aspects are not pathognomonic and the diagnosis requires a cerebral biopsy with histological and bacteriological confirmation. The histopathological examination usually discloses a central region of caseous necrosis surrounded by a capsule with a granulomatous structure. This aspect evolves dynamically as follows: 1) noncaseating granuloma; 2) caseating granuloma with a solid center; 3) caseating granuloma with a liquid center. This dynamics could also be detected at the contrast enhanced MRI or MRI spectroscopy as opposed to the images induced by a cerebral abscess. The MRI examination indicates a correspondent evolution with the histopatological examination as: 1) hypointense lesions on T1-weighted images (T1W) and hyperintense T2W lesions with nodular enhancemen postgadolinium administration; 2) hypointense lesions on T1W and T2W with peripheral rim enhancement postgadolinium ;3) hypointense T1W and hyperintense T2W with hypointense rim postgadolinium. Difussion weigthed images indicate diffusion restriction within the tuberculoma. The lesions are surrounded by edema. The lesions in HIV patients often appear as ring-enhancement lesions under 1 cm and the mass effect is rarely seen [97]. The CT/MRI aspect should be distinguished from other ring-enhancing lesions including bacterial cerebral abscesses, cerebral toxoplasmosis, CNS cryptococcosis, neurocysticercosis or CNS lymphomas.

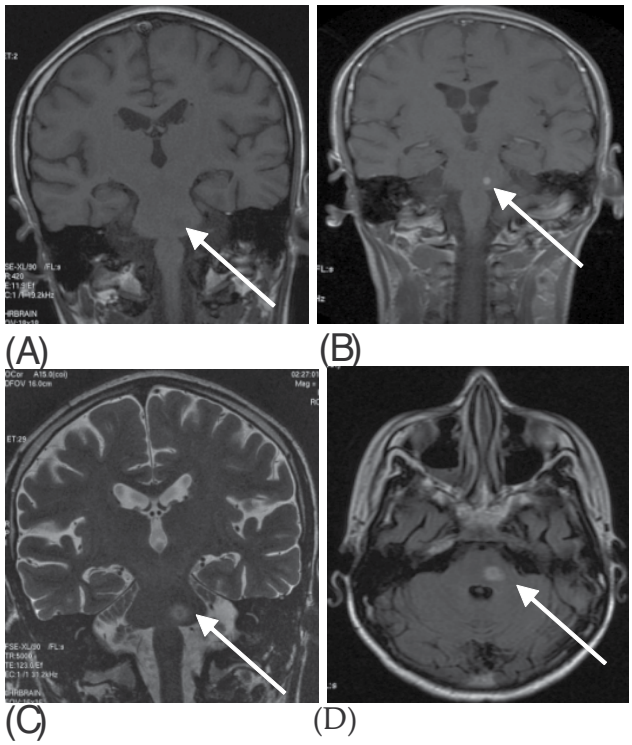

Figure 3. Cranio-cerebral *MRI,* showing left pontine tuberculoma in a 16 year-old patient previously diagnosed and undergoing treated for lymph node TB for the past 2 months and recently diagnosed with HIV infection.The patient also associated HBV and CMV infection and oral candidiosis.On admission the patient was in coma. The laboratory data displayed a CD4 count of 24 cells/mm^3 and RNA HIV 1064973copies/ml. Final diagnosis was NeuroIRIS TB (tuberculoma).The CSF disclosed no changes.The clinical evolution was favourable. *A:* coronal T_1 weighted image demonstrating left pontine paramedian nodular lesion of 4 mm surrounded by perilesional edema (discrete hyposignal). *B:* coronal section T_1 postcontrast shows hypersignal; C- coronal section T_2 and D- axial FLAIR section show intense contrast uptake and no diffusion restriction.

6.3.2.Tuberculous abscess

The tuberculous abscess represents a purulent collection delineated by a capsule with a granulomatous structure. This is a rare finding in immunocompetent patients as well as in the early stages of AIDS but common in severe immunodeficiency states with CD4+T cell count under 100/mm^3 [96]. The tuberculous abscess results from the liquefaction of tuberculomas [13] or from the necrotic evolution of granulomas in the setting of severe immunodeficiency [98].The necrotic centre is invaded by mycobacteria. The CSF is unchanged. The evolution is more acute than tuberculomas with neurologic deficit, fever and headaches [96, 99-100]. The CT/MRI aspect resembles the images in caseous tuberculomas but the lesion is larger (>3cm), multilobulated, surrounded by a thick capsule and ring enhancement. The perilesional edema and the mass effect are the most important features. The histological and bacteriological exam the cerebral biopsy confirm the diagnosis. The differential diagnosis includes other intracranial

space-occupying lesions especially cerebral toxoplasmosis and lymphoma [19].In such cases PCR techniques could increase the diagnostic yield [101,102].

7. Infections with non-tuberculous mycobacteria in HIV patients

Nontuberculous mycobacteria induce CNS lesions especially in AIDS patients with advanced stages of immunodepression. Sporadic cases triggered by M. avium, M. kanssasi, M. fortuitum, M gordonae, M. genavense and M. terrae were reported [105,106]. As a rule CNS infections with non-tuberculous mycobacteria are the result of MAC infection. Nevertheless infection with MAC shows no predilection for the CNS as it frequently colonises the respiratory and gastrointestinal tract. Disseminated infections occur as a result of a severe immune dysfunction at a CD4 count under 60 cells/mm^3 [57]. Under 10 cells/ mm^3 the neurological dissemination is also possible [107]. However a case study reported by Fletcher disclosed a cerebral abscess with a double etiology involving M tbc and MAC in an AIDS patient with a CD+4 count of 140 cells/mm^3 [108]. Higher values of the CD4+ count were also found in cases of MAC–related IRIS in the absence of a systemic infection [109]. Most MAC neurologic manifestations in HIV infected patients are cerebral abscesses and meningoencephalitis. Localized mass lesions (including single or multiple abscesses) contain a large number of mycobacteria in the absence of the typical granulomatous structure. These findings are frequently accompanied by pleocytosis and an occasionally high protein level on CSF examination. The diagnosis should be confirmed by a histological exam (in cerebral localized forms) or by using minimum 2 hemocultures (in disseminated forms). MAC was also isolated in the CSF in disseminated forms. NeuroIRIS-MAC associated manifestations were sporadically reported in HIV patients [110].

8. The treatment of NTB in HIV patients

The treatment of NTB in HIV patients should be combined, controlled and individualized.

1. The antituberculous and antiretroviral medication must be *combined* according to the synergistic drug interactions; the doses in the combined scheme must be adjusted to prevent treatment resistance.
2. The drug regimen must be *controlled* for adherence, drug interactions, toxicities, clinical response and treatment resistance
3. Treatment must be *individualized* and adapted to other co-morbidities, associated therapies and hypersensitivity reactions of the patient

The main antituberculous and antiretroviral classes, their corresponding representative drugs, pharmacological interactions, adverse reactions and treatment efficacy are shown in table 3. The NTB treatment principles in HIV patients are presented in accordance with the European AIDS Clinical Society guidelines, CDC and American Thoracic Society recomandations [111-113].

8.1. The antituberculous treatment

Treatment of tuberculous meningitis. TBM is a curable disease. Response to treatment in patients with NTB and HIV is similar to patients diagnosed with TB only. The elevated mortality is a result of the belated diagnosis, resistant mycobacteria and severe immunodeficiency

- **The main characteristics of the antituberculous treatment in HIV patients with NTB**

1. Treatment should be urgently started based on clinical and biological data, CSF modifications, the history of TB, other tuberculous lesions and imaging studies. The CSF specimens should be collected for culture and for resistance detection before treatment starting. The bacteriological confirmation should not delay the treatment as the treatment delay accounts for a poor prognosis. Advanced stages of the disease with irreversible complications (hydrocephalia, adherences, cerebral infarcts) are related to high mortality rates.

2. The antituberculous therapy must have increased CSF penetration (table 3) [114-120].

3. Corticosteroid therapy should be initiated as early as possible and continued for 6–8 weeks.

4. A long course of therapy for a minimum of 12 months is strong recomended.

- **Factors to consider**

1. *Combined treatment* must include an *initial phase* of 2 months, with 4 first-line antituberculous drugs having high CSF penetration (ussualy isoniazid, rifampicin, pyrazinamide, ethambutol) administered daily; the initial phase is followed by a *second phase* of another 10 months with only 2 first-line antituberculous drugs (isoniazid, rifampicin) administered 3 times per week [121]

2. *Controlled treatment* should approach:

- treatment adherence

- drug interactions and toxicities taking into consideration the followings (see table 3):a) the side effects to the antituberculous treatment are three times more frequent in HIV than non HIV patients; b) the interactions between the antituberculous and antiretroviral therapy may impede the administration of the most efficient regimen or a simultaneous therapy; the most important interaction involves the protease inhibitors (important class of antiretrovirals) and rifampicin (first line antituberculous drug). Rifampicin accelerates the hepatic metabolism of protease inhibitors decreasing their blood levels and increasing the risk of HIVdrug resistance. In addition protease inhibitors delay the metabolism of rifampicin increasing its serum concentration and toxicity. Izoniazid and rifampicin also decrease the concentration of fluconazole, an antifungal frequently used in the HIV patients. Additionally there are many other interactions between rifampicin and antiretrovirals, corticosteroids or trimetoprim/sulfamethoxazole (table 3). For this reason rifabutin is preferred to rifampicin in HIV patients along with a prolonged treatment.

- neurological/extraneurological complications

Monitoring for ensuing complications includes a complete physical examination, laboratory data, CSF aspects and imaging studies. It is important to consider the followings: a) neurological complications are more frequent in HIV patients (mostly due to immune exacerbation as tuberculous vasculopathy or IRIS); b) neurological complications may occur during treatment: hydrocephalus and arachnoiditis could sometimes occur even in the presence of a correct treatment; c) complications are frequently associated with other undetected TB localizations.

- drug resistance.

The risk of resistance is increased in non-adherent patients, large bacillary load and patients who start less efficient regimens. The glucocorticoid therapy reestablishes the low permeability of the blood-brain barrier and could therefore decrease the CSF diffusion of antibiotics. Inadequate doses of antituberculous therapy or low CSF antituberculous concentration may induce drug resistance. An unfavourable clinical evolution and decreasing CD4+T cell count require repeated CSF collection for culture and drug resistance. Close surveillance for drug resistance is essential throughout the entire course of therapy.

3. *Individualized treatment*. The patient's co-morbidities (like viral hepatitis or other risk factors for hepatotoxicity, ocular diseases, renal failure, allergic reactions,other medications and pregnancy) must be investigated before establishing the drug regimen and should continue to be closely monitored.

Treatment of tuberculomas. Cerebral tuberculomas are potentially curable tumor-like masses. There is a low number of tuberculoma cases reported in HIV patients [94- 95, 122-125]. Treatment is based on the same principles as TBM but with the following mentions:

- The perilesional granulomatous vasculitis decreases the penetration of antituberculous drugs; the lesions heal progressively and require 12 to 30 months of antituberculous treatment, or even longer;
- The recommended regimen is based on rifampicin, izoniazid and pirazinamide for 4 to 5 months and then rifampicin and izoniazid for 12 to 16 additional months. Other active drugs include rifabutin, fluoroquinolones, kanamycin, ethionamide;
- Surgical treatment is rarely needed; it is indicated in tuberculomas with mass effect, increased intracranial hypertension and hydrocephalus. The antituberculous treatment should be started before surgery. The recurrence after surgical ablation is unsual.
- Glucocorticoid therapy is an important part of the treatment regimen as it reduces the edema and improves the clinical manifestations. It should be maintained for at least 4 to 8 weeks.

Treatment monitoring requires the clinical and radiological follow-up on the long term. The evolution of other tuberculous localizations if present should also remain under observation. Response to therapy is favorable despite large lesions or immunodeficiency.

Treatment of tuberculous abscesses requires surgical and pharmacological treatment similar to the regimen recommended in tuberculoma but for an interval of 18 months to 2 years. The *prognosis* is unfavourable due to severe imunodeficiency and large lesions [99, 101].

Treatment of NTB with resistant strains of M.tbc. The risk of resistance is higher in geographic areas with high prevalence of resistant mycobacteria and in the case of recent TB improperly treated. Resistance could occur against one or more antituberculous drugs. The association between HIV and multidrug resistance (MDR-TB) or extensive drug resistance (XDR-TB) is not well documented [126,127].The antituberculous treatment should be undertaken according to the advice of an experienced specialist only and should include at least 4 antituberculous drugs with an increased diffusion in the CSF [128].

Treatment of CNS TB with nontuberculous myobacteria. Data related to infections with nontuberculous mycobacteria is scarce and insufficient for establishing definite treatment guidelines. Therefore treatment regimens are largely undefined and the subsequent outcome remains disappointing. The severity of the evolution appears to be related to the variable sensitivity to the antituberculous antibiotics and the advanced stages of immunodeficiency which predispose to a disseminated disease. Therapeutic regimens should be individualized to include complex drug associations (5-6 drugs) on longer periods of time. A close consultation with an experienced specialist is required. Mycobacteria belonging to the MAC display increased resistance against most antituberculous drugs and therefore a large variety of therapeutic regimens was evaluated. The repeated therapeutic failure is apparently linked to the diverse sensitivity to antituberculous drugs associated with M. avium species. Moreover there is the alternative that some HIV patients could be simultaneously infected with more than one species of M avium. Macrolides proved efficient but cannot penetrate to the CSF. Chlaritromycin is involved in several drug interactions with the antiretroviral therapy. Considering the increased risk for disseminated forms induced by the MAC it is recommended to add azithromycin, ethambutol and rifabutin to therapy. Other drugs that could be associated in such cases include fluoroquinolones, streptomycin, amikacin. Treatment should always be based on the results of susceptibility testing. After 12 months of treatment, prophylaxis with macrolides is recommended until the CD4+ count raises above 100/mm3.M. scrofulaceum, M. simiae, M. malmoense reveal the same sensitivity pattern as MAC. In the case of M. kansasii recommended drugs include: rifabutin, streptomycin, HIN, ethambutol, amikacin.

Treatment during Pregnancy. The antituberculous treatment is urgently instituted according to classic treatment regimens. Among prohibited drugs are streptomycin, fluoroquinolones and ethionamide.

Treatment of NeuroIRIS-TB. Neurologic TB-IRIS is a rare manifestation of TB-IRIS. It generally occurs within 2-3 months after initiating the combination of antiretroviral and the antituberculous therapy [42].The risk of IRIS increases with the early starting and high efficacy of antiretroviral therapy. Delaying the antiretroviral therapy with a minimum of 2 weeks after antituberculous therapy is recommended to avoid IRIS complication. Usually IRIS is self-limited and requires symptomatic or anti-inflammatory treatment without stopping the antiretroviral treatment. Severe forms benefit from treatment with prednisone or methylprednisolone 1 mg/g gradually tapered within the 2 following weeks [129,130]

8.2. The antiretroviral therapy

The antiretroviral (ARV) treatment ought to be started as soon as possible after the antituberculous treatment. The urgency of the ARV therapy increases with the degree of immunodeficiency. Three important studies (CAMELIA performed in Cambodgia, SAPiT conducted in South Africa and STRIDE a multinational study) established that an earlier start of the ARV therapy significantly decreases the mortality in AIDS patients and especially in patients in which the CD4+ cell count is below <50 cells /mm^3. Although the development of IRIS is more frequent if the ARV treatment is more precocious, the gravity of the IRIS manifestations in the 3 studies above cannot justify a longer delay of the antiretroviral therapy. Most guidelines recommended that HIV patients start the antiretroviral treatment at least 2 weeks after the antituberculous treatment if the CD4+ count is below 50 cells per mm^3 ; the antiretroviral treatment can be delayed until 4 weeks if the CD4+ count > 50 cells/mm^3. Note that NTB in HIV patients could be shadowed by the possible reactivation of other neurotropic agents (cytomegalovirus, toxoplasma, JV virus) or cerebral tumors (cerebral lymphoma, Kaposi sarcoma).The diagnosis in these cases could be difficult and if these associations are not excluded from diagnosis, treatment should also address these conditions with the risk of multiple drug interactions. Such is the case of cerebral toxoplasmosis.

- **The main characteristics of antiretroviral treatment in HIV patients with NTB**
  - Therapeutic regimens must contain antiretroviral drugs with a high penetration in the CSF. The main ARV drugs used in the co-infection with TB are listed in table 3 along with their adverse reacions.
  - The antiretroviral therapy in NTB is based on reverse transcriptase inhibitors represented by 2 important classes: nucleoside reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI). The highest drug penetration into the CSF is assigned to zidovudine, abacavir, nevirapine, delavirdine. Although efavirenz (a NNRTI) does not display high levels in the CSF some studies advocate a very good response in the treated adults [131]. Protease inhibitors should not be used due to their interaction with rifampicin and low diffusion in the CSF. If their use is required (as a result of resistance or toxicity to other antiretrovirals) rifampicin is to be replaced with rifabutin with similar results.
  - The doses of antiretrovirals should be changed according to the antituberculous drug interference.
- **Factors to consider**

1. *Combined treatment* includes 3 NNRTIs with a preferred option for trizivir (combination of zidovudine, abacavir and lamivudine) or 2 NRTIs + 1 NNRTI (ussualy efavirenz).
2. *Controlled treatment* should approach:

- The adherence (especially if a large number of drugs are introduced at the same time) [132]. Nevertheless adherence to trizivir is high (the number of capsules is low, there are few adverse reactions).

- Drug interactions and toxicities (see table 3). The clinician should recognize the overlapping toxicities, drug interactions and also the occurrence of IRIS (paradoxical reactions) [133].The interactions between NNRTI or NRTI and antituberculous drugs are few. The risk of toxicity is minimal but adverse reactions are possible with some NRTIs (see table 3). Regarding the toxicity the ARV could interfere not only with antituberculous drugs but also with other drugs used in the prophylaxis or treatment of other opportunistic infections (such as fluconazol for Candida or Criptococcus neoformans or sulphametoxazole/trimethoprim for Penumocystis jirovecii).
- The efficiency and complications of treatment. The efficiency is to be monitored on a clinical, virologic and immunological basis. The best control in HIV infections is the virologic (RNA HIV viral load) and immunologic control (CD4+ cell count).Treatment control could be undertaken at 14 days, one month, three and six months respectively. If the HIV RNA load does not become undetectable after 3 months of treatment virologic failure should be considered. If this is the case investigations on the underlying cause should focus on the lack of adherence, acquired resistance (especially to NNRTIs) or a wrong treatment regimen (doses, antagonistic associations or the lack of drug penetration to the CSF). Nevertheless the intracerebral load of HIV could be hard to evaluate since the viral load detection in the serum does not always reflect the intracerebral levels of HIV.
- Drug resistance. In case of virologic failure drug-resistance testing should be obtained during treatment with the failing ARV regimen or within 4 weeks of treatment discontinuation. Resistance to antiretrovirals generally applies to most compounds in the same class.A new regimen with other fully active drugs preferably from other new classes must be restarted.

3. *Individualized treatment*: the treatment options should address other opportunistic infections and the patient's medical history. A CD4+ count under 200 cells/mm^3 urges the prophylaxis against fungal infections (cryptococcus, pneumocytsis). Prophylaxis against toxoplasmosis should be started at a CD4+ cell count under 100 cells/mm^3 due to an increased risk of reactivation. Pregnant patients require urgent ARV treatment after 14 days of antituberculous treatment.

9. Conclusion

The failure of the antituberculous/antiretroviral treatment is generally a result of the low compliance, inadequate treatment regimen (length, doses, low penetration into the CSF, adverse reactions impeding the use of certain efficacious drugs), delays in the diagnosis or treatment resistance. Any changes in the clinical examination, imaging studies and CSF aspect during treatment or at follow-up require further investigations. Despite the immunodeficiency the prognosis of CNS TB in HIV patients resembles that of non-HIV patients.

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ANTITUBERCULOUS DRUGS		
Drug	Pharmacologic aspects	Drug interactions/Adverse reactions
Isoniazid (NIH)*** (first-line agent)	Interferes with mycolic acids synthesis. Bactericidal to rapidly-dividing extracellular mycobacteria, bacteriostatic against the slow-growing intracellular mycobacteria. CSF peak concentrations exceed 30 times the minimal inhibitory concentration	Peripheral neuropathy (requires pyridoxine supplementation). Hepatotoxicity (reversible) depending on the dose and association with rifampicin and alcohol consumption. Rare cases of fulminant hepatitis. Rare allergic reactions.
Rifampicin* (first-line agent) Associations of rifampicin: rifamate, rifater Rifabutin* Rifapentine is a semi-synthetic rifamycin derivate with longer half-time (not recommended in HIV patients)	Rifampicin acts against intra and extracellular bacilli, especially on slow-growing mycobacteria (bactericidal). The metabolism is primarily hepatic; because of its ability to induce certain microsomal hepatic enzymes (CYP3A4) it interferes with the metabolism of other drugs. Poorly penetrates the CSF in the absence of meningeal inflammation. In meningitis CSF level is up to 10-20% of the serum levels. Rapid emergence of resistant mycobacteria. Rifabutin is bactericidal.The level of rifabutin in the serum is 7-10 times lower than the concentration of rifampicin. It easily diffuses through the uninflammed meninges.	Renal failure. Digestive and allergic reactions. Hepatotoxicity (cholestatic hepatitis) especially in drug associations. Hemorrhagic manifestations due to thrombocytopenia. Sulfamethoxazole/ trimethoprim enhances the effect of rifampicin and could increase its toxicity. Corticosteroids decrease the level of rifampicin. Rifampicin could singnificantly reduce the plasma concentrations of most PIs and some NNRTIs; it could be associated with NRTI and some NNRTIs. Adverse reactions to rifabutin mirror those of rifampicin; in addition rifabutin could induce uveitis, arthralgias, leucopenia, asymptomatic hepatitis. Rifabutin does not interact with PIs. Because rifabutin is a less potent inducer, it is generally considered a reasonable alternative to rifampicin. Doses should be adjusted in the coadministration with an PI ; underdosing of rifabutin can result in selection of rifamycin resistance, whereas overdosing of rifabutin might result in toxicities.
Pyrazinamide*** (first-line agent)	Active against intracellular bacilli only at acid pH. Bactericidal/bacteriostatic (dose dependent). Is well absorbed and crosses the blood-brain barrier leading to CSF concentrations almost as high as those in the blood	Hepatotoxicity Hypersensitivity reactions
Ethambutol* (first-line agent)	Bactericidal with low activity. Ethambutol could increase the activity of other antituberculous drugs affecting the cellular permeability of MAC strains and possibly of multiresistant M.tbc strain. Low CSF level (moderate rise above the minimum bactericidal concentration)	Optic neuropathy especially after prolonged treatments. Rarely triggers allergic reactions and hyperuricemia. No hepatotoxicity reactions.
Streptomycin* (second-line drug)	Belongs to aminoglycosides class. Bactericidal. Active only on replicating extracellular bacilli. Poor CSF level even in patients with meningitis. High rate of resistance	Nephrotoxicity. Neurotoxicity. Ototoxicity. Contraindicated in pregnancy. No recorded hepatotoxic reactions
Amikacin* (second-line drug)	Belongs to the class of aminoglycosides. The same characteristics as streptomycin. Low CSF concentrations	Less toxic than streptomycin.Contraindicated in pregnancy

ANTITUBERCULOUS DRUGS		
Drug	Pharmacologic aspects	Drug interactions/Adverse reactions
Ofloxacin** Levofloxacin** Moxifloxacin** Ciprofloxacin *	Belongs to fluorochinolones class. Bactericidal. Active on rapidly multiplying bacilli. Acts on nontuberculous mycobacteria. Good CSF penetrations. except for ciprofloxacin	Rare adverse reactions. To be avoided in pregnancy. Interferes with antiacids
Azithromycin Clarithromycin	Belongs to macrolides class. Bacteriostatic. Active on nontuberculous mycobacteria. High intracellular levels Do not cross the blood brain barrier.	Clarithromycin interfers with PIs and efavirenz; azithromicin does not display these interferences.
Ethionamide*** (second-line drug)	Bacteriostatic/bactericidal (dose depending). Effect on extra/intra cellular bacilli. Good CSF penetration (equal to those in serum).Active on resistant mycobacteria.	Allergic reactions. Digestive reactions. Hepatotoxicity. Neurotoxicity. Teratogenic effects
Cycloserine*** (second-line drug)	Bactericidal/bacteriostatic (dose depending). Effect on intra and extracellular bacilli, including resistant mycobacteria. Good CSF penetration	Neuropsy-chic reactions. Rash. Not recommended with efavirenz. No hepatotoxicity; indicated in patients with acute hepatitis in combination with other nonhepatotoxic drugs.
CCR5 antagonist: maraviroc (MVC) **	Belongs to the entry inhibitor class (chemokine receptor antagonist); it blocks HIV entry into the host cell. Substrate of CYP3A enzymes.	Hepatotoxicity. Rash. Caution and dose adjustment is necessary when MVC is used in combination with CYP3A inducers agents (such as EFV or rifampin).
Fusion inhibitor: enfuvirtide (EFV) *	Belongs to the entry inhibitor class. It is not affected by the CYP enzymes	Hypersensitivity reactions. Can be used with the rifamycins
Integrase inhibitor: RAL**	HIV-1 integrase inhibitor. Blood-brain-barrier restrict RAL entry; meningeal inflammation enhances drug entry.	Hypersensitivity reactions. Rifampin and rifabutin can significantly reduce the concentration of RAL.
Protease inhibitors (PI): SQV*;ATV***;DRV*;FPV***; AMP ***; IDV***; LPV***; NFV*;RTV*; TPV*	Interfere with the protease enzyme that HIV uses to produce infectious viral particles. PI are CYP P450 inducer and substrate	Hepatotoxicity (requires monitoring of hepatic enzymes). Rash. Prolonged QT interval. PIs are not recommended with rifampicin. Adjust the dose of PIs when combined with rifabutin
Non-nucleoside reverse transcriptase inhibitors (NNRTI): EFV**;NVP*** ETV; DVR***	NNRTI bind to revers transcriptase, interfering with its ability to convert the HIV RNA into HIV DNA The NNRTIs are also substrates of CYP3A4 and can act as an inducer/inhibitor or mixt NNRTIs are related with an increased risk of resistance if the therapeutic regimen is not respected.	Hepatotoxicity. Hypersensitivity reactions. Fewer interactions with RIF; nevirapine does not affect the levels of RIF; efavirenz or nevirapine-based regimen are preffered when using associated therapy with RIF; etravirine not recommended with RIF. Adjust the doses in the combination of EFV and rifabutin /rifampicine
Nucleos(t)ide reverse transcriptase inhibitors (NRTI): ZDV***; 3TC** ABC ***; d4T ** ddI* ; FTC**TDF*; ZAL*	Interfere with reverse transcription and conversion of HIV RNA to HIV-DNA. Do not use the CYP metabolic pathway. No significant interaction with rifampicin or rifabutin	Hepatitis. Neuropathy (only stavudine, didanosine). Optic neuritis (didanosine)

***very good ability to cross the blood-brain barrier; ** moderate ability to cross the blood-brain barrier; * low ability to cross the blood-brain barrier

Table 3. The most important antituberculous and antiretroviral drugs used in the treatment of CNS tuberculosis [113-118]

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Miscellaneous

Chapter 16

Research and Development of New Drugs Against Tuberculosis

Juan D. Guzman, Ximena Montes-Rincón and Wellman Ribón

Additional information is available at the end of the chapter

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1. Introduction

Tuberculosis (TB) is a contagious infectious disease caused byspecies belonging to the *Mycobacterium tuberculosis* complex. At present, it is a re-emerging disease, due to co-infection with the Human Immunodeficiency Virus (HIV), but also to global bacterial resistance, and lack of adequate treatment in some places in the world. Approximately one third of the world's population is infected with *M. tuberculosis*, and out of these people, about 1.1 million people die every year of TB [1], making this disease the main cause of bacterial infectious death in adolescents and adults all around the world. In 2010 there was an estimation of 8.8 million incident cases and 12.0 million prevalent cases of TB worldwide. *M. tuberculosis* drug-resistant isolates have appeared giving origin to multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains. XDR-TB has been identified in every continent of the planet. By 2010, the World Health Organization (WHO) was notified of the existence of 53.018 cases of multi-drug resistant TB (MDR-TB) worldwide; figure that only represents 18% of the TB-MDR estimated cases among reported pulmonary TB cases around the world [1]. Currently, there is global alarm since the infection with these strains is cured only in 66% of MDR cases and in 60% of the XDR cases [2].

More than sixty years ago, the introduction of the first anti-TB drugs for the treatment of TB (streptomycin (STR), *p*-aminosalcylic acid (PAS), isoniazid (INH) and then later ethambutol (EMB) and rifampicin (RIF)) gave optimism to the medical community, and it was believed that the disease would be completely eradicated soon. After a 30-year halt of anti-TB drug Research & Development pipeline, the Global Alliance for TB Drug Development (TB Alliance) started to fill the gap between the existing chemotherapeutics and the clinical need. Despite the efforts carried out with candidates in clinical trials such as PA-824 and bedaquiline, there is an urgent need of in-depth medicinal chemistry discovery studies for assuring enough leads

and candidates feeding the pipeline within the next decade[3]. Emerging chemical entities must shorten the time of treatment, be potent and safe while effective facing resistant strains and non-replicative, latent forms, and not interfere in the antiretroviral therapy [4]. In this review, we explore why we require to work continuously on the development of novel anti-TB agents, the stages necessary for the development of new anti-TB agents, breakthroughs in the discovery of new active principles and targets, the preclinical and clinical development of drugs, as well as the new approaches for the search of anti-TB active principles.

2. Targets and action mode of active principles currently used in the treatment of TB

Current TB chemotherapy is based on the combination of four anti-TB drugs which inhibit the bacterial metabolism, particularly the cell wall synthesis [5]. During the therapy, the goal of this drug combination strategy is to prevent effectively the mutational events [6]. According to their action mode, first and second line anti-TB drugs are grouped into cell wall inhibitors (INH, EMB, ethionamide (ETH), and cycloserine (DCS)), protein synthesis inhibitors (RIF, fluoroquinolones, STR, kanamycin (KAN)), and membrane energy metabolism inhibitors (PZA).

Current chemotherapy principally inhibits cell processes such as cell wall biosynthesis and DNA replication, and they only turn to be active regarding bacteria in active growth [5]. This implies that the chemotherapeutic agents in use are efficient bactericides but are poor sterilizers, not able to kill "dormant" *M. tuberculosis* which persists in macrophages after the death of the active bacteria [5]. RIF and PZAhave a partial sterilizing activity and they play an important role in the decrease of therapy from 18 to 6 months, even though there is a persistent population surviving these two agents. Consequently the current therapy ensures a clinical cure but fails to obtain a bacteriological cure [5].

3. Why we need new active anti-TB agents?

Whereas it is true that TB can be cured with the current active principles, treatment is complex and long, involving four drugs for two months and two drugs for four months more as a minimum.

	Active principle (year of discovery)	Source	MIC (μM)	Action mechanism	Target site	Genes involved in the resistance
First Line	Isoniazid (1952)	Synthetic	0.182	Mycolic acids synthesis inhibition, multiple effects on DNA, lipids and carbohydrates	Enoylreductase (InhA)	katG, inhA, ndh

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	Active principle (year of discovery)	Source	MIC (μM)	Action mechanism	Target site	Genes involved in the resistance
	Rifampicin (1966)	Semi-synthetic	0.486	RNA synthesis inhibition	RNA polymerase β sub-unit	rpoB
	Pyrazinamide (1952)	Synthetic	490 pH 5.5	Breakage of transport membrane and energetic depletion	Membrane energy metabolism	pncA
	Ethambutol (1961)	Synthetic	2.45	Aarabinogalactanbiosynthesisinhibition	Arabinosyltransferase	embCAB
	Streptomycin (1944)	Natural	1.72	Protein synthesis inhibition	rRNA ribosomal proteins S12 and 16S	*rpsL, rrs*
Second Line	Kanamycin (1957)	Natural	3.43	Protein synthesis inhibition	rRNA ribosomal proteins S12 and 16S	*rpsL, rrs*
	Amikacin (1972)	Semi-synthetic	0.85-1.7	Protein synthesis inhibition	rRNA ribosomal proteins S12 and 16S	rpsL, rrs
	Fluoroquinolones (1980s)	Synthetic	0.6-1.4	DNA replication and transcription inhibition	DNA gyrase	gyrA, gyrB
	Ethionamide (1956)	Synthetic	1.5	Mycolic acid biosynthesis inhibition	Enoylreductase (InhA)	inhA, etaA/ethA
	Prothionamide	Synthetic	2.77	Mycolic acid biosynthesis inhibition	Enoylreductase (InhA)	*inhA, etaA/ethA*
	p-aminosalicilic acid (1946)	Synthetic	1.9-6.5	Inhibition of thymidilate synthase and iron acquisition	Thymidilate synthase	thyA
	Cycloserine (1952)	Natural	245	Peptidoglycan synthesis inhibition	D-alanine racemase	alrA,ddl

Table 1. Reported MIC and molecular targets drugs of first and second-line drugs used in the treatment of TB [7].

Since the start of chemotherapeutic era, physicians have realized the slowness and difficulty of achieving effective cure. McDermott et al proved in 1956 that the *in vitro*efficacy of first-line TB drugsdo not correlate to their *in vivo* efficacy [5]. Cultures of *M. tuberculosis* in exponential growth are sterilized *in vitro* in a few days by firstline agents such as INH and RIF, while the same combination requires months to achieve the same result in host tissue. It has been stated that mycobacterial persistency is due to the physiologic heterogeneity of bacillus in the tissues, the existence of subpopulations with completely different rate-determining factors. Despite an urgent need for new therapies targeting persistent bacteria, our knowledge of bacterial metabolism throughout the course of infection remains rudimentary [8].Mitchison and colleagues proposed in 1979 that, in lesions, *M. tuberculosis*exists under at least four different population stages listed below [9] and showed in Figure 1:

1. Bacteria in active growth, susceptible to INH.
2. Bacteria with intermittent metabolism period, susceptible to RIF.
3. Low metabolic activity bacteria residing in acidic pH, susceptible to PZA.
4. "Dormant" or "persistent" bacteria, non susceptible to any current active principle.

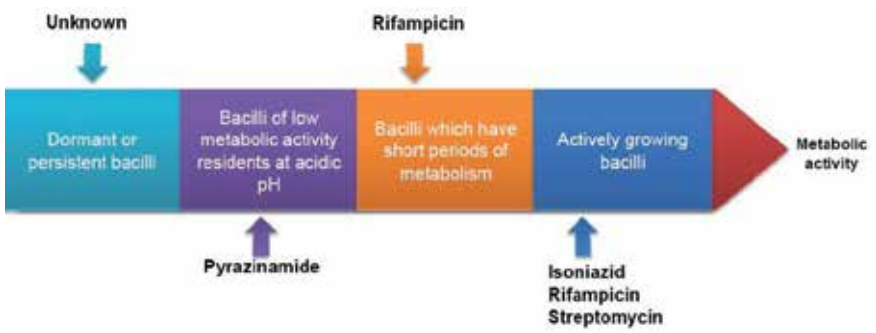

Figure 1. Spectrum of *M. tuberculosis*physiology.Extent of variation of physiological cell subpopulations of *M. tuberculosis* on an *in vivo* environment. Notice that first-line drugs mainly inhibit actively dividing bacteria, while there is not a single agent targeting the lower physiologically active stages.

During the initial chemotherapy phase (2 months), actively dividing bacilli rapidly die mostly because of INH bactericidal activity. Thereafter bacilli of low metabolic activity suffer from a slow death under the effects of RIF and PZA. There is evidence that persistentbacillarpopulation existing in the lesions usually determines the duration oftherapy [9]. Therefore efforts need to be made to target every physiological state of *M. tuberculosis* thus shortening the time of therapy and the appearance of drug resistance.

That brings us to the second reason why we need new anti-TB drugs. Drug resistance has emerged as a phantom from the dark, threatening today every corner of the world. RIF-resistance often correlates to MDR category (resistant to INH and RIF). XDR *M. tuberculosis* is an MDR strain also resistant to any fluoroquinolone and at least one injectable agent. Prognosis is less favorable for patients harboring XDR-bacilly compared to patients with MDR, with five times higher risk of death, require longer hospitalization or treatment times. However it has been shown that within an aggressive treatment, XDR-TB patients have been successfully cured in 60% [10,11]. Treatment of M/XDR-TB usually takes more than two years, and requires the use of more toxic, less effective and more expensive drugs. In resource-limiting countries, supplies of second-line drugs cannot be guaranteed. In an attempt to improve the conditions for millions of patients, Jim Yong Kim and Paul Farmer from Partners in Health brought down the price of second-line drugs has by more than 80%. Unfortunately the latest reports from Italy, India and Iran, facing the extremely (XXDR) or totally (TDR) super-bug, have made imperious the essential necessity of new drugs targeting novel mechanisms of action [12].

TB infection in immune-compromisedpopulation leads tosevere cases,possibly affecting other parts of the body, such as the pleura, meninges, the lymphatic system, the genitourinary

system, and the bones [13]. It has been estimated that HIV infected patients are 100 times more likely to develop TB [14]. Although the studies support a decrease of mortality for TB patients after the introduction of antiretroviral therapy, evidence of the existence of interactions between Highly active antiretroviral therapy (HAART)and TB chemotherapy. HAAR is based on a combination therapynormally involving two reverse-transcriptase inhibitors and a non-inhibitor [15]. P450 Cytochrometypicallymetabolizes reverse-transcriptase inhibitors,however this cytochrome is also induced by RIF. TB chemotherapy may reduce significantly the concentrations of anti-retroviral drugs which may lead to treatment failure or resistance. An increase of the nevirapine dose to compensate for this interaction increases the risk of toxic effects and hepatotoxicity in patients who already present a low body mass index and high level of CD4 lymphocytes [16]. Physicians prefer to avoid the concomitant use of nevirapine and RIF; consequently there is a clinical need for mycobactericidal agents devoid of P450 catabolism.

4. A 50-year wait

Antibiotic discovery began in the early 1930s when different classes were discovered [17]. At the end of the 1950 decade, the combined regime was established and was thought to eradicate the disease completely. In the following thirty years after the introduction of the last first-line anti-TB drug, RIF, the regimen remained unchanged. The landscape changed in 1993 when the WHO declared TB a global health emergency [18]. Until recently, research in development of new anti-TB drugs was poor. These days, the TB Alliance has emerged as a non-profit organization promoting and funding anti-TB drug development by creating consortia over a defined project involving oftenbig pharmacompanies, institutes of research, and universities. Interest in drug discovery has placed on both phenotypic and target-based approaches to set in motion strong pipeline. With the joint effort of the Working Group on New TB Drugs, Stop TB Partnership and other societies.gatifloxacin, delamanib, PA824, rifapentine, sutezolid, SQ-109, bedaquiline and linezolid are candidatesin clinical trial [19]. There are other promising compounds (CPZEN-45, BTZ043, AZD5847, DC-159a and others), but a handful ofscientists believe that the gap is large and there is no certainty whether there will be a full new regimen in the next decade [3].

Neglected diseases affect mostly the poorest population on Earth, predominantly those who live in remote, rural areas, in depressed urban settings, or in regions of conflict. Together with malaria, leishmaniasis,filariasis and Chagas disease, TB makes part of the high impact neglected diseases, which unfortunately represent an insufficient market to attract enough investment on research by the pharma industry [20]. Whereas the most advanced societies have increased their life expectation thanks to technological development of medicine, in developing countries these diseases (some of which are preventable, treatable, and curable) still devastate the frailest populations. However, governments, multilateral organizations, and foundations spend billions of dollars in the procurement of treatments; and with the current situation of the disease, world health care organisms applaud recent efforts to develop new anti-TB drugs, even though the panorama is not that promising yet [3,21].

5. Platform for the development of active principles in the treatment of TB

Both basic and clinical pharmacology have contributed to the progress in the discovery of drugs applying their experience to the development and validation of hypotheses of new action targets in order to produce novel drugs. In this sense, researchers need to be innovative and they must have a wide vision over the interpretation of the results [20]. The choice of a therapeutic candidate is probably the most important decision to make in the discovery and development of a medication. The chemical structure of a drug confers its biologic, pharmacokinetic, physicochemical, and toxicological properties [22]. On the other hand, the discovery and development of new drugs is a complex and costly process requiring large amount of resources and time. The cost of launching a new drug to the market ranges from US$ 800 million and 1000 million, and it may take between 8 and 17 years depending on the disease and the treatment (Figure 2) [23].

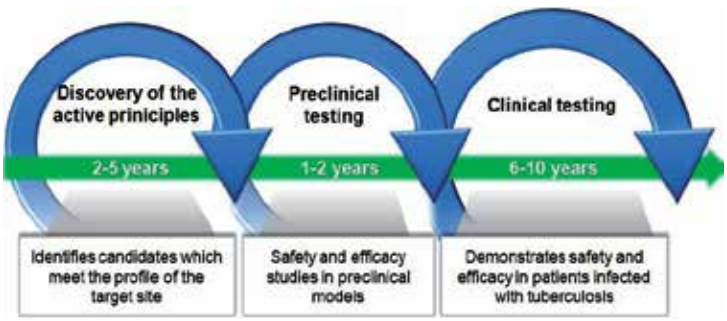

Figure 2. TB drug pipeline. From the discovery bench through preclinical and clinical studies for novel anti-TB agents, a process that could last more than 15 years.

The term "hit" is used to describe a small number of structurally related molecules possessing an established biologic (antituberculosis) activity [24,25]. The term "lead" is defined as a molecule belonging to a series, which shows a substantial structure – activity improvement around a determined *"hit"*, and from which other important factors have been obtained such as evidence of selectivity and pharmacokinetic data or *in vivo* activity [24].

Once these terms are defined it is important to knowthe biochemical target on which a certain structural type of a chemical compound exerts a biological action. The determination of the mechanism can be carried out *in vitro* by generating drug-resistant mutants which are examined on their whole-genome sequences analysis. The transcriptional profile using cutting edge mycobacterial microarrays or *q*PCR) can be interrogated among the whole transcriptome for potentially distinguishing a defined set of genes involved in the response against chemical injury. Once a determined protein or receptor has been identified, cloning, over-expressing and purifying the proteins is usually performed with the aim of examining its biochemistry and its possibility of affinity or interaction in the tube test is always possible option. Gene deletions and over-expressing systems in *Mycobacterium* are also used for confirming the mechanism of action of a defined candidate [26].Ideally, an antibacterial agent must show bactericidal activity often impeding an essential function for the survival of the microorganism.

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Another more classical possibility is monitoring of microbiologic parameters such as growth rate, CFU counting and chemical analysis of metabolites in the treatment with sib MIC, MIC and over-MIC values of the agent. Currently, many active principles are identified as the result of a rational design, supported by genomics inspired hypotheses or from another perspective, by automated high-throughput screening (HTS) using compounds libraries [23].

6. Discovery of active compounds

The parameter most commonly determined to examine the in vitro antibacterial activity of a specific moleculeis the minimum inhibitory concentration (MIC) which represents the concentration required to inhibit 99.9% of the growth of bacilli The main limitations of these trials is that do not describe the percentage of dead bacteria (which critically depends on cell density) or the metabolic state of the bacteria, if we aim to examine the persistent antimicrobial effects of a certain drug [27]. Most publications include at least a compound with a MIC lower than 6.25 mg/Ll [24,26]. It is recommended that active compounds under a colorimetric assay (Resazurin, Alamar Blue, MTT) are reconfirmed usingagar-based techniques or MGIT. A simple and easy to use, agar-based method using Middlebrook 7H10 was introduced in 2004 by Bhakta et al for measuring MIC values [28,29]. The spot culture growth inhibition assay (SPOTi) has now being used to screen more at least more than 1000 compounds. Simultaneously, the cytotoxicity in different type of mammalian and/or macrophages is carried out. The selectivity index (SI) is determined by dividing the growth inhibitory concentration 50 (GIC_{50}) corresponding to the concentration of compound capable of killing half of the mammalian cellsby the MIC using the same concentration units. If the SI is larger than 10, infection of a macrophage with a selected strain of mycobacteria and treating with the drug candidate can help to determine its intracellular potential (Figure3)[26].

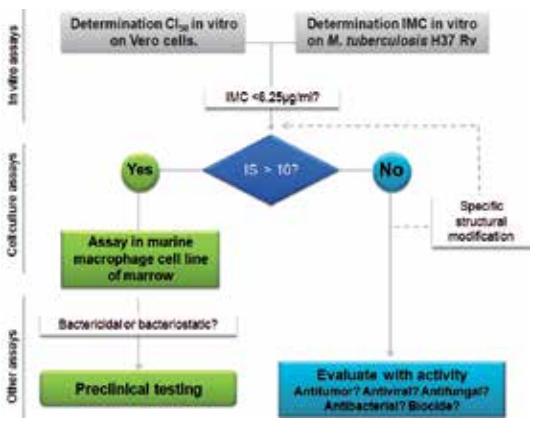

Figure 3. Research and development of new TB active compounds. In an attempt to promote quickly pre-clinical studies of early leads, Orme propose this rapid diagram based on the selectivity ration between a bacteria and mammalian cell line [26].

The macrophage infectionmodel offers the possibility of evaluating the compound in a physiologically challenging intracellular space. By plotting a viability curve fordifferent concentrations of the active principle, The $EC_{90,}$ EC_{99} and $EC_{99.9}$values of are determined verifying the concentration that is able to reduce the bacterial load by 1, 2 and 3 logarithmic units. MIC is most usually defined as EC_{99}.(or EC_{95}). Bactericidal compounds are generally associated with a 3-fold reduction in CFU logarithmic units. In addition the infection assay determines the activity of a compound in an intracellular medium which does not always correlate with *in vitro* media-based inhibition measurements. For instance, transport mechanisms in the cell may influence the intracellular concentration of the drug regardless of the external fluid concentration [30].

The success of a discovery program of antibacterial principles is founded on three factors: identification of key elements contributing to pathogenicity of the microorganism, the understanding of the existing relationships between the microbe and the host, and importantly, the properties of the chemical compound [30]. Two pathways have been traced with the aim of discovering active principles. One is the empirical pathway, mainly based on chemistry and phenotypic screening; and the more modern is the mechanistic, based on genomics, biochemistry and molecular biology. The former begins with the identification of an active principle with potent antimicrobial activity on *in vitro* conditions. The active principle is discovered by chance or by random screening. Then, it is subject to trial on rigoroustoxicological assays before using animal models. Some candidates may eventually beselected for human trials. The limitation of the empirical pathway is the lack of information on the specific target or the action mode, sometimes this lack of understanding can led to high failure rates mostly for toxicity problems [30]. On the other hand,the mechanistic pathway started with the age of molecular biology and genomics which allowed the identification of specific targets of the microbe, absent or structurally different in human hosts. This strategy can be upgradeto high-throughput screening (HTS) platform and to evaluate a large amount a substances in little time. Crystallization of the target proteins and X-ray diffraction spectroscopy, together with an analysis of the active site in the presence of the natural substrate and inhibitors allow the detailed study of the crucial structural interactions.

In the mechanistic approach discovery usually involves firstly the identification and validation of amycobacterial target macromolecule to be inhibited or interrupted. Obtaining the small molecules which inhibit such a target is another story. Large collections of compounds can be screened directly against the protein if a high-throughput method of assay is available. Alternatively if there is structural information it is possible to computationally interrogate the target against a defined set of computer-based compounds (docking). The preferred targets are generally the ones ocurring in *M. tuberculosis* and not represented in the human genome. By means of comparative genomics, the targets are present in the human genome. For example, nicotinamide adenine dinucleotide (NAD)is generated in humans either by *de novo*biosynthesis, or by DNA and RNA degradation. However*M. tuberculosis*can only synthesize NAD using the*de novo* synthesis. This allows to rationally explorequinolinatephosphoribosyltransferase (QAPRTase) inhibition (*de novo* pathway) for the developing of microbial selective inhibitors [30].

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Targets existing in *M. tuberculosis*whileabsent in other bacteria would seem ideal since active compounds against this target will be harmless to bacteria beneficial for the human being. However, selecting targets complying with this requisite leads to restrict extensively the likely targets: for the most of it only the biosynthesis of the mycobacterial cell- wall or those implied in specific mycobacterial process (virulence, detoxification, others).

The validation of a target, involves the examination of bacterial viability when decreasingthe protein expression. If reducing the enzyme level,led to lose in bacterial viability, then the target is known as "vulnerable", and it is meant to be attacked [30]. The elimination or knockout of the gene that codifies an essential protein is difficult (or impossible) to produce by homologous recombination if the gene is essential in the conditions of growth, and therefore inducible promoters are a better chance to show the effect of tightly reducing its expression. Over-expression of the target is also possible, the growth of the over-expressed mutant being rescued under higher concentrations of inhibitor. These studied have led to many targets that have been identified and validated. The studies of Sasettiet al identified which enzymes were essential in vitro and in vivo using a transposon site hybridization analysis (TraSH) using both in vitro or in vivo [31,32].

Another approach is related to the genomics of virulence. Some mycobacterial genes are only expressed in granulomabut not inside the macrophages. Isocitratelyase enzyme is fundamental in the persistence of bacilli in chronic infection in mice and its function is related to obtaining carbon during its persistence in the host [8,33]. The extracellular repetition protein (Erp) is another essential protein involved in *M. tuberculosis* virulence that was the first discovered virulence factor. The mutant Δ-erp that does not express correctly the extracellular repetition protein does not show any alteration in standard *in vitro* culture, but maintains an essential function for *in vivo* survival [34,35]. This protein is also a potential target for the development of anti-TB active principles. Two independent proteins (fadD28 and mmpL7) have been identified contributing to the early growth of *M. tuberculosis* in mice lungs and are related to the synthesis and transport of a complex lipid associated to the cell wall, i.e. phthioceroldimycocerosate (PDIM) [34]. Although the function of this lipid is unknown, it is suspected that itplays a role in the decrease of the host's immune response. There is no doubt about the remarkable progress that the sequencing of the*M. tuberculosis*genomehas brought to the anti-TB drug discovery area of research. The functional annotation of all these genes remains a considerable amount of experimental work. Sequencing of other related organisms such as *M. marinum*, *M. leprae*, *M. aurum*and othersoffer often clues about the essentiality of specific set of genes and operon distribution.

7. Target or compound type in discovery stage

Analogues of thiolactomycin: Thiolactomycinwas the first natural thiolactonedisplaying antibiotic activity. The compound showed moderate *in vitro* activity of 56 μM against *M. tuberculosis*[36]. Thiolactomycin analogues have been synthesized and some hits were found [5]. Analogues of thiolactomycin seem to inhibit mycolatesynthetase, an enzyme involved in the cell wall biosynthesis.

Nitrofuranilamides:*M. tuberculosis*has been found to be susceptible to compounds containing a nitro group. Nitrofuranilamide was identified in a screening for inhibitors of UDP-galacto-semutase [5]. A set of compounds structurally related to nitrofuranilamides was synthesized and tested for antimicrobial activity. All resulted active both to sensitive and resistant strains with a MIC ranging from 0.0004 – 0.05 mg/L [37]. Four nitrofuranilamide type compounds showed significant activity in the tuberculous infection in mice models [37].

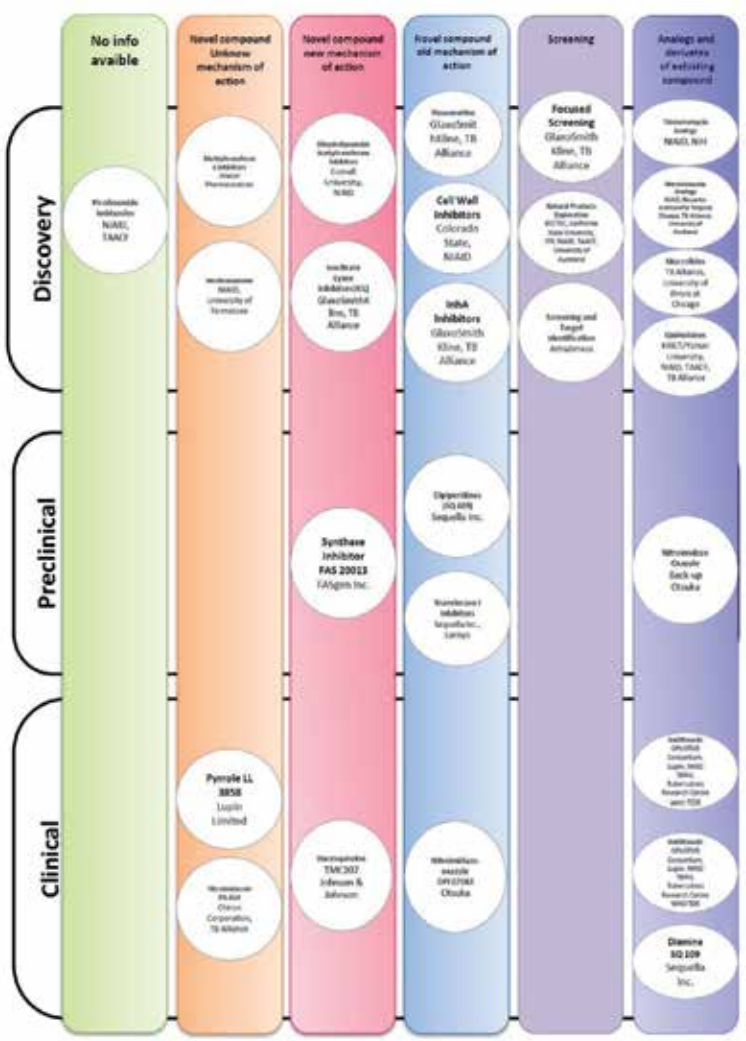

Figure 4. Development of new active compounds targeting *M. tuberculosis*

Analogues of nitroimidazole: While the PA-824 product is developed, the TB Alliance started a project to maximize the potential of this class, by identifying the improved versions of PA-824 [38,39].

Dihydrolipoamideacetyltransferase inhibitors: Dihydrolipoamideacetyltransferase (dlaT) enzyme of *M. tuberculosis* is a potential target for TBdrug discovery [5]. This enzyme is a component of the pyruvate dehydrogenase sub-unit, an enzyme catalyzing acetyl-CoA synthesis and also contributes to peroxinitritereductase, adefense enzyme against oxidative/ nitrosative stress. Some heterocyclic compounds have been found to be inhibitors of the dlaT enzyme, displaying non-replicative bacterial killing [40].

"Focused Screening": TB Alliance is helping to develop a set of projects identify chemical compounds which are active against specific molecular targets including DNA gyrase inhibitors (fluoroquinolones targets), peptidedeformylase inhibitors, and quinone-analogous electron transport inhibitors [5].

InhA inhibitors: InhA is the well-known enoyl-reductase of *M. tuberculosis*being an essential-biocatalyst forlong chain fatty acids biosynthesis (FAS-II) [41]. INH resistance is mainly mediated by mutations on KatG, the enzyme activating the prodrug. Consequently, InhA inhibitors that do not require activation by KatGcould be interesting candidates. The main goal of the screening is directInhA inhibition. Some compounds of the biphenylether type have proven to be inhibitory of InhAin a degree correlatingwith*in vitro* growth inhibition [42]. A possible limitation of this class is the possibility of cross resistance with INH and potentially with ETH [5].

Isocitratelyase inhibitors: The isocitratelyase enzyme (ICL) has been found to be essential for the long term persistence of *M. tuberculosis* in mice but not in culture medium or under hypoxia conditions. McKinney and colleagues have proved recently that inhibition of the ICL1 and ICL2 isoforms block bacterial growth and survival in macrophages [8]. The absence of orthologues in mammals for this enzyme, makes it a good target for the development of inhibitors [5]. A screening of more than 900,000 compounds has been performed without satisfactory results. The potential of traditional Chinese medicine has also been researched in obtaining specific inhibitors of this enzyme [43].

Pleuromutilins: Pleuromutilins represent a new kind of antibiotics derived from pleuromutiline, a bioactive diterpene initially isolated from edible *Clitopilusscyphoides*fungus [44]. These molecules interfere with protein synthesis associating to the 23S rRNA unit. Despite the structural novelty of these compounds, recent studies have pointed out cross-resistance among pleuromutilins and oxazolidinones[5]. Pleuromutilins have proved to inhibit *M. tuberculosis* growth of *in vitro.*

Macrolides: This project aimed to optimize the anti-TB activity of the macrolide class through the synthesis of modified derivates of erythromycin [5]. Derivatives of erythromycin such as 11, 12-diol, 11, 12-carbamates, and 11, 12-carbazates have been found to be to most promissory [45].

Quinolones and DNA gyrase inhibitors. The goal of this projectwasto synthesize and assess the potential of novel quinolones trying to decrease the time of treatment. More than 450 compounds were synthesized and assessed [5]. The 2-pyridones class has proven to be active DNA gyrase of *M. tuberculosis*, being KR1-10018 an interesting lead for the development of anti-TBdrugs [46].

Survey of natural products: Natural products represent an alternative for the search of new compounds. Different research institutescontinuously carry out screening of natural products (products from plants, fungi, and bacteria) with the hope of identifying compounds with anti-TB activity [5]. Some natural substances have shown significant anti-TB activity: saringosterol 24-epimers, esgosterol-5,8-endoperoxide, micromolide, ascididemin, the manzamines, and engelhardione, among others; however, there is lack of more research regarding selectivity and toxicity [47-50].

Plants: Drugs based on plants extracts have been used worldwide for the treatment of several diseases from ancient times.A great interest in phytomedicine and natural product structures are screened in order to measure their pharmacologic activity. In Colombia, there has been a resurgent interest in the discovery of novel natural anti-TB drugs [50-54].

Natural sea products: oceans are outstanding sources of natural products, not only in invertebrate species such as sponges, mollusks, bryozoos, but also in marine bacteria and marine sediments. The alkaloid (+)-8-hydroxymanzamine A was initially isolated from the *Pachypellina*sp sponge [55]. In the same way, irciniol A was found in sponges from the Indian Pacific proving to be a good candidate for further studies [56]. Aerothiononine isolated from the marine sponge *Aplysinagerardogreeni*marine sponge was active against clinical isolates of MDR-TB, despite of the resistance patterns, with MIC from 6.5 to 25 mg/L [57]. The alkaloid (+)-8-hydroxymanzamine A alkaloid showed potent inhibitory activity against *M. tuberculosis* H37Rv [58].

Insects. The immune system of invertebrates and vertebrates is made up by cytolitic peptides which act as antimicrobial agents during the invasion of eukaryotes and prokaryotes microorganisms. Poison from arachnids (spiders and scorpions) contains toxic peptides of high molecular weight (2 – 12 kDa) with high specificity against prokaryote cells [59]. This type of compounds may be very promising as a drug in the treatment of tuberculosis.

Microorganisms. Most of the major antibiotics drugs have been isolated frommicroorganisms. Streptomycin,the first effective anti-TB drug was identified in *Streptomyces griseus*. Besides streptomycin, aminogyicosides kanamycin, amikacin, and capreomycinhave been very important therapeutically as second-line agents [59]. Other important anti-TB drugs in TB treatment are the rifamycins, which constitute a group of semi-synthetic antibiotics isolated from *Streptomyces mediterrani*[59].

8. Preclinical and clinical development for new anti-tuberculosis drugs

8.1. Preclinical development of anti-TB active principles

Preclinical tests involve the use of animal models to prove the efficacy and safety of a given candidate before being tested in humans. Because of its management in terms of size, offer, maintenance, strength, and reproducibility, the mouse constitutes the preferred animal model for *in vivo* research of the TB infection [60]. Other possible animal models include rats, guinea pigs, and macaques. The amounts of viable mycobacteria and mortality and the possibility of organomegaly in the pulmonary tissue are evaluated during therapy, at the end of therapy

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and in the post-therapy period. Post antibiotic effect, relapses, and resistance development are examined. Antagonists, additives, or synergistic effects are also evaluated when the compound is administered in combination with other active principles, as well as its capability to sterilize lesions in experimentally infected animals. Finally, toxicological studies, which must be highly controlled and documented, are carried out forthe determination of the safety window in order to perform the subsequent clinical trials in humans [60].

The drugs regime must be administered for several months, using commonly between 100 – 150 mice per test, therefore requiring large amounts of space and resources for maintaining the animals. Model in mice is more effective regarding the cost-benefit relation, and most of the data obtained can be reproduced in clinical studies. The model of infection by TB in mice has served to predict the sterilizing potential of new compounds, the effectiveness of the combination of drugs, success in intermittent therapy and the duration of therapy necessary to avoid relapse. The effectiveness of the active principle is measured mainly the reduction of the colony forming units (CFU) in the lung and spleen. Several varieties of mice have been used in laboratories conducting this type of test and, to this date, no comparisons have been reported [61].

Genetically modified mice have been used in the in bioassay of compounds with anti-mycobacterial activity [62]. A mouse that does not express the interferon-γ gene (knock-out) is incapable of producing cytokine Th1 and therefore suffers a more acute infection. Bioassay with this mouse allows determining the initial efficacy of a chemical compound in six days. Because of their statistical power, substances with low antimycobacterial activity can be detected by a small decrease in the CFU count. The model has great usefulness in initial trials, when there is a limited amount of the chemical compound. Another model, still under development, has been proposed to study relapse. An animal that cannot produce the granulocyte-macrophage-colony stimulation factor (GM-CSF) is used.

Wayne's model, which indicates the effect of compounds against persistent bacilli, has also been used. Bacilli under anoxia conditions are used and they are directly inoculated in the mouse. The guinea pig model also allows observing the destruction of tissue by caseous necrosis where there is not oxygen contribution and bacteria go into a hypoxia state [61].

Pharmacokinetics and pharmacodynamics range from *in vitro* tests, *in vivo* tests in animal models, and finally clinical trials in humans [57]. The simplest pharmacodynamic measure is determining the MIC *in vitro,* used widely in the primary discovery of active principles. This measure can be roughly related to the maximum cut point of the active principle concentration in plasma (C_{max}) and can aid in the prediction of *in vivo* pharmacodynamics among a series of structurally related agents. However, it does not represent the concentration at which the growth ceases, and, as we have already seen, does not allow distinguishing between bactericide and bacteriostatic activity. Moreover, it does not allow obtaining information regarding the dynamic relationships *in vivo* either, since the growth conditions do not represent the ones of persistent organisms in the living tissue.

Animal models enable to evaluate the *in vivo* efficacy of novel active principles regimens. Protection experiments using monotherapyfor a certain amount of time and then performing

lethal intravenous or aerosol inoculation can prove the efficacy and selection of a preliminary dose. Studies on the short term using colonies count in different homogenized organs allow estimating the bactericide capability of a medication or a combination of drugs, as well as the likely appearance of resistance [57]. However, in order to describe the sterilizing activity of a given compound, a larger study time is required as well as other techniques since negative cultures finalizing the therapy do not necessarily indicate that there was sterilization. Three months are required after the end of treatment to determine a durable cure and success of the sterilization. Cornell's mouse model uses an intensive therapy in order to obtain negative cultures and then evaluate the ability of individual active principles or their combinations to prevent relapse when the mouse is left untreated or when it is maintained immunocompromised[57].

The following are the PK and PD parameters which are calculated in the trial with mice: the C/MIC quotient, defined as the ratio of the serum maximum concentration (C_{max}) over the MIC; the AUC/MIC quotient, defined as the ratio of the area below the concentration-time curve (AUC) over the MIC in the serum during the total time of treatment(144 h) divided by 6 in order to obtain a daily value (AUC_{24}/MIC); and the percentage of time above the MIC (T > MIC) estimated by the first order kinetic equation $C = C_0 e^{-kt}$, where C_0 is the concentration to time 0, k is the constant and t the time, and it is defined as the percentage of the 144-hour time in which the medication concentration surpasses the MIC in the serum [63].

Recent studies of the PK and PD parameters for INH, RIF, and fluoroquinolones have improved the understanding of PK and PD properties of these drugs. Although the PK and PD parameters are characterized for antibacterial agents, a clear description of the efficacy is still lacking [63]. The parameter that best describes the bactericidal activity of anti-TB drugs in the mice model is AUC_{24} / MIC, with a correlation of 0.83. For INH when the value of AUC_{24} / MIC reaches 500, the maximum effect is observed with a decrease of 1.3 log CFU per mouse lung. In other words, the INH effect was the same when the total doses were administered into 6, 12, or 18 doses divided equally during one week [63]. Mitchison observed that the administration of a single total dose of INH in infected guinea pigs had the same effect than if administered daily, every other day, or every four days during a six-week period. Therefore, the efficacy of INHwas dependent on the size of the doses but not on the regime [63]. Preclinical trials that establish pharmacodynamic and pharmacokinetic properties enable to obtain information regarding the optimal doses and regimens.

Despite of the large use of the mouse model,this rodentdoes not develop the typical human lesions observed in pulmonary TB such as caseous necrosis or cavitations[57]. Also, one has to be very prudent conducting escalation in the doses of the agents between the mouse and the human due to the metabolic differences and possible pharmacokinetic interactions. The histological characteristics of guinea pigs in a TB infection are more similar to human pathology; but there is little experience in the chemotherapeutical use of this model. Preliminary studies suggest that the guinea pig model is capable of differentiating the sterilizing activities of INH and RIF [57].

A good model to study latent forms of TB is the cynomolgus macaque (*Macacafascicularis*) [61, 64]. All primates infected by bronchial instillation developed the infection, based on the tuberculin test and immune responses to *M. tuberculosis* antigens. Differences in the progres-

sion of the disease for the 17 macaques studied were observed. Between 50 – 60% of infected primates developed active and chronic infection, characterized by clear signs of disease in thoracic x-rays and other tests. Approximately 40% of the initially infected macaques did not develop the disease in the 20 months of study. These primates showed clinical signs of latent TB. In summary, the study proves that it is possible to use the cynomolgus macaque in infection by *M. tuberculosis* because it presents the complete spectrum of infection in humans (rapid and lethal infection, chronic infection, and latent infection). This animal model is the only one that enables to study the latent forms of the infection. Its great advantage is the high pathologic similarity of the infection in macaques and humans, whereas the disadvantages are the cost and maintenance of the animals, particularly since they require facilities with Biosafety Level 3 [64]. This model has been proposed in final preclinical trials for the development of active principles for latent forms of TB [61].The following are examples of promising compounds in preclinical phase. Some of these substances are protected by patents and therefore access to information is restricted.

The following are examples of promising compounds in preclinical phase. Some of these substances are protected by patents and therefore access to information is restricted.

SQ609 dipiperidine: this compound is a completely novel anti-TB active principle. It acts by interrupting the biosynthesis ofcell wall, but its specific mechanism is unknown [5]. It demonstratedantymycobacterial activity in an*in vivo* mouse model.

FAS20013 synthetase ATP inhibitor: Inhibition of bacterial fatty acids synthesis (FASII) still represents a valid, target for the discovery of anti-TBdrugs. However, this novel compound was identified by Fasgen and it has as action target the inhibition of enzymes for biosynthesis of fatty acids in *Mycobacterium* [65]. It belongs to the β-sulfonylcarboxamides class.

Translocase inhibitors SQ641: The pharmaindustryis developing a series of translocase inhibitors for the treatment of TB. The mycobacterial translocase I is an enzyme required for the biosynthesis of the cell wall, and the SQ641 compound has been reported as a selective inhibitor of this enzyme [66,67].

8.2. Clinical development of anti-TB active principles

Identifying new anti-TB is a complex and highly regulated process carried out around a critical moment: when the new compound is tested in humans [5]. Currently, clinical images offer a support method for generation of drugs which enables to establish information about the biodistribution of the molecule, interaction of the target, and pharmacokinetics[68]. Clinical development of a promising substance is usually divided into four phases. The first phase is carried out in healthy human beings and it provides information regarding the chemical compound pharmacokinetic profile, and some preliminary information regarding safety [69]. Phase I trials are conducted in a small size, usually 15 to 30 subjects, and can be of single or multiple doses. Besides the phase I trials, researchers may consider incorporating the pharmacokinetics and safety studies to a wider population size (200 to 300 subjects). Phase II studies are conducted on patients diagnosed with active TB. The efficacy in monotherapy and

combination therapy is evaluated. One of the objectives of trials in phase I/II is to determine the optimal dose for the phase III studies.

Early Bactericidal Activity (EBA) is one of the fundamental parameters to determine the clinical efficacy of active principles [70]. It consists on a large trial conducted on patients recently diagnosed with pulmonary TB who are treated with active principles or combinations for a period of 2 to 14 days. Patients must not have used anti-TB drugs previously. During the treatment period, the amount of viable bacilli appearing in sputum samples is determined quantitatively. The traditional EBA unit is the logarithmic decrease of colony forming units (CFU/mL sputum/day during the first 48 hours). EBA studies have shown that there are differences between the fall of viable bacteria counts in the first two days of treatment in comparison with the following twelve [71]. Differences among several treatments were also more significant during the first two days. In the early therapy, the activity of INH was superior and dominant regarding the other active principles administered in combination. Any addition of INH to a regime leads to an increase of EBA but never higher than INH on its own. The addition of PZA to a regime of STR, INH, and RIF increased 0-2 days EBA from 0.415 to 0.472 [71]. The greatest disadvantage of determining the EBA is its inability to detect sterilizing activity. Some researchers have concluded that extended EBA trials (2 to 14 days) do not correlate to the sterilizing activity [72]. For example, the potent sterilizing activity of PZA was not detected in an extended EBA trial. STR showed potent activity in extended EBA, and it is known it has a very low sterilizing activity in randomized clinical trials. In extended EBA, EMB appears as antagonist; however, there is no clinical evidence that this drug interferes with the sterilizing effects of RIF and PZA [72].

In order to determine the sterilizing activity of the anti-TB active principles, and 8-week study has been proposed, and the ratio of patients whose sputum be negative is determined; this parameter correlates to the ratio of patients who suffer relapse after the treatment [73]. In these studies, frequent counts of the number of viable bacilli are carried out being known as “serial sputum CFU counts” or SSCC. This method enables to distinguish between differences in the organisms that divide rapidly from the persistent ones.These studies turn out to be appropriate to determine the possibility of a regime to decrease the time of treatment [73].

Phase III clinical trials are carried out at large scale; they are randomized and they are conducted to demonstrate the improved or equivalent efficacy of a new treatment against standard treatments [60]. Around 1000 patients are enrolled per study for TB and the cure on treated patients is bacteriologically observed during certain amount of time as well as the ratio of relapse. The accepted end point to demonstrate efficacy is 2 years. The experimental design of Phase III trials must be designed cautiously clearly definingcritical primary and secondary end points, the size of the sample, the intervalsof confidence, and the statistical methods that will be used to obtain the data [60]. It is fundamental that microbiologic assessments are being conducted during an appropriate time, with the aim of determining the real activity of the researched agent. To ensure a sufficient population in Phase III studies, trials may be conducted in countries with high incidence rate of TB. Countries possessing a robust and expansive TB control program that provides essential information such as annual incidence (location of the disease, comorbility, resistance) are preferred. A reference laboratory is required for most of

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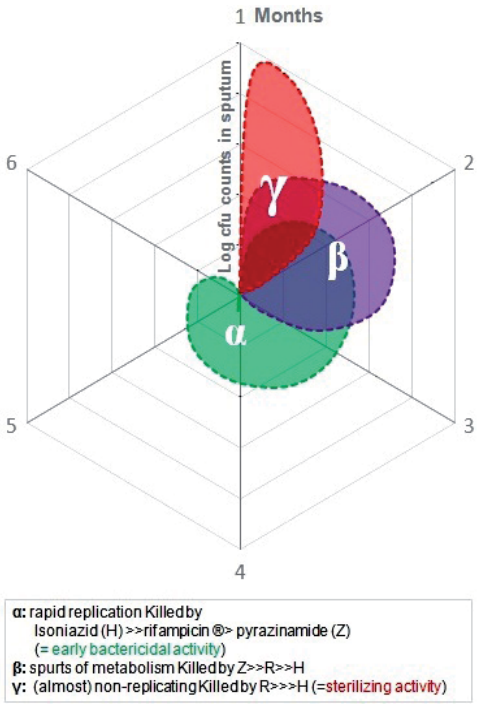

Figure 5. Pharmacological activity of RIF, INH and PZA targeting *M. tuberculosis* subpopulations. Differential population of *M. tuberculosis* in the lesions, observed after 6 months of treatment.as log CFU in sputum

the trials, but there is also need to extend duplicates to local laboratories. Finally, validated relapse markers, which provide evidence of the sterilizing activity of an active principle or regime, are used. To this end, the most used method is determining the ratio of patients who have a negative sputum culture, 8 weeks after the beginning of the treatment compared to the standard treatment. Molecular methods using relapse markers require greater study and validation in order to be employed successfully [60].

Phase IV studies include product development efforts such as patents, description of biologic activity, toxicity, safety profile in humans and demonstrated clinical efficacy. Best manufacturing practices studies are conducted, as well as laboratory and clinical practice to ensure the marketing needs of the product. Post-marketing studies duringPhase IV are typically assessment of new regimens in comparison to thenormally used, and surveillance of likely adverse effects, including the development of resistance. The acceptance for use of the new active principle must be subscribed by the patient, and the economic benefits of the new drug must also be established [60].

SQ109 diamine: The lead was identified in a screening conducted in 2003 using a combinatory library based on EMB as pharmacophore. It shows an MIC value of 0.11 μg/ml. t remains equally active as EMB at 100 mg/kg when administered in the mouse model at a dose of 1 mg/kg. However, SQ109 did not increase its effectiveness at higher doses (10 mg/kg, 25 mg/kg) and it was clearly less effective than INH[5]. Its effectiveness has been proved against MDR

strains. Preclinical toxicology studies have been completed and further phase II clinical studies are underway [67].

TMC-207 diarylquinoline: This agent is a promising agent as a new kind of antimycobacterial agent. Twenty diarylquinolines have been reported on the series with MIC lower than 0.5 mg/L against $H_{37}Rv$. Antimycobacterial activity was confirmed *in vivo* for three compounds of this class. The most effective agent was TMC-207, which had a MIC of 0.06 mg/L against $H_{37}Rv$ and its spectrum was unique in specificity against mycobacteria [74]. TMC-207 inhibits the ATP synthetase leading to a decrease of ATP and a pH imbalance. This compound has a potent EBA in the murine infection model, superior or similar to INH. The combinations TMC-207, INH, and PZA cleansed the bacilli present in the lungs of all mice after two months. Trials have also been conducted in mice with combinations of second line agents. Preliminary studies have proved *in vivo* sterilizing activity in the mice model, and decrease in the treatment time. Currently it is in phase IIa. Therefore, it is the most promising drug candidate in the last 30 years.

Gatifloxacin: It has been reported bactericidal activity *in vitro* and *in vivo* against *M. tuberculosis* to this compound. Its MIC has been reported between 0.25 mg/L and 0.03 mg/L against $H_{37}Rv$ [76]. In an *in vitro* study on bacilli in stationary phase, gatifloxacin showed the greatest bactericidal activity in the first two days, but none afterwards. In mice studies, the combination of gatifloxacin with EMB and PZA cleansed the lungs of infected animals after two months of treatment. Currently, gatifloxacin is under phase III to prove the efficacy and safety of a four-month regime for the treatment of pulmonary TB supervised by the European Commission Oflotub Consortium, WHO-TDR, Tuberculosis Research Unit (TBRU), National Institute of Allergy and Infectious Diseases (NIAID), Tuberculosis Research Centre [5,67].

Moxifloxacin: Moxifloxacin is the most promising fluorquinolone against *M. tuberculosis.* Its activity *in vitro* seems to affect bacilli unaffected by RIF. Its MIC reported *in vitro* is 0.5 mg/L [77]. It seems that moxifloxacin interferes with the protein synthesis on bacteria with low metabolic activity by a mechanism different to the one of RIF. However, the specific action mechanism is unknown. In the mouse model, the effectiveness of moxifloxacin is comparable to INH. When administered in combination with PZA, moxifloxacin has a greater bactericidal activity tan the INH + RIF + PZA regime. In fact, the combination RIF + moxifloxacin + PZA decreases completely bacilli count within four months, whereas the combination RIF + INH + PZA requires 6 months. It is likely that there is synergism among the three drugs, and the alternative regime replacing INH by moxifloxacin has been proposed. Moxifloxacin is under phase III [67]. Clinical studies have not proved a greater sterilizing activity of a regime containing moxifloxacin in comparison with the standard regime; however, it has increased activity in early points [5,75].

Nitroimidazole PA-824: This bicyclical nitroimidazole is under development by the TB Alliance, which has the proprietary rights. The *in vitro* MIC of the PA-824 compound is between 0.15 and 0.3 μg/ml [78].After an activation by the F420 factor of *M. tuberculosis,* the PA-824 compound inhibits biosynthesis of the cell wall components by means of mechanisms still to be established. It has proved bactericidal activity against replicative and static bacilli *in vitro.* Although PA-824 was more efficient than INH or moxifloxacin, during the continuation phase it was not better than the RIF + INHH combination. On the long term, the 12-month treatment

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did not achieve total eradication in any of the mice treated. The 6-month regime of PA-824 in combination with RIF, INH, and PZA in mice proved to be superior to the standard regime regarding quickness of eradication and lower relapse rate. This compound has been widely evaluated in animals and humans; currently it is under phase II clinical trials as part of an initial scheme (PA-824, moxifloxacin, and PZA) containing new anti-TB drugs[79].

Nitroimidazole-oxazol OPC67683: There is very little public information regarding this compound. It belongs to a subclass of mycolic acids synthesis inhibitors. It has shown *in vitro* activity against standard and resistant strains, showing a MICof 12 ng/ml [80]. It has not shown cross resistance with any other medication. The compound shown activity against bacilli residing within human macrophages and type II pneumocytes. The chronic TB trial in mice demonstrated an activity 6 – 7 times more effective than the one observed for first line INH and RIFdrugs. Favorable oral absorption and distribution have been reported. Currently it is under phase II clinical trials.

Pyrrole LL-3858: Some pyrroles derivatives have been found to have *in vitro* activity against *M. tuberculosis.* The MIC of pyrrole LL-3858 is between 0.025 and 0.12 μg/ml. The LL-3858 derivate identified by Lupin Limited showed greater bactericidal activity in the lungs of mice infected in monotherapy than INH. The trial of LL-3858 in combination with INH and RIF, or with INH, RIF, and PZA sterilized the totality of mice in 3 months [5]. Currently, the compound is under phase II clinical trials [67].

8.3. New approaches for the development of anti-TB active principles

In the dawn of the 21st century, pathogenesis of the infectious disease appears as a competition between the host and the pathogen involving short term adaptations and co evolution of the genomes [81]. The pathogen and the host constantly exercise selective pressure over each other, making the environment in test tube completely different from that within the host.

In latent tuberculous infection (LTBI), most of bacilli are not replicating, whereas in a phase of active disease most of the population is on active growth. Chemotherapy must take this metabolic adversity to favor the host.A durable cure must eliminate both the replicative and the persistent bacilli.Eradication of the persistent bacilli onchemotherapylasting between 6 – 24 months has been proposed in order to avoid relapse. However, such a long treatment is difficult to sustain and there is always resistance-associated risks in interrupted regimens [81].A philosophy of mycobacterial infection states that the essential genes for infection in mice include genes that are not essential *in vitro.*

The proteasome of *M. tuberculosis* is a set of proteins that provide a quick adaptation to changing conditions [82]. Two genes, *mpa (Mycobacterium* proteosomal ATPase) and *pafA (*proteasome accessory factor) were identified as important in the survival of *M. tuberculosis*to exposure against reactive nitrogen species (RNS) *in vitro* and required for active disease *in vivo* [82]. These genes codify for proteins involved in the bacteria proteasomal function. Proteasomes are barrel-shaped proteases consisting of 14 α units and 14 β units [82]. Mpa is similar to ATPases found in the proteosome of eukaryotes cells, and chemical inhibition of the protease activity of the *M. tuberculosis* proteosome causes sensitization of the wild strain to

reactive nitrogen species (RNS). The PafA protein does not share homology with any protein of known function [82]. Two specific proteasomeinhibitors, epoxomicin and a peptidic-boronate prevented the growth of *M. tuberculosis* and turned out to be bactericidal during the recovery of the mycobacterium against exposure to RNS [81]. The operon that codifies for the proteasome was knocked out by using conditional gene silencing and it was proved that bacteria require it to survive during the chronic infection in mice and its silencing allowed the mouse to be free of the persistent infection [83]. Whereas the proteasome of the mycobacterium is essential for the infection of a host, it is not required to grow in a rich and aerated medium such as Middlebrook 7H9 broth [81].

Unlike other bacteria, *M. tuberculosis* possesses a unique defense system that relates the antioxidant and metabolic activities [81]. The system includes a peroxyredoxin, the C subunit of an alkylhydroperoxy-reductase (AhpC), a thioredoxin type protein (AhpD), dihydrolipoa-mideacyltransferase (DlaT), and lipoamide dehydrogenase (Lpd), and the four enzymes together work as peroxydases and peroxynitroreductases and peroxynitroreductasesdepend-ant of NADH [81]. The dual functionality of these enzymes is interesting as potential targets for the development of anti-TB active principles.

Moreover, the DosRsystem, discovered 15 years ago, regulates the development of a form of non-eplicative survival without morphologic differentiation in *M. tuberculosis* (known as latency state). This state of physiologic quiescence maintained viable the microorganism for long periods of time, contributing with two key characteristicsof TB: the symptom-free latent infection state and the persistence of the active disease of the tubercular infection in spite of the prolonged therapy time. Due to the importance of the bacilli latency state in the pathophysiology and chemotherapy of the disease, researchers have set their interest in the DosR system. Drugs that attack the latent state of the bacterium not only would be the key for eradication of the latent infection, but also shortening the time of treatment of active infection [84].

9. A new approach to research processes

Traditionally, the focus of research is the evaluation of a single drugin extensive and costly trials. This process may take a lot of time and reduces the possibility of developing a combination of new drugs that is effective. For this reason, a new approach to research has been led by the Critical Path to TB Drug Regimens (CPTR) organization created in March 2010 by the Bill & Melinda Gates Foundations, the Critical Path Institute, and the TB Alliance. This strategic partnership has the strength of reducing the time necessary to develop a new TB treatment scheme, as well as reducing significantly the research costs. This initiative has been endorsed by the US Food and Drug Administration and other regulatory entities, as well as the World Health Organization [67].

As a result of the 41st Union World Conference in Berlin, Germany, on November 2010, the TB Alliance announced the launch of the first clinical phase to test a new TB treatment scheme which expedites the treatment of patients. The combination of three drugs has been promising for the treatment of drug sensitive (DS-TB) and MDR-TB, thus changing the course of the TB

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pandemics through simplification and shortening in the treatment time of the disease worldwide. The combination is currently in phase II of clinical trials and contains PA-824 and moxifloxacin together with PZA. Researchers have reported that preclinical data reveal a decrease in the treatment time both for DS-TB and MDR-TB patients, and possibly XDR-TB ones with a simple three-drug treatment scheme [67].

10. Nano-particles: A projection towards the future

Nanoparticles can create new directions in the diagnosis, treatment, and prevention of TB. A significant application in the progress of this technology is using drug carriers.This system has been found to be advantageous, as it gives high stability of the drug, high load capacity (many molecules of the medication can be incorporated in the matrix of the particle), easiness to incorporate hydrophilic and hydrophobic substances, possibility of being administered orally or via inhalation. Perhaps more importantly, the anti-TB drug release in a controlled manner from the matrix enables to improve the bioavailability and reduction of the doses frequency. Load or delivery systems such as liposome or microspheres have been developed for the sustained release of anti-TB drugs, and better chemotherapeutical efficacy has been found when the system is researched in animal models (e.g. mice) [85,86].In 2005, the efficacy of nanoparticles was assessed in the distribution of anti-TBdrugs administered every 10 days versus the non capsulated form of aerosol administration of drugs against *M. tuberculosis* in guinea pigs; in both cases the treatments reduced the bacteria count. These findings suggest that the distribution of drugs by nanoparticles has a great potential in the treatment of TB [86].

11. Conclusions

Currently, devastating diseases in the world such as tuberculosis get the attention of authorities with the aim of supporting breakthroughs which provide alternatives for their control.

The development of active principles against *M. tuberculosis* is nowadays a worldwide priority due to the appearance of strains resistant to medications used in current therapeutic schemes, thus existing the need to articulate in an expedite manner the basic research looking for new therapeutic choices, along with clinical research and its articulation with the industry in order to guarantee a quick production of novel alternatives which overcome the limitations of current treatment schemes.

The concern in many sectors devoted to tuberculosis control is that there are not sufficient alternatives that can join rapidly the treatment against tuberculosis, and they convey discouraging estimations regarding the degree of resistance that each one of these molecules will have at the moment of entering the therapeutic scheme deduced from natural resistant bacilli. These justifications have promoted research around the world towards finding new molecules, based on investigations of natural sources such as plants, insects, marine microorganisms, synthetic molecules deduced from the modification or substitutions made on the structure of already

existing molecules with the aim of potentiate their effect; or from new sources such as nanoparticles, computing studies, among many others.

The results expected at the end of each process producing a new alternative of treatment against tuberculosis is that these drugs may shorten the duration of the current treatment, be active against resistant strains and non-replicative conditions of *M. tuberculosis* as well as not interfering with HIV antiretroviral therapy, reduce adverse side effects, and that it is of easy administration to facilitate the patient's compliance. For the management of tuberculosis as a public health event worldwide, these new drugs must be produced easily in large amounts; they must be stable under minimum storage conditions, and they must be of low cost so that all governments may guarantee access of all the diseased population.

For these expectations to become true in the short term, more basic and applied research is required to generate new ideas in the development of anti-tuberculosis drugs, as well as stronger financial support in research and greater commitment from the pharmaceutical industry and public health entities.

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Chapter 17

Web Resources on TB: Information, Research, and Data Analysis

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Additional information is available at the end of the chapter

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1. Introduction

Since its creation in the middle of the 20th century, the Internet has become the universal language of the digital world. All the capabilities it offers, such as electronic mail systems, information distribution, file sharing, multimedia streaming services and online social networking, have already been of service to billions of people around the world. In fact, if the Internet were to disappear tomorrow, most people would struggle to manage their lives without it.

By providing millions of people with information that is constantly updated (24 hours a day, seven days a week) Internet has become the second source of information for the whole world, television still being the first one in most countries. It has also provided a unique way of communication, where a person in an isolated geographical location can instantly be in touch with thousands and maybe millions of other individuals around the world.

Scientists were among the first ones to explore all these capabilities. Now, we talk about data mining, terabytes and petabytes, algorithms – terms related to what we call "Big Data", the large volume of information generated by a variety of new technologies, ranging from Astronomy (telescope data), the Internet itself (more and more Facebook users every day) to Biology (cheaper and more efficient DNA sequencing technologies), among other areas of study and research. Some technologies and experiments, like the Large Hadron Collider at CERN, Switzerland (perhaps the most important scientific tool ever built), produce an incredible volume of information, on the order of terabytes per second.

Databases containing DNA and protein sequences were created; institutions around the world developed websites to expose their work to the world; scientific magazines started their online versions. The world is connected as never before. This connection transcend the virtual realm

of the Internet: today, it is possible to travel from one side of the world to the other in just one day. Unfortunately, this has presented us a negative side: infectious agents may also cross the world in just about the same time.

Tuberculosis is a global disease, with an estimated one-third of all people in the world contaminated by the bacillus, *Mycobacterium tuberculosis*. Although treatable, the large period of treatment (many abandon the therapy as soon as they feel better) together with the indiscriminate use of antibiotics is causing the spread of new, drug-resistant strains. Actually, as those familiar with epidemiology have already noticed, that is a remarkable similarity between the patterns of an epidemic or outbreak with the spread of a new piece of information throughout the internet.

However, there has also been a revolution in other areas: new high-throughput technologies, like genomics, transcriptomics and proteomics, offer a new, more integrated view of the metabolism and genetics of the organism studied, and of course *M. tuberculosis* was among the first to have its genome sequenced. Today, more than 30 different strains have been sequenced, as well as other organisms from the *Mycobacterium* genus. By comparing the genomes of virulent and non-virulent strains of TB, scientists may pinpoint particular genes and/or polymorphisms involved in this process; by examining transcriptome data, researchers may have an idea of the effects of a given drug in the bacillus' metabolism.

The purpose of this chapter is by no means to offer an exhaustive list of all the resources available on the Internet about TB, the topic of this book. This would be a massive and perhaps futile work, since the evolution of the internet occurs at a very fast pace. Rather, this chapter concentrates on a selection of the most important, relevant and stable websites with relevance to several aspects of TB, such as research, treatment, main Institutions, funding, and specialized platforms. We think this should complement all the other information already presented in this book, offering the reader a more integrated view of the disease, and also access to new platforms and systems specialized in the analysis of data generated by a series of new technologies such as DNA sequencing.

2. Tuberculosis facts information and treatment research

Most of the selected sites presented in this section have information about several aspects of TB, like history, epidemiology, transmission and pathogenesis, diagnosis, treatment, infection control, besides offering other services such as courses, guidelines, fact sheets and links to related sites. We have chosen an alphabetical classification to avoid conveying a false impression of importance to some sites in detriment of others. In fact, we think that every effort is worthy in this global battle against this terrible disease.

Centers for Disease Control and Prevention. The mission of the Division of Tuberculosis Elimination (DTBE) is to promote health and quality of life by preventing, controlling, and eventually eliminating tuberculosis from the United States, and by collaborating with other countries and international partners in controlling global tuberculosis. URL: <http://www.cdc.gov/tb/>

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Global Tuberculosis Institute. Located at the New Jersey Medical School, the institute provides expertise in program development, education, training and research to ministers of health, national TB programs and healthcare providers around the globe. URL: <http://www.umdnj.edu/globaltb/home.htm>

	Agency	URL
Americas	American Lung Association (ALA) Lung Disease Programs	http://www.lung.org/
	American Public Health Association	http://www.apha.org/
	Bill & Melinda Gates Foundation	http://www.gatesfoundation.org/
	CREATE: Consortium to Respond to the AIDS/TB Epidemic	http://www.tbhiv-create.org/
	Food and Drug Administration (FDA)	http://www.fda.gov/default.htm
	Institute for Tuberculosis Research	http://www.tuberculosisdrugresearch.org/
	National Institute of Allergy and Infectious Diseases (NIAID)	http://www.niaid.nih.gov/
	National Library of Medicine, PubMed	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi
	Tuberculosis Net	http://tuberculosis.net/
Africa	AllAfrica.com: TB News from Africa	http://allafrica.com/tuberculosis/
	Desmond Tutu TB Centre	http://sun025.sun.ac.za/portal/page/portal/Health_Sciences/English/Centres/dttc
	South African Tuberculosis Vaccine Initiative	http://www.satvi.uct.ac.za/
Asia and Oceania	JATA - Research Institute of Tuberculosis	http://www.jata.or.jp/eindex/home.html
	National Institute for Research in Tuberculosis	http://www.trc-chennai.org/
	Pakistan Anti TB Association	http://www.patba.org/
	TBC India	http://www.tbcindia.nic.in/
Europe	European Tuberculosis Surveillance Network	http://www.ecdc.europa.eu/en/activities/surveillance/european_tuberculosis_surveillance_network/Pages/index.aspx
	International Union Against Tuberculosis and Lung Disease (UNION)	http://www.theunion.org/
	Max Planck Institute for Infection Biology	http://www.mpiib-berlin.mpg.de/
	Pasteur Institute	http://www.pasteur.fr

Table 1. Additional websites covering tuberculosis facts information and treatment research

Pan American Health Organization (PAHO). Serving as the regional office for WHO, PAHO has been working for more than one century to improve health and the living standards of the countries of the Americas, being recognized as part of the United Nations' system. URL: <http://new.paho.org/hq/>

StopTB Partnership. The StopTb Partnership operates through a secretariat hosted by the World Health Organization (WHO) in Geneva, Switzerland, and seven working groups whose role is to accelerate progress on access to TB diagnosis and treatment, research and development for new TB diagnostics, drugs and vaccines, and tackling drug resistant- and HIV-associated TB. URL: <http://www.stoptb.org/>

Tb Alliance. Established in the year 2000, its main objective is to discover and develop better, faster-acting, and affordable drugs to fight tuberculosis. Today, the organization and its partners manage a portfolio of new anti-Tb drugs. URL: <http://www.tballiance.org>

World Health Organization (WHO). Created in 1948, WHO is the directing and coordinating authority in international health within the United Nations' system, composed of 193 countries and two associate members. It supports and promotes health research in several areas, Tb being one of them. URL: <http://who.int/topics/tuberculosis/en/>

3. Tuberculosis databases and platforms

Since the emergence of Bioinformatics and Computational Biology back in the 1960's, numerous databases and computational tools have been created in order to provide the scientific community the necessary means to access and interpret a range of biological data.

Actually, the contribution of these disciplines became particularly evident in the 1990's and 2000's, when the development of supercomputers, powerful personal computers, and computer networks at global scale, as well as of high-throughput technologies, collectively referred as *omics* – *e.g.*, genomics, transcriptomics, and proteomics –, revolutionized the field of Biology.

Nowadays, a number of web resources are publicly available aiming to organize, integrate, and provide efficient access to the ever-increasing amount of biological information produced over decades of research, particularly in recent years, with numerous projects applying the aforementioned high-throughput technologies worldwide. Accordingly, diverse options to visualize, search, retrieve and analyze this wealth of data are offered, providing the opportunity to acquire more detailed knowledge about genomes and their respective organisms, among many others opportunities.

However, the creation and maintenance of such web resources is a challenge by itself, not only because they usually have to deal with large amounts of data, but mostly because they require the designing of schemes and frameworks that accurately represent the complexity of biological systems, which is frequently a hard task to be accomplished. Another difficulty is the development of efficient data retrieval systems, implemented in user-friendly interfaces and intended for complex and massive database searching. It is worth noting that, in many

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circumstances, the authors and curators of such resources receive little or no remuneration for their productive efforts, and the access to financial support for creation and maintenance of biological databases is still a difficult task.

In this section we present the main web resources fully or partially dedicated to mycobacterial species with relevance for readers interested in TB. Each database or platform, categorized according to its purpose and functionality, is quickly reviewed, and references to the original paper describing it, as well as its electronic site, are provided, serving as a guideline for researches or students working on TB. Notably, the computational resources presented here are all publicly available as online services and can potentially be applied to the identification of new drug targets, vaccine antigens, or diagnostics for TB, among many others applications.

3.1. Generic and multifunctional

MyBASE. The Mycobacterial Database [1] is an integrated platform for functional and evolutionary genomic study of the genus *Mycobacterium*, comprising extensive literature review and data annotation on mycobacterial genome polymorphism, virulence factors, and essential genes. URL: <http://mybase.psych.ac.cn/>

TBDB. The TB Database [2] provides a comprehensive genomic data repository for *M. tuberculosis* and related bacteria, combining (*in silico*) genome sequence and annotation data and (experimental) gene-expression data. It also provides an analysis platform with suitable computational tools to assist (comparative) genomic and gene-expression studies of these microorganisms. Annotated features of genes and genomes, predicted orthologous groups, operons and synteny blocks, as well as predicted and curated immunological epitopes and gene-expression patterns are available. URL: <http://www.tbdb.org/>

The MycoBrowser portal. The Mycobacterial Browser portal [3] is an extensive genomic and proteomic data repository for four related mycobacteria: *M. tuberculosis* H37Rv, *M. leprae* TN, *M. marinum* M, and *M. smegmatis* MC2. The system provides *in silico* generated and manually reviewed information on the complete genome sequence of these organisms. As part of this portal, the **TubercuList** database [4] integrates a range of information on the *M. tuberculosis* genome, such as genomic and protein annotations and features, drug and transcriptome data, mutant and operon annotation, and comparative genomics. It represents a complete redesign of the database with the same name provided by the GenoList genome browser (also described in this chapter). URL: <http://mycobrowser.epfl.ch/>

3.2. Genomic mapping and data mining

TubercuList, **BoviList**, **BCGList**. The GenoList [5] is a collection of databases dedicated to microbial genome analysis, providing a complete data set of protein and nucleotide sequences for selected species, as well as annotation and functional classification of these sequences. The TubercuList, BoviList, and BCGList databases are devoted to collect and integrate various aspects of the genomic information of *M. tuberculosis* H37Rv, *M. bovis* AF2122/97, and *M. bovis* BCG Pasteur 1173P2, respectively. URL: <http://genolist.pasteur.fr/>

TBrowse. The TBrowse [6] is a genomic data resource, based on the Generic Model Organism Database (a collection of open source computational tools for creating and managing genome-scale biological databases); the browse provides the scientific community an integrative genomic map of *M. tuberculosis* with millions of data-points representing different genomic features and computational predictions systematically collected from online resources and publications, including gene/operon predictions, orthologs, gene expression data, non-coding RNA, pathway/networks, regulatory elements, variation and repeats, subcellular localization, among others. URL: <http://tbrowse.osdd.net>

3.3. Comparative genomics

GenoMycDB. The GenoMycDB [7] is a relational database for large-scale comparative analysis of completely sequenced mycobacterial genomes based on their predicted protein content. Currently, the database comprises six mycobacteria – *M. tuberculosis* (strains H37Rv and CDC1551), *M. bovis* AF2122/97, *M. avium subsp. paratuberculosis* K10, *M. leprae* TN, and *M. smegmatis* MC2 155 – providing for each of their encoded protein sequences the predicted subcellular localization, the assigned cluster of orthologous groups (COGs), features of the corresponding gene, and links to several important databases; in addition, pairs or groups of homologs between selected species/strains can be dynamically inferred based on user-defined criteria. URL: <http://www.dbbm.fiocruz.br/GenoMycDB.html>

MycoDB. The xBASE [8] is another collection of databases, this one dedicated to bacterial comparative genome analyses. It provides precomputed data of comparative genome analyses among selected bacterial genera, as well as inferred orthologous groups and functional annotations. It also provides precomputed analyses of codon usage, base composition, codon adaptation index (CAI), hydropathy, and aromaticity of the protein coding sequences in these bacteria. As part of this multi-microbial system, the MycoDB currently comprises comparative data from 61 completely sequenced or unfinished mycobacterial genomes, including 40 strains of *M. tuberculosis*, *M. bovis* AF2122/97, *M. bovis* BCG strains Pasteur 1173P2 and Tokyo 172, among others mycobacteria. URL: <http://www.xbase.ac.uk/>

Mycobacterium tuberculosis Comparative Database. This Broad Institute's database comprises precomputed comparative genome analyses data of eight *M. tuberculosis* patient isolates with relevant clinical phenotypes and disease epidemiology (varied degree of spread, drug resistance, and clinical severity): *M. tuberculosis* F11, *M. tuberculosis* Haarlem, *M. tuberculosis* KZN 4207 (DS), *M. tuberculosis* KZN 1435 (MDR), *M. tuberculosis* KZN 605 (XDR), *M. tuberculosis* C, *M. tuberculosis* 98-R604 INH-RIF-EM, and *M. tuberculosis* W-148. Among the comparative data provided by this TB resource we can cite: inferred families of orthologous genes, genomic two-dimensional dot plot matrices, comparative genome mapping and browsing, and several comparative gene annotations and features. URL: <http://www.broadinstitute.org/annotation/genome/mycobacterium_tuberculosis_spp/MultiHome.html>

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3.4. Genetic diversity and epidemiology

MGDD. The Mycobacterial Genome Divergence Database [9] comprises a data repository of genetic variations among different organisms belonging to the *M. tuberculosis* complex. The MGDD system provides quick searches for precomputed single nucleotide polymorphisms (SNPs), insertions/deletions, repeat expansions, and divergent sequences (inversions, duplications, and changes in synteny) in genomic regions of fully sequenced *M. tuberculosis* complex species and strains genomes. URL: <http://mirna.jnu.ac.in/mgdd/>

MIRU-VNTRplus. The Mycobacterial Interspersed Repetitive Unit – Variable Number Tandem Repeat (MIRU-VNTR) database [10,11] comprises a collection of 186 well characterized strains representing the major *M. tuberculosis* complex in which, for each strain, species, lineage, and epidemiologic information are provided together with 24 MIRU loci, Spoligotype patterns, Regions of Difference (RD) profiles, Single Nucleotide Polymorphisms (SNPs), susceptibility data, and IS6110 Restriction Fragment Length Polymorphism (RFLP) fingerprint images. The system enables users to analyze genotyping data of their own strains alone or in comparison with the reference strains in the database; analyses and comparisons of genotypes can be based on Multiple Locus VNTR Analysis (MLVA), Spoligotypes, Large Sequence Polymorphism (LSP) and SNPs data, or on a weighted combination of these markers. Tools for data analysis include: search for similar strains, creation of phylogenetic and minimum spanning trees and mapping of geographic information. URL: <http://www.miru-vntrplus.org>

MTCID. The *M. tuberculosis* Clinical Isolate Genetic Polymorphism Database [12] consists in a repository of genetic polymorphisms, providing Single Nucleotide Polymorphism (SNPs) and Spoligotype profiles of clinical isolates of *M. tuberculosis*, based on published literature and manual curation. URL: <http://ccbb.jnu.ac.in/Tb/>

SITVITWEB. The SITVITWEB [13] is a multi-marker database, comprising three major types of molecular markers: Spoligotypes, Mycobacterial Interspersed Repetitive Units (MIRUs) and Variable Number Tandem Repeat (VNTRs); this webserver is dedicated to the investigation of *M. tuberculosis* genetic diversity and molecular epidemiology. Currently, this international resource provides genotyping information on 62,582 *M. tuberculosis* complex clinical isolates from 153 countries of patient origin. URL: <http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/>

Additionally, a few relevant computational tools are currently available as web services dedicated to analyze the genetic diversity of *M. tuberculosis* complex strains and characterize TB dynamics using molecular epidemiological data:

The **spolTools** [14] comprise a collection of browser programs designed to manipulate and analyze Spoligotype data of the *M. tuberculosis* complex, consisting in an online repository of Spoligotype isolates collected from various published data sets (currently, 1179 Spoligotypes and 6278 isolates across 30 datasets), and online tools for manipulating and analyzing these data (computation of basic population genetic quantities; visualization of clusters of Spoligotype patterns based on an estimated evolutionary history; and a procedure to predict emerging

strains/genotypes associated with elevated transmission). URL: <http://www.emi.unsw.edu.au/spolTools/>

The **TB-Insight** [15] is a collection of computational methods (based on different models and datasets) for both lineage classification of *M. tuberculosis* complex strains, and for visualization of genetic diversity in *M. tuberculosis* complex population and distribution by lineage, as well as visual representation of associations between patient and strain groups, providing perception on differences in phenotypic characteristics, and phylogeographic associations of *M. tuberculosis* complex strains with host populations. URL: <http://tbinsight.cs.rpi.edu/>

3.5. Gene expression and regulation

MTBRegList. The MTBRegList [16] is dedicated to the analysis of gene expression and regulation data in *M. tuberculosis*, containing predicted and characterized regulatory motifs cross-referenced with their respective transcription factor(s), experimentally identified transcription start sites, and DNA binding sites. URL: <http://www.usherbrooke.ca/vers/MtbRegList>

MycoperonDB. The MycoperonDB [17] is a repository of known and computationally predicted operons and transcriptional units of (currently) five different mycobacteria – *M. tuberculosis* (strains H37Rv and CDC1551), *M. bovis* AF2122/97, *M. avium subsp. paratuberculosis* K10, and *M. leprae* TN – whose genomes have been completely sequenced. Presently, it comprises 18,053 genes organized as 8,256 predicted operons and transcriptional units, providing literature links for experimentally characterized operons, and access to known promoters and related information. URL: <http://cdfd.org.in/mycoperondb/home.html>

MTBreg. The MTBreg is part of the online services provided by the UCLA-DOE Institute for Genomics and Proteomics (http://www.doe-mbi.ucla.edu/), and consists in a repository of conditionally regulated proteins in *M. tuberculosis* grown under several different conditions mimicking infection; the database provides information on proteins that are regulated by selected transcription factors or other regulatory proteins, as well as on the experimental condition, the experimental dataset and a literature reference. URL: <http://www.doe-mbi.ucla.edu/Services/MTBreg/>

MycoRegDB. The Mycobacterial Promoter and Regulatory Elements Database [18] is part of a user-friendly web interface (**RegAnalyst**) that integrates a motif prediction program (MoPP), a pattern detection tool (MyPatternFinder), and a database of promoter and regulatory elements from various mycobacterial species (MycoRegDB). Currently, the MycoRegDB comprises the following species: *M. tuberculosis* (strains H37Rv and CDC1551), *M. bovis* BCG, *M. leprae*, *M. smegmatis*, *M. avium* subsp. *paratuberculosis*, *M. marinum*, *M. ulcerans*, *M. gilvum*, and *M. vanbaalenii*. For each database entry, a variety of useful information is provided, such as, gene annotation, CDS positions, promoter/regulatory sequence (with Transcription Start Point (TSP) or binding site explicitly marked), TSP-CDS/Motif-CDS distance, among others. The first release of MycoRegDB contained 290 annotated DNA motifs (174 promoters and 116 transcription factor binding sites) described in 81 research papers, according to the authors.. URL: <http://www.nii.ac.in/~deepak/RegAnalyst/MycoRegDB>

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3.6. Structural biology

MtbSD. The *M. tuberculosis* Structural Database [19] is a resource dedicated to 3D protein structures of *M. tuberculosis*, providing relevant information on description, reaction catalyzed, domains, active sites, structural homologs and similarities between bound and cognate ligands. Currently, the database comprises 876 structures for 332 mycobacterial genes. URL: <http://bmi.icmr.org.in/mtbsd/MtbSD.php>

3.7. Drug targets and resistance

TDR Targets database. The Tropical Disease Research (TDR/WHO) Targets database [20] comprises extensive genetic, biochemical and pharmacological data related to tropical disease pathogens, including *M. tuberculosis*, as well as computationally predicted druggability for potential targets and compound desirability information; the goal is to exploit the availability of diverse datasets to facilitate the identification and prioritization of drugs and drug targets in neglected disease pathogens, such as the tubercle bacillus. URL: <http://tdrtargets.org/>

TB Drug Resistance Mutation Database. The Tuberculosis Drug Resistance Mutation Database [21] is a comprehensive database that catalogs mutations associated with TB drug resistance and the frequency of the most common mutations associated with resistance to specific drugs, providing a resource for the development of molecular diagnostics for TB, as well as structural mapping of mutations to investigate mechanisms of resistance for drug discovery purposes. URL: <http://www.tbdreamdb.com/>

4. Conclusion

As outlined in this chapter, Informatics has acquired a great importance not only in the biological sciences, but in all areas of knowledge. Internet has become one of the most important tools for most people, from a dedicated researcher interested in the latest advances in his/her particular field of work to the teenager trying to contact his friends. Companies, industries and research institutes developed sites, where they expose their work to laymen.

The large number of publicly available databases and computational tools that have been developed, dedicated to organize, integrate, and provide efficient access to the ever-increasing amount of biological information produced over decades of research, have benefited researchers all over the world, especially those from low-income countries.

One important drawback, that still has to be overcome, is that the wealth of biological information available is presently fragmented, dispersed across numerous computational resources, and is redundant in many circumstances, clearly requiring unification in order to provide a global and integral picture of the biological systems they are dedicated to.

Ideally, the upcoming databases and computational tools should offer: data integration, providing multi-perspective analyses; combine *in silico* generated and manually curated data, improving the quality of our research; present efficient data structure, storage and processing,

providing dynamic, flexible and fast data visualization, data searching, data retrieval and data analysis, via user-friendly graphical interfaces; implement a consistent and controlled vocabulary to describe the data and standardized data formats, providing full data interchanging and integration with other data sources. We believe that only in this way, a fruitful field for interactions and cooperation among researches from distinct areas might emerge, providing the required support to interpret and analyze this wealth of data according to a truly multidisciplinary approach.

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Chapter 18

Peadiatric Tuberculosis: Is the World Doing Enough?

Claude Kirimuhuzya

Additional information is available at the end of the chapter

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1. Introduction

Until recently, tuberculosis (TB) had become a neglected disease, particularly in children. It was after the emergence of multi drug resistant TB, and the complications brought about by the HIV/AIDS coinfection, that TB took centre stage again. The common perception has been that children rarely develop severe forms of TB and that they do not contribute much to the spread of the epidemic. Although this could be the case in non-endemic areas, where diligent contact tracing is enforced, in children from endemic areas it is a different story as revealed by an autopsy study conducted in Zambia which demonstrated that tuberculosis was a major cause of death from respiratory disease (Chintu et al., 2002).

Children are particularly vulnerable to more rapid development of severe disease and death after infection, and those with latent infection become the reservoir of disease reactivation in adulthood, fueling the future epidemic (Nicol et al., 2011). Since focus has been on reducing transmission, previous TB control strategies have not prioritized childhood TB (Zar and Pai, 2011). Due to the difficulty in establishing an accurate diagnosis of childhood tuberculosis, the true extent of the tuberculosis-related morbidity and mortality suffered by children in endemic areas is rarely appreciated (Chintu et al., 2002). For example, Regional Data from the WHO in 2007 showed that smear-positive TB in children aged under 14 years accounted for 0.6–3.6% of reported cases but since about 95% of cases in children under 12 years of age are smear negative, these data underestimate the true burden of TB. Furthermore, in countries with a high prevalence of HIV infection, there has been a marked increase in the incidence and a decrease in the peak age prevalence of infectious TB; thus, most cases now occur in young adults, who are often parents of young children (WHO Report, 2009). This finding suggests that children in developing countries will emerge as a group at high risk. In industrialized countries, most childhood TB cases are detected through contact tracing and have good outcomes. This is in contrast to the situation in low- and middle-income countries, where

childhood TB is closely associated with poverty, overcrowding, and malnutrition, with consequently higher death and lower treatment success rates (Nelson and Wells, 2010).

Studies have revealed that children contribute a significant proportion to the disease burden and suffer severe tuberculosis-related morbidity and mortality, particularly in endemic areas. TB is now among the 10 major causes of mortality among children, with a global estimate of 130,000 deaths per year (WHO Report, 2009). Mortality has a strong correlation with socioeconomic status, underlying nutritional status and immunocompetence (Palme, 2002). TB has also been reported to be the third most common cause of death in HIV-infected children with a clinical diagnosis of acute severe pneumonia (Palme, 2002). With roughly a million cases estimated globally each year (Walls and, Shingadia, 2004) and a much higher risk of severe disease and death among young children than adults, paediatric TB remains a public health emergency and this is particularly evident in developing countries with poor public health infrastructure.

As in adults, the majority of cases occur in the 22 high burden countries, where a combination of high transmission rates and a large proportion of the population under the age of 15 years mean children account for up to 25-40% of cases, with incidence rates for paediatric TB ranging from 60-600 per 100,000 per year (Nelson and Wells, 2004). Increasing rates of childhood TB have also been reported in Eastern Europe in the wake of the explosive TB epidemic which followed the break up of the Soviet Union (Walls and Shingadia, 2007). Even traditionally low-burden countries have seen a rise in cases, mainly due to immigration from TB endemic areas. In most countries of Western Europe and North America, where children account for 4-7% cases, paediatric incidence rates vary from about 1 to 15 per 100,000 per year, although much higher rates are observed in some cities, such as London (Newton et al., 2008).

Despite this huge disease burden, children's access to anti-tuberculosis treatment in most endemic areas remains poor, as tuberculosis control programs focus predominantly on the treatment of sputum smear–positive adults (Starke, 2002). Recent technological advancements in diagnosis of TB in adults have not been validated in children and, similarly, trials of new drugs and development of pediatric formulations of standard first- and second-line drugs are lagging behind. As a result both research and surveillance data in the field of childhood TB have been greatly limited. Further research into the epidemiology, immune mechanisms, diagnosis, treatment and prevention of childhood TB is urgently needed to enhance our understanding of TB in children which may provide wider insights and opportunities to facilitate efforts to control TB in the population.

Another problem is that most programs for TB control are limited because they target and treat only active cases (Graham, et al., 2004) when most TB cases in children present as latent tuberculosis infection (LTBI) with active disease occurring mainly in developing countries (Dogra, et al., 2007). Without treatment, the majority of infants aged under 1 year die due to TB. Even with effective antimicrobial therapy, severe TB continues to occur in young children (Ávalos and Montes de Oca, 2012). Priorities for future research should, therefore, enhance collaborations between developing and developed nations.

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This chapter addresses some of the unique features of TB in children; presents existing and novel diagnostic, therapeutic and preventative measures; and outlines important areas of future research. The main challenges for future research are highlighted and in conclusion it is emphasized that well-targeted interventions, improved resources, and improved political commitment, may lead to a dramatic reduction in tuberculosis-related morbidity and mortality among children.

2. Epidemiology of paediatric TB

2.1. Global disease burden of paediatric TB

Poor case ascertainment, lack of resources for active case finding in most settings, and limited paediatric surveillance data from TB control programs all hamper efforts to define accurately the global burden of childhood TB (Nelson and Wells, 2004). Until recently, under the WHO Directly Observed Treatment Short Course (DOTS) strategy, only smear positive cases were being reported for children, yet smears are seldom performed in many high burden settings and most disease in children is smear negative.

Although limited surveillance data prevent reliable estimates of the contribution of TB to childhood mortality, available data indicate that pneumonia is the commonest cause of childhood death globally (Nelson et al., 2004) an implication that TB, being an important cause of pneumonia in many settings (Scott et al., 2008), may contribute significantly to these global childhood deaths. A necropsy study in Zambia found evidence of TB in 18% of HIV-positive and 26% of HIV-negative children dying of pneumonia (Chintu et al., 2002) although more robust regional data on the epidemiology of childhood TB are urgently needed to define the true burden of disease, and to characterize current transmission rates and circulating strains.

2.2. Pathophysiology of TB in children

2.2.1. Natural history of TB in children

The natural history of TB in children and pediatric patients follows a series of steps in which phase 1 occurs after an incubation period of 3–8 weeks after primary infection. This is followed by appearance of well-defined signs that include fever, erythema nodosum, a positive tuberculin skin test response, and formation of the primary complex visible on chest radiography. Phase 2 occurs 1–3 months after the phase 1 in which period, the bacillus can migrate to other parts of the body via the blood and this represents the period of the highest risk for the development of tuberculous meningitis and miliary tuberculosis in young children. This is the phase where dissemination of the bacillus most frequently occurs. Phase 3 occurs 3–7 months after primary infection and is the period of pleural effusions in children greater than 5 years old and bronchial disease in children less than 5 years. Phase 4 presents after 1–3 years after phase 1 and is during which the osteoarticular tuberculosis in children 5 years and below, appears. Phase 5 occurs up to 3 years after phase 1 and it is presented after calcification has

been completed. It is after this stage that manifestations of classical adult tuberculosis appear (Marais et al., 2004).

Extrapulmonary tuberculosis or miliary TB is a complication of primary TB in young children. It includes peripheral lymphadenopathy, TB meningitis, skeletal TB, and other organ involvement. Other unusual sites for TB include the middle ear, gastrointestinal (GI) tract, skin, kidneys, and ocular structures (Marais et al., 2006). Lymph node involvement typically occurs 6-9 months following initial infection by the tubercle bacilli. More superficial lymph nodes commonly are involved. Frequent sites of involvement include the anterior cervical, submandibular, and supraclavicular nodes. TB of the skeletal system may lead to involvement of the inguinal, epitrochlear, or axillary lymph nodes. Typically, infected lymph nodes are firm and non- tender with non erythematous overlying skin. The nodes are initially non-fluctuant. Suppuration and spontaneous drainage of the lymph nodes may occur with caseation and the development of necrosis (Marais et al., 2006).

Bone or joint TB or skeletal TB may present acutely or sub-acutely. Vertebral disease may go unrecognized for months to years because of its indolent nature. Common sites involved include the large weight-bearing bones or joints, including the vertebrae (50%), hip (15%), and knee (15%). Destruction of the bones with deformity is a late sign of TB. Manifestations for skeletal TB may include angulation of the spine (gibbus deformity) and/or Pott disease (severe kyphosis with destruction of the vertebral bodies). Cervical spine involvement may result in allantoaxial subluxation, which may lead to paraplegia or quadriplegia.

2.2.2. TB risk factors

Following infection children have a higher risk not only of progression to disease, but also of extrapulmonary dissemination and death. Infants have a particularly high morbidity and mortality from TB (WHO, 2007). While many factors including host genetics, microbial virulence and underlying conditions that impair immune competence (as is the case with malnutrition and HIV infection) determine the outcome of infection, it is likely that the high rate of progressive TB seen in young children is largely a reflection of the immaturity of the immune response. Risk factors for the acquisition of tuberculosis (TB) are usually exogenous to the patient. Thus, the likelihood of being infected depends on the environment and the features of the index case. However, the development of TB disease depends on inherent immunologic status of the host. For example, tuberculosis has been reported in patients treated for arthritis, inflammatory bowel disease, and other conditions with tumor necrosis factor (TNF)-alpha blockers/antagonists.

2.2.3. Factors for acquiring paediatric TB disease

Neonatal CD4 cells appear intrinsically deficient in their capacity to express Th1 effector function, partially attributed to hypermethylation of the proximal promoter of the IFN-γ gene, (White et al., 2002) and this results in a highly restricted pattern of IFN-γ response to a variety of stimuli (Kampmann et al., 2006). CD154 (CD40 ligand) expression is also significantly reduced compared with adult cells. These findings of generally impaired cell-mediated

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immune responses in the neonate and young children raise the question of whether antigen-specific immune responses to mycobacteria are equally affected. Delayed type hypersensitivity (DTH) to purified protein derivative (PPD) may be absent in up to 40% of HIV negative children presenting with extrapulmonary TB, (van der Weert et al., 2006) compounding the difficulties of diagnosis in young children. However, studies measuring responses to neonatal vaccination with M. bovis BCG demonstrate potent Th1 responses, possibly related to the activating properties of BCG vaccine on the potent antigen-presenting cells (APC). Indeed while the long term efficacy of BCG vaccination may be limited, it does offer protection against disseminated disease in infants and young children. The risk of serious and potentially devastating disease is nevertheless still high in the first two years of life, underscoring the need for a better understanding of the determinants of host protection particularly in this vulnerable age group.

In the natural history of childhood intrathoracic TB, primary infection before 2 years of age frequently progresses to disease within the first 12 months (Marais, et al., 2004). Young age and HIV infection are the most important risk factors for severe or disseminated disease; the risk of disease progression decreases during childhood, least at 5–10 years of age, and increases again during adolescence. Pulmonary parenchymal disease and intrathoracic adenopathy are the most common clinical manifestations of pediatric TB, accounting for 60%–80% of all cases (Jensen, 2002). Among extra-pulmonary manifestations, lymphadenopathy is the most common (67%), followed by central nervous system involvement (13%) and pleural (6%), disseminated (5%), and skeletal (4%) TB (Marais, 2006). Disseminated disease and TB meningitis are usually found in very young children (age, under 3 years) and/or HIV-infected children (Starke, 2003). More research is required to identify better strategies for case detection and contact tracing, especially in high-burden settings, and to study the role of genetic and nutritional factors that protect children from TB infection and disease.

HIV-infected children are at risk of both atypical pulmonary presentation and extra-pulmonary disease, which comprises up to 60% of TB in this population (Starke, 2003). Symptom-based diagnostic approaches perform poorly, because other HIV-related conditions, such as lymphocytic interstitial pneumonitis, broncho-ectasis, and respiratory infections (including viral pneumonitis), mimic the clinical and radiographic features of TB (Marais, 2007). Lymphocytic interstitial pneumonitis tends to occur in children aged less than two years, presents with recurrent respiratory symptoms, and is associated with clubbing and generalized lymphadenopathy and a miliary TB-like picture on chest radiograph. Although these patients improve temporarily with antibiotic therapy, antiretroviral treatment is required for sustained benefit and to avoid development of chronic lung disease. In the short term, there is little prospect of achieving a widely available gold standard diagnosis of TB in children either by means of culture, microscopy, PCR, or serological testing. Consequently, clinicians must rely on clinical criteria, chest radiography, and tuberculin testing, and attempts must be made to improve the predictive power of available tools (Swaminathan and Rekha, 2010).

2.2.4. Host genetic susceptibility to paediatric TB

The immunological responses to MTB are due to the interaction between the immature immune system in children, the host, bacterial and environmental factors (Meya and McAdam, 2007). Genetic as well as acquired defects in host immune response pathways greatly increase the risk of progressive disease (Kampmann et al., 2005). Results from genome wide linkage studies suggest that TB disease susceptibility is highly likely to be polygenic, with contributions from many minor loci (Hill, 2006) and a large number of TB susceptibility markers have been identified from candidate gene studies as 'disease-causing' genes which include TIRAP, HLA DQB1, VDR, IL-12β, IL12Rβ1, IFN-γ, SLC11A1 and MCP-1. However, to date the greatest evidence to support an underlying genetic basis for TB has come from the discovery of single gene defects predisposing to disseminated and often lethal mycobacterial disease. Most observations were initially made in children with reduced ability to activate macrophage antimycobacterial mechanisms through defects in the IFN-γ (Kampmann et al., 2005) /IL-12 pathway resulting in severe mycobacterial infection. However, subsequent studies have led to description of mutations in five susceptibility genes (Ottenhoff et al., 2005) confirming that up-regulation of the macrophage through the IL-12/23-IFN-γ pathway is a fundamental step in the containment of infection with mycobacteria. However, despite a growing adult literature on the role of candidate genes from this pathway, data from children is scarce. This is surprising given the marked differences in TB pathophysiology in children, which may also reflect differences in genetic factors. Further studies of TB genetics in well-defined paediatric populations are therefore needed.

2.3. Impact of HIV epidemic paeditric TB

Studies demonstrating a higher risk of TB among HIV- children (Jeena et al., 2002) highlight the essential role of cell mediated immunity (CMI) in preventing mycobacterial dissemination (Tena et al., 2003). Poor CMI in HIV co-infection often results in disseminated disease, especially in advanced stages of HIV-infection, resulting in poorer survival compared to HIV-negative children (Palme et al., 2002). Risk of active TB in HIV co-infected children is related to both CD4 count and more indirectly also to viral load (Elenga et al., 2005).Conversely, restoration of cellular immunity with anti-retroviral therapy partially reverses TB susceptibility (Kampmann et al., 2006).

The impact of the Human Immunodeficiency Virus (HIV) epidemic on the burden of childhood TB has been less well characterized than for adults (Corbett et al., 2003). However, the observed shift in disease burden to younger adults it has caused, suggests that children are at particularly high risk of exposure as well as disease (Graham et al, 2001). Reported prevalence of HIV co-infection among children with TB range from below 5%, in industrialized settings, to over 50% in some high burden African settings (Nelson and Wells, 2004). However, it is often difficult to draw reliable inferences about the effect of HIV on TB incidence or risk from these observational data due to ascertainment bias or diagnostic bias; incomplete ascertainment of HIV status and because denominator population data on the proportion of all children infected with HIV are usually lacking. (For example, children with HIV are more likely to be investigated for TB and diagnosis is unreliable because it is affected by HIV status). Nevertheless an

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increased TB incidence and poorer outcome have been observed among HIV infected children in a variety of settings (Palme et al., 2002) including an estimated 20-fold increased TB incidence associated with HIV infection in a study from South Africa. Methodological constraints in some studies may explain why this has not been a universal finding (Marais et al., 2007).

2.4. Nutrition and paediatric TB

Several observational studies from adults and children show an association between malnutrition and TB, (Cegielski and McMurray, 2004) although proving the direction of a causal link is challenging, as TB in itself causes wasting. Diagnosis is further complicated by frequently false negative TST in malnutrition, reverting to positivity only once nutrition has improved. Nevertheless these observational data, coupled with experimental animal data and impaired CMI observed in malnutrition, support its role as a risk factor for childhood TB (Cegielski and McMurray, 2004). However the effect of differing types and degrees of malnutrition, and the population at risk due to malnutrition in communities where both are endemic, are yet to be defined.

Among micronutrients, vitamin D deficiency has been most extensively studied, and shown to be associated with TB in UK immigrants. Its active metabolite 1-alpha, 25-dihydroxy-vitamin D modulates the host response to TB infection in numerous ways, including the induction of antimicrobial peptides such as Cathelicidin LL-37 (Martineau et al., 2007).

2.5. Host-pathogen interactions in paediatric TB

The relationship between MTB strain genotype and clinical manifestation of disease is poorly documented in children. A study in the Western Cape Province of South Africa demonstrated that the Beijing and Haarlem genotype families are significantly associated with drug resistant TB in children (Marais et al., 2006). The high prevalence of Beijing and Latin American Mediterranean (LAM) strains in children reflects considerable transmission of these genotype families in this setting (Marais et al., 2006).

Genetic markers of virulence and transmissibility, (Lopez et al., 2003) and the ability to modulate host cellular immunity have been described for the Beijing strain, HN878 (Reed, et al., 2004). Similarly the East African-Indian lineage is characterized by an LSP (Large Sequence Polymorphism) conferring an immune subverting phenotype that contributes to its persistence and outbreak potential of this lineage (Newton et al., 2006). Strain differences in immunogenicity may result in reduced detection by TST (Anderson et al., 2006) as documented in a London school contact tracing investigation - an extremely worrying phenomenon which may lead to underestimates of the true global burden of TB and underscores the need for new diagnostics. Most studies of strain-specific responses are derived from adult TB cases, and it remains to be established whether results are equally applicable to children. Further research to characterize strain differences in pathogenicity and induction of immune responses should include children as well as adults.

2.6. Clinical spectrum of paediatric TB disease

The clinical spectrum of childhood TB also reflects differences in the balance between the pathogen and the host immune response, with more severe disease resulting from either poor or 'over-exuberant' attempts to contain the disease. Many cases of primary TB infection in children are asymptomatic, self-healing and remain completely unnoticed or accidentally discovered at a later stage (Marais et al., 2004). In previously healthy children, what determines the differences in the host/pathogen interactions that lead to successful containment as opposed to progressive disease remains largely unknown. However, age and immunodeficiency are important factors. Thus, while an exuberant immune response, in immunocompetent adolescents, tends to result in adult-type, cavitating disease, (Marais et al., 2005) in young children and/or HIV co-infection, poor CMI is thought to allow unrestrained proliferation of bacilli with progressive parenchymal lung damage (with or without cavity formation) dissemination (Marais et al., 2005)

While dissemination can occur to almost any site, TB meningitis (TBM) is one of the commonest consequences of extra-pulmonary TB and develops three to six months after primary infection (Donald and Schoeman, 2004). It is also the most severe and potentially devastating form of childhood TB with mortality or significant long term neurological sequelae occurring in almost 50% of cases (Thwaites and Tran, 2005). Anatomical differences in children, compared with adults, also modify the presentation of TB. Complications arising from enlarging lymph nodes and small airways are common in children less than five years of age (Marais, et al., 2004; Marais et al., 2005). Post-primary TB can result in upper-lobe pulmonary consolidation and cavitation with highly infectious patients, more likely to be seen in older children.

HIV infection often mimics TB associated signs and symptoms, such as weight loss, failure to thrive and chronic pulmonary symptoms, corroborating the diagnostic difficulties (reviewed in references (Marais et al., 2007). In turn, the treatment of HIV with ART can result in unmasking signs and symptoms of underlying LTBI or active TB in the form of immune reconstitution disease (IRD) (Walters et al., 2006) in young children is largely a reflection on the immaturity of the immune response.

2.7. Differences in childhood immune responses to TB

The alveolar macrophage is the first line of defense in the innate immune response to TB and plays a critical role in amplifying the response to infection. Studies in the animal and human host have consistently demonstrated reduced microbial killing and diminished monocyte recruitment to the site of infection in infants compared to adults. Thus impairment of innate pulmonary defenses in the neonate and infant may allow mycobacteria to overwhelm the effects of the innate immune system prior to the initiation of an antigen-specific immune response.

Antigen presentation by dendritic cells (DC), the major antigen-presenting cell (APC) in the lung, and the efficiency with which naïve T cells respond to antigen, also appears less effective in infants and may contribute to the delay in initiating an appropriate antigen-specific response, resulting in development of active disease. Blood derived DCs are functionally

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immature at birth relative to adult DCs and continue to express a less differentiated phenotype throughout early childhood (Upham et al., 2006). Some studies also suggest that neonatal APCs lack the capacity to deliver important Th1 polarising signals to T-cells. Their capacity to synthesise interleukin (IL)-12, a key APC-derived cytokine, matures slowly during childhood (Upham et al., 2002) and neonatal, monocyte-derived DCs have a specific defect in IL-12p35 expression (Goriely et al., 2001). IL-12 is critical for the initial phases of Th1 polarisation and also for maintaining the efficiency of the interferon (IFN)-γ transcription machinery in Th1 effector cells (Goriely et al., 2001).

2.8. Latent tuberculosis in children

2.8.1. Detection of the infection

Transmission within a community is measured by the Annual Risk of Infection (ARI) (Rieder, 2005). Infection rates rise with increased exposure in toddlers, around the ages of school entry and with increased social mobility in late teens and early adulthood (Marais et al., 2004). ARI is traditionally estimated using childhood tuberculin surveys, although this has limitations due to the poor specificity of the tuberculin skin test (TST), particularly where Bacille Calmette Guerin (BCG) vaccine is given at birth and non-tuberculous mycobacteria (NTM) are endemic. T-cell based interferon gamma release assays (IGRAs) may offer a more specific alternative (Dinnes et al., 2007), but have not yet found a use in this context due to their cost, ethical concerns about venepuncture in healthy children (Rieder, 2005), and uncertainty about the significance of a positive result for later development of active disease. Threfore, differences in the pathophysiology and clinical presentation of TB in children make diagnosis more challenging than in adults (Shingadia and Novelli, 2003) and the definitions of latent infection and disease, are less clear cut (Marais et al., 2004).

2.8.2. Activation from infection to disease

Following infection, several factors appear to influence the balance of risk between latent TB infection (LTBI) or progression to active disease, including age (Marais et al., 2004) and nutritional (Cegielski and McMurray, 2004), vaccination and immune status (Chen, 2004). Children are at much higher risk of progression to active disease than adults. This risk is greatest for infants and children under 2 years of age (Marais et al., 2004). Active surveillance data from the pre-chemotherapy era suggest that the majority of children develop radiological abnormalities following infection, including 60-80% of children less than two years. However, less than 10% of those are notified, suggesting the disease is controlled by the host immune response in most cases (Marais et al., 2004). This has implications for case definitions based on radiological findings. Overall the risk of disease is highest among infants and in late teens, with the lowest risk between 5 and 10 years - the so-called “safe school years” (Marais et al., 2004). Most disease occurred in the first year following infection (Marais et al., 2004). Thus because disease in young children reflects recent infection, rather than secondary reactivation, the paediatric disease burden potentially provides a useful measure of current transmission

within a community, Marais et al., 2005) including multi-drug resistant (MDR) (Schaaf et al., 2006) and extensively drug resistant (XDR) strains.

3. Challenges presented by paediatric TB in the field of diagnosis and treatment

3.1. Concepts from the natural history of disease

The pre-chemotherapy literature documented the natural history of tuberculosis in children. Unfortunately, clinicians and researchers have limited access to these important studies as they were conducted before 1950 and are not included in modern electronic databanks. Since the discovery of safe and effective antituberculosis treatment, conducting studies on the natural history of disease became unethical and therefore these historic disease descriptions remain invaluable today. The pre-chemotherapy literature provides a strong body of evidence; multiple studies monitored large cohorts of children for prolonged periods of time and carefully documented the development of disease after primary infection with Mycobacterium tuberculosis. A critical review of the natural history of disease identified three central concepts that are important to consider when addressing current and/or future challenges in the field of childhood tuberculosis: (1) the need for accurate case definitions, (2) the importance of risk stratification, and (3) the diverse spectrum of disease pathology, which necessitates accurate disease classification (Marais et al., 2004).

3.2. Challenge of case definition

Accurate case definition revolves mainly around the ability to differentiate primary infection from active disease. Primary infection is believed to occur when a previously uninfected child inhales a single infectious aerosol droplet, which may contain fewer than five bacilli that penetrate into a terminal airway. A localized pneumonic process, referred to as the primary parenchymal (Ghon) focus, results at the site of organism deposition. For the first 4–6 weeks, unrestrained multiplication occurs within the Ghon focus and bacilli drain via local lymphatics to the regional lymph nodes and beyond. The upper lobes drain to ipsilateral–paratracheal nodes, whereas the rest of the lung drains to perihilar and subcarinal nodes, with dominant lymph flow from left to right (Marais et al., 2006). The Ghon complex is represented by both the Ghon focus, with or without some overlying pleural reaction, and the affected regional lymph nodes (Marais et al., 2006).

Occult dissemination frequently occurs during this early proliferative phase before cell-mediated immunity is fully activated. Bacteriologic cultures collected at this time may be positive; Wallgren demonstrated in the 1930s that M. tuberculosis is sometimes recovered from recently infected children who are not diseased. Therefore, with active contact tracing and aggressive screening that includes the collection of mycobacterial cultures in asymptomatic children it is not unexpected to find some positive cultures in recently infected children who are not diseased. This illustrates the overlap that exists between recent primary infection and

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case definitions of disease that rely exclusively on bacteriology. It is important to consider this overlap when case definitions are formulated for research purposes, particularly within the contact setting, although it is less relevant in everyday practice where there is no reason to obtain cultures from completely asymptomatic children.

Uncomplicated hilar adenopathy remains the most common disease manifestation in children and is usually regarded as the hallmark of primary tuberculosis. However, the prechemotherapy literature documented transient hilar adenopathy in the majority (50–60%) of children after recent primary pulmonary infection, of whom only a few progressed to disease (Marais et al., 2004). The natural history of disease illustrates that progression to disease is indicated by the onset of persistent, nonremitting symptoms, referred to as the breakpoint of clinical significance whereas the complete absence of symptoms usually indicates good organism containment (Marais et al., 2004). By convention, asymptomatic hilar adenopathy is currently treated as active disease, although early experience with isoniazid alone demonstrated that one-drug therapy was sufficient in these cases. In terms of pathophysiology, microbiology, and natural history, asymptomatic hilar adenopathy is more indicative of recent primary infection than active disease (Marais et al., 2004).

This indicates that radiologic signs should be interpreted with caution in the absence of clinical data. The entity of so-called asymptomatic tuberculosis, where the case definition rests exclusively on radiographic criteria, is a case in point. High-resolution computed tomography is the most sensitive tool available to detect hilar adenopathy (Andronikou et al., 2004), as demonstrated by the fact that in children with recent M. tuberculosis infection and a normal chest radiograph, prominent intrathoracic nodes are frequently demonstrated by high-resolution computed tomography. Particular caution is required when interpreting the relevance of these radiologic signs in the absence of clinical data. It is important to point out that there is no role for high-resolution computed tomography in the evaluation of asymptomatic, immune-competent children exposed to M. tuberculosis.

In reality, differences in patient selection may result in the use of different functional case definitions even though the definitions appear similar on paper. In non-endemic areas where active contact tracing is diligently enforced, more children with transient radiologic signs indicative of recent primary infection will be identified, and those with active disease will be diagnosed at an earlier, less advanced stage. Active contact tracing is rarely enforced in endemic areas and children usually present to health care facilities with suspicious symptoms and more advanced disease (Marais et al., 2006). Unlike asymptomatic contacts in which visible radiologic signs probably indicate recent primary infection only, radiologic signs in symptomatic children indicate active disease. From a research perspective it is important to be aware of these differences, as inconsistent case definitions may confound the scientific interpretation of results. In everyday practice, distinguishing between the signs and symptoms of recent primary infection and active disease is less relevant in high-risk children (less than 3 years of age and/or immune compromised) in whom infection frequently progresses to disease, sometimes with rapid disease progression.

3.3. Problems of risk stratification

The natural history of disease demonstrates that age is the most important variable that determines the risk to progress to disease after primary M. tuberculosis infection in immune-competent children (Marais et al., 2004). Infants are at the highest risk (Marais et al., 2004) and the risk drops but stays appreciable in the second year of life, to reach its lowest level in children infected between 5 and 10 years of age (Marais et al., 2004). Children with human immunodeficiency virus (HIV) infection and/or other forms of immune compromise, such as severe malnutrition, seem to experience a similar high risk as the very young (less than 2 years of age), immune-immature children (Marais et al., 2004). The vast majority (more than 95%) of children who progress to disease do so within 12 months of primary infection and, therefore, it seems prudent to categorize all children less than 3 years of age and/or immune-compromised children as high-risk. Because of the frequency and rapidity with which disease progression may occur, exposure to and/or infection with M. tuberculosis warrants treatment intervention in this high-risk group (Marais et al., 2004).

Immune-competent children of at least 3 years of age are at low risk of progression to disease after primary infection. However, as the vast majority of children in endemic areas become infected after 2 to 3 years of age, these low-risk children still contribute a significant percentage to the total disease burden. In addition, although these children are at low risk to progress to disease, latent infection with M. tuberculosis does pose the risk of future reactivation of the disease. In non-endemic areas, where transmission rates are low and eradicating the pool of latent infection is an achievable aim, the provision of preventive therapy to these low-risk children is warranted. In endemic areas, where the majority of disease in immune-competent adults results from ongoing transmission and not from reactivation (Saiman et al., 2001), the provision of preventive therapy after exposure and/or infection becomes less relevant. The major diagnostic challenge in this low-risk group is the differentiation between latent infection and active disease (Marais et al., 2004). Fortunately, active disease is accompanied by persistent, non-remitting symptoms and disease progression is slow, which provides a window of opportunity for symptom-based diagnosis (Marais et al., 2004).

3.4. Difficulties in classifying disease diversity

Childhood tuberculosis is often reported as a single disease entity, although it represents a diverse spectrum of pathology (Marais et al., 2004), and one of the obstacles has been the lack of standard descriptive terminology. Accurate disease classification is important, because of its prognostic significance and to facilitate scientific communication and optimal case management. Within the Ghon focus, containment is usually successful, but disease progression may result from either poor or "excessive" containment. Poor containment and unrestrained organism proliferation may cause progressive parenchymal damage, with ultimate breakdown of the Ghon focus. Infants (Dinnes et al., 2007) and HIV-positive children (Pai et al., 2004), who have poor cell-mediated immune responses, are most vulnerable to this type of cavitation. In contrast, immune-competent adolescents seem to mount an "excessive" (damaging) immune response in an attempt to contain the organism. The exact immune mechanisms underlying adult-type disease remain uncertain, but it is a striking observation that it emerges

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only as children enter into puberty (Marais et al., 2004). It is important to remember that children with adult-type disease are frequently sputum smear–positive and that they do contribute to disease transmission (Cegielski and McMurray, 2004), particularly in congregate settings such as schools.

Complications that arise from affected lymph nodes are most common in children less than 5 years old, because of exuberant lymph node enlargement and small airway size (Marais et al., 2004). Extraluminal compression results when the airway is encircled by enlarged lymph nodes and associated inflammatory edema (Marais et al., 2004). Intraluminal obstruction results from polyps or granulomatous tissue that develops secondary to inflammatory changes in the bronchial wall, or when caseous material is deposited into an airway after lymph node eruption (Marais et al., 2004). Radiologic signs vary from segmental or lobar hyperinflation with partial obstruction and a check-valve effect (Marais et al., 2004), to segmental or lobar collapse with total obstruction and resorption of distal air (Marais et al., 2004). The pathology that results from the aspiration of caseous material is influenced by the dose and virulence of the bacilli aspirated. The pathology may range from transient parenchymal consolidation, resulting from a pure hypersensitivity response to dead bacilli and/or toxic products, to an expansile pneumonic process with progressive caseating pneumonia in the affected segment or lobe (Marais et al., 2004). Expansible caseating pneumonia frequently leads to parenchymal destruction and cavity formation.

Thus, cavitary disease in children may result from three distinct pathologic processes: (1) poor containment at the site of organism deposition (very young and/or immune-compromised children); (2) aspiration of live bacilli when a diseased lymph node erupts into an airway, with destructive caseating pneumonia in the distal segment or lobe (children less than 5 year of age); and (3) adult-type disease (mainly children greater than 10 year of age). The fact that immune-competent children 5 to 10 yr of age experience the lowest risk to progress to disease after primary infection with M. tuberculosis is an interesting immunologic phenomenon that is poorly understood. A better understanding of age-related differences in the immune response to M. tuberculosis may provide important insight into immune correlates of disease and protection.

Disseminated disease occurs predominantly in very young (immune-immature) and/or immune-compromised children, such as the HIV-infected or severely malnourished (Pai et al., 2004; Shingadia and Chen, 2004). These children have suboptimal cellular immune responses and demonstrate poor containment of the organism, both within the regional lymph nodes and at the multiple sites of occult dissemination. TB meningitis (TBM) is the most dangerous complication of disseminated disease, occurring in 20 to 30% of cases (Chen, 2004).

3.5. Challenges in diagnosis

3.5.1. Overview of diagnostic challenges

Diagnostic difficulties pose the greatest challenge to childhood TB management (Marais and Pai, 2007). There are diagnostic complications because: (i) TB can mimic many common childhood diseases, including pneumonia, generalized bacterial and viral infections, malnutrition,

and HIV (Marais and Pai, 2007); (ii) the absence of a practical reference test or gold standard (Marais et al., 2006); (iii) of the inability of child patients to expectorate sputum (Nelson and Wells, 2004) (iv) of the nonspecific clinical presentation (Nicol. et al., 2009); (v) of the lower bacillary load in children which is often smear negative (Detjen et al., 2007) (vi) confirmation by culture of Mycobacterium tuberculosis, usingthe gold standard of diagnosis in adult TB, rarely exceeds 30–40% sensitivity (although it may be considerably higher in children with advanced disease) (Hesseling et al., 2002) even when using gastric aspirates, induced sputum, liquid media, and polymerase chain reaction (PCR) (Edwards et al., 2007); vii) distinguishing between recent primary infection and active disease is highly difficult (Gomez-Pastrana et al., 2001); viii) gastric aspirates continue to be the best specimens for testing for suspected pulmonary TB in children (Ling et al., 2011) with 30–40% sensitivity (Hesseling et al., 2002).

Bacteriologic confirmation, the accepted gold standard, is of limited use in children because of the paucibacillary nature of their disease and poor bacteriologic yields. Sputum smear microscopy, often the only diagnostic test available in endemic areas, is positive in less than 10 to 15% of children with probable tuberculosis (Schaaf et al., 2006). However, the yield is high in children with adult-type disease and sputum smear microscopy has definite diagnostic value in older children (more than 10 year of age) (Cegielski and McMurray, 2004). Culture yields are also low; reported yields in children with probable tuberculosis are less than 30 to 40% (Schaaf et al., 2006). However, the bacteriologic yield depends on the specific intrathoracic disease manifestation. A study from South Africa reported a yield of 77% in children with advanced intrathoracic disease, whereas the yield in those with uncomplicated hilar adenopathy was only 35% (odds ratio, 6.3; 95% confidence interval, 3.2–12.8) (Graham et al., 2001). This indicates the potential value of sensitive bacteriology-based diagnostic approaches, particularly in endemic areas where children frequently present with advanced disease.

Most children with TB are classified as smear-negative pulmonary TB (PTB) for the reasons mentioned above, which is an inappropriate term as a smear or culture has not usually been done. This leads to difficulties in determining the true extent of PTB in children in different areas and circumstances. Extrapulmonary TB (EPTB) accounts for up to 20–30% of the total caseload of TB in children, and the diagnosis is usually easier than PTB because of the characteristic clinical features like lymphadenopathy with or without scrofula, spinal deformity, disseminated disease, meningitis, effusions (pleural or pericardial), or painless ascite (Lewinsohn et al., 2004). The isolation of Mycobacterium tuberculosis takes several weeks. Consequently, the diagnosis of TB in children is often supported only by epidemiological, clinical, and radiographic findings in the presence of a positive tuberculin skin test (López Ávalos and Montes de Oca, 2012).

The value of the classic diagnostic is based on: (1) exposure to an adult index case; (2) chronic respiratory symptoms that do not respond to broad-spectrum antibiotics; (3) documented weight loss or failure to thrive; (4) a positive tuberculin skin test (TST); (5) the presence of suggestive signs on the chest radiograph (CXR), which is greatly reduced in endemic areas where exposure to and/or infection with M. tuberculosis is common (Marais et al., 2006). These criteria are less helpful in endemic areas where a positive TST result is common and exposure to M. tuberculosis is often undocumented (Hesseling et al., 2002). For all these reasons, many

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children with TB are never diagnosed or registered as cases of TB (Nelson and Wells, 2004). Furthermore, the consequences of missed diagnosis in children are severe, as untreated children have a high probability of developing active TB, usually within two years of infection (López Ávalos and Montes de Oca, 2012).

The difficulty to obtain samples for TB diagnosis in children has led researchers to create smart approaches as "la cuerda dulce" (sweet string), reported by Chow et al., (2006). They provide a technique which consists of a coiled nylon string inside a gel capsule. The string unravels through a hole in the end of the weighted capsule as it descends into the stomach and the capsule then dissolves in it, allowing the string to become coated with gastrointestinal secretions containing whatever pathogens are present. After about four hours, the capsule is passed in the feces. This methodology is well tolerated by children and is less invasive than the gastrointestinal lavage (López Ávalos and Montes de Oca, 2012).

In addition to poor bacteriologic yields, the collection of bacteriologic specimens is often problematic. Two or three fasting gastric aspirates collected on consecutive days, usually requiring hospital admission, are routinely performed in young children who cannot cough up phlegm. A retrospective study from California compared the bacteriologic yield achieved in gastric aspirates collected from hospitalized and nonhospitalized children. Although the yield in hospitalized children was higher (percentage of positive cultures, 48 compared to 37%), this difference was not statistically significant, which suggests that hospitalization may not be a prerequisite for the collection of a good gastric aspirate specimen. Bronchoalveolar lavage, using flexible fiberoptic bronchoscopy, has additive value when used in combination with gastric lavage, but this technique is highly specialized and is unavailable in most endemic areas. In a study from Peru, midmorning nasopharyngeal aspiration was compared with early morning gastric aspiration; gastric aspiration provided a slightly better yield than nasopharyngeal aspiration (38 compared to 30%), but the results were comparable (Nelson et al., 2004). Nasopharyngeal aspiration is minimally invasive, does not require hospitalization or fasting, and can be performed any time of the day. A study from South Africa demonstrated that a single specimen, using hypertonic saline–induced sputum collection, may provide the same yield as three gastric aspirate specimens (Corbett et al., 2003). However, the overall yield in this study remained poor (15% with one and 20% with three induced sputum specimens) and the technique has not been used outside the hospital setting. Additional studies are awaited to confirm the feasibility and diagnostic value of collecting induced sputum specimens in primary health care settings.

Because of the difficulty in achieving bacteriologic confirmation, the diagnosis of childhood tuberculosis in non- endemic areas is usually based on (1) known contact with an adult index case (frequently within the household), (2) a positive tuberculin skin test (TST), and (3) suggestive signs on the chest radiograph. This triad provides a fairly accurate diagnosis in settings where exposure to M. tuberculosis is rare and well documented. However, its diagnostic accuracy is greatly reduced in endemic areas where exposure to M. tuberculosis is common and often undocumented, as exposure frequently occurs outside the household. Despite reservations about the specificity of the TST response after Bacille Calmette-Guérin (BCG) vaccination and/or exposure to environmental mycobacteria, a positive TST reaction

remains a fairly accurate measure of M. tuberculosis infection in immune-competent children. Current U.S. guidelines recommend the use of three different cutoff points to define a positive TST reaction. In endemic areas a positive TST is not uncommon in randomly selected healthy children (Jeena et al., 2002), which limits its diagnostic value. Consequently, the diagnosis of tuberculosis in children from endemic areas depends mainly on clinical features and the subjective interpretation of the chest radiograph (Marais et al., 2007). However, chest radiography is unavailable in many endemic areas and it has well-known limitations that may result in both under- and overdiagnosis of disease (Brent et al., 2007). Despite these limitations it provides an accurate diagnosis in the majority of symptomatic children with tuberculosis and the interpretation of the chest radiograph remains the most widely used diagnostic criterion in clinical practice (Palme et al., 2002).

Various clinical scoring systems have been developed. A critical review of these clinical scoring systems concluded that they are limited by a lack of standard symptom definitions and adequate validation (Walls and Shingadia, 2007). Developing standard symptom definitions through consensus of expert opinion is a difficult and subjective exercise; better guidance may be provided by objectively measuring the potential diagnostic value of different symptom definitions. A community-based survey demonstrated that the poorly defined symptoms traditionally associated with tuberculosis (such as a cough greater than 3 weeks in duration) are frequently reported in a random selection of healthy children (Bryce et al., 2005). Of 1,397 children without tuberculosis, 253 (26.4%) reported a cough during the preceding 3 months and 66 (6.9%) reported a cough greater than 3 weeks in duration (Bryce et al., 2005). In addition, nearly 50% of children with visible hilar adenopathy on the chest radiograph (diagnosed with tuberculosis) reported no symptoms at all (Bryce et al., 2005). These observations demonstrate the limited diagnostic value of poorly defined symptoms and the need for improved symptom and case definitions. In a follow-on study the use of well-defined symptoms with a persistent, nonremitting character showed greatly improved diagnostic accuracy (Scot et al., 2008). However, the potential diagnostic value offered by the use of these well-defined symptoms requires further validation in a prospective, community-based study that includes children from all relevant risk groups. It is expected that symptom-based diagnostic approaches would have less value in high-risk children (less than 3 years of age and/or immune compromised) where disease progression may occur rapidly, emphasizing the need for preventive chemotherapy and early diagnosis of disease in this group (Chintu, et al., 2002). Other diagnostic modalities may hold promise, but have not shown convincing results to date (WHO, 2006).

Serologic tests are currently unable to diagnose childhood tuberculosis with accuracy, and sputum-based polymerase chain reaction (PCR) tests have shown variable results and limited utility (WHO, 2007). Good results were reported with the use of a heminested PCR technique in Peru, but the study used uninfected children as the control group and therefore could not evaluate the ability of this novel PCR-based test to differentiate latent infection from active disease (Upham et al., 2006), which is important, as specific concerns have been raised regarding the specificity of PCR-based tests.

The diagnostic dilemma is even more pronounced in HIV-infected children. The specificity of symptom-based diagnostic approaches is reduced by the presence of chronic HIV-related

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symptoms, while the potential window for symptom-based diagnosis is limited by the rapidity with which disease progression may occur. Chest radiograph interpretation is complicated by HIV-related comorbidity and atypical disease presentation. These difficulties increase the potential diagnostic value of sensitive bacteriology-based approaches, to identify HIV-infected children with tuberculosis (Upham et al., 2002). However, as HIV-infected children are in the high-risk group the detection of M. tuberculosis infection is also highly relevant. Disease progression may occur soon (less than 12 months) after primary or reinfection, or latent infection may be reactivated at a later date because of a decline in immunity. The traditional TST has poor sensitivity to detect M. tuberculosis infection in HIV-infected children; 50% or less of HIV-infected children with bacteriologically confirmed tuberculosis are TST positive, despite using an induration size of at least 5 mm (Upham et al., 2002). This is a major limitation and development of a more reliable measure of infection will be valuable to identify HIV-infected children who may benefit from preventive chemotherapy; it may also provide supportive evidence to establish a diagnosis of active tuberculosis.

3.5.2. Challenges presented by diagnosis of latent infection in children

LTBI, in children as in adults, lacks a diagnostic gold standard. The diagnosis is usually pursued after a documented household exposure, or to evaluate if chemoprophylactic therapy is indicated in the context of immunosuppression. In this setting, pre-existing MTB specific host immune responses are measured to confirm previous infection. Data in adults have confirmed that IGRA are more sensitive and specific than the TST (Pai et al., 2004; Ferrara et al., 2006) in this context. Preliminary data suggest IGRA also perform better in children but age-related data are still sparse. Longitudinal studies assessing their positive predictive value for the development of active TB are required in both TB-endemic and low-incidence countries, as the continued exposure in TB endemic settings might yield very different results, compared to the "one-off" exposure more typically encountered in non-endemic countries.

3.6. Challenges presented by drug resistance

There were an estimated 0.5 million adult cases of MDR-TB in 2007. By the end of 2008, 55 countries and territories had reported at least 1 case of extensively drug-resistant TB (WHO Report, 2009). Latest research reports published in The Lancet at the end of August 2012, indicate that researchers have found rates of both multi drug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) higher than previously thought and that they are threatening global efforts to curb the spread of TB. They contend that most international recommendations for TB control have been developed for MDR-TB prevalence of up to around 5 percent but that now the world faces a prevalence up to 10 times higher in some places, where almost half of the patients are transmitting MDR strains. The Researchers who studied rates of the disease in Estonia, Latvia, Peru, the Philippines, Russia, South Africa, South Korea, and Thailand are reported to have found that almost 44 percent of cases of MDR TB were also resistant to at least one second-line drug outline goes here (Dalton et al., 2012)

Comprehensive studies on resistance to anti-TB drugs in children are lacking, because they are not included in global surveys. Surveillance of anti-TB drug resistance during 1995–2007

among children from South Africa showed a significant increase in resistance to INH or RIF from 6.9% to 15.1% and an increase in multidrug resistance from 2.3% to 6.7% (Schaaf et al., 2009). Drug resistance among children has been documented in clinical trials of both pulmonary and extrapulmonary TB (Rekha and Swaminathan, 2007). Management of MDR-TB is a challenge, because it requires prolonged treatment for 24 months with second-line drugs, which are more toxic and expensive than first-line drugs. According to the 2006 WHO guidelines for programmatic management of MDR-TB, an optimal regimen should include a fluoroquinolone, an injectable (capreomycin, kanamycin, or amikacin), and at least 2 of the following drugs: cycloserine, thiomides, para-amino salicylic acid, and first-line agents other than INH and RIF (WHO, 2008). Experience with second-line TB drugs in children is limited; 38 children in Peru were treated with supervised, individualized regimens consisting of 5 drugs in the national program. Despite half of these children being anemic and malnourished, treatment was well tolerated and resulted in a 95% cure rate (Drobac et al., 2006).

There is little published information on optimal treatment of latent TB infection in children in contact with patients with MDR-TB. In a 30-month follow-up of contacts of patients with MDR-TB, 5% of children who received appropriate chemoprophylaxis and 20% of those who did not receive prophylaxis developed disease (Schaaf et al., 2007). Regimens used included INH, PZA, and ethionamide or EMB. Currently, the best approach may be to perform a complete risk assessment and clinical evaluation and to individualize therapy, while keeping these children under close observation. Multicentric trials are urgently required to determine the most effective drug combinations and optimal duration of chemoprophylaxis for contacts of patients with MDR-TB.

TB is often not considered in the differential diagnosis in children, especially in low endemic settings. TB can mimic many common childhood diseases, including pneumonia, generalised bacterial and viral infections, malnutrition and HIV. However, the main impediment to the accurate diagnosis of active TB is the paucibacillary nature (containing just a few bacilli) of the disease in children. Younger children also produce smaller amounts of sputum, which is usually swallowed rather than expectorated. Bacteriological samples may be collected by conducting early morning gastric washings, a fairly unpleasant procedure that requires hospital admission and overnight-fast for up to three consecutive nights. Consequently bacteriological confirmation is the exception rather than the rule with only 10-15 % of sputum samples revealing acid fast bacilli (AFB) and culture remaining negative in around 70% of cases with probable TB (Zar et al., 2005). Without a definitive diagnosis treatment is therefore often initiated on clinical judgment, aided by algorithms based on exposure history, clinical features, chest x-ray (CXR) and TST (Marais et al., 2006). Several approaches have been taken to improve the diagnosis (Marais and Pai, 2007).

3.7. Improving bacteriological detection and rapid resistance analysis

Recent advances in bacteriological and molecular methods for the detection of MTB in patient samples aim to identify drug-resistance in parallel with detection of MTB. These include the Microscopic Observation Drug Susceptibility assay (MODS) (Moore et al., 2006), more sensitive PCR techniques (Sarmiento et al., 2003) or phage-based tests such as FASTPlaque

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(Kalantri et al., 2005). This represents laudable progress, particularly in the context of increasing drug resistance. Calorimetric culture systems such as the TK medium (Kocagoz et al., 2004) and electronic-nose technology (Fend et al., 2006) are also under investigation. Among adults MODS appears to be at least as sensitive as gold standard liquid culture methods (Moore et al., 2006), Data comparing its performance in children is more limited, but MODS has been evaluated in a paediatric hospital setting and found to be more sensitive than solid media in one study (Oberhelman et al., 2006). Data validating other new methods in paediatric specimens are also lacking, yet performance may be affected by the paucibacillary nature of childhood TB. The lowest limit of detection of TB by the electronic nose for example has been reported to be 104 CFU/ml of sputum for example which is just within the range of the expected bacillary burden in paediatric specimens (Fend et al., 2006). Validation of these assays on paediatric samples is a research priority (López Ávalos G G and Montes de Oca, 2012). The introduction of GeneXpert which includes use of integrated DNA extraction and amplification systems and utilizes real-time PCR (rt-PCR) technology to both diagnose TB and detect rifampicin resistance, has given a ray of hope with paediatric TB diagnosis and rifampicin resistance (Gordetsov et al., 2008).

4. Diagnosis and treatment: Current state of affairs

4.1. Classical diagnosis

4.1.1. Clinical symptoms approach

The use of well-defined symptoms improves diagnostic accuracy of pulmonry tuberculosis (PTB) (Imaz et al., 2001). With clinical symptoms approach only, the status can be classified in two; suspected TB or probable TB. Two situations lead the clinician to suspect that a child has tuberculosis. The first is a history of chronic illness with clear symptoms: cough and/or fever, weight loss or failure to thrive, an inability to return to normal health after measles or whooping cough, fatigue, and wheezing; second, is when one or more of the following: malnutrition, lymphadenopathy, chest signs, hepatomegaly and/or splenomegaly, meningeal signs, and/or ascites is/are observed. For probable TB, in addition to suspected TB, the child presents with a positive TST, a suggestive radiological chest appearing as pleural effusion, caseation of biopsy material, poor response to 2 weeks of antibiotic treatment, and/or favourable response to antituberculous treatment (weight gain and loss of signs) (Hesseling et al., 2002).

In pediatric TB, the most common symptoms are pulmonary parenchymal disease and intrathoracic adenopathy accounting for 60–80% of all cases. Among extrapulmonary manifestations, lymphadenopathy is the most common (67%), followed by central nervous system involvement (13%), pleural (6%), miliary and/or disseminated TB (5%), and skeletal TB form (4%). Disseminated disease and TB meningitis are usually found in very young children who are below the age of 3 years, and/or HIV-infected children (Nelson et al., 2004). TB meningitis occurs when the child has contact with a suspected or confirmed case.

In general, there is a sense of skepticism regarding the potential diagnostic value of symptom-based approaches but nevertheless, the natural history of childhood tuberculosis demonstrates that symptoms may have diagnostic value if appropriate risk stratification is applied. Marais et al., (2005), evaluated whether well-defined symptoms have a diagnosis value in children and a standard symptom-based questionnaire was completed and reported symptoms were individually characterized. A tuberculin skin test (TST) and chest radiograph (CXR) were performed in all children. In this study,well-defined symptoms had excellent diagnostic value.

4.1.2. Radiologic studies

Radiography became available after the First World War, and since that time, PTB detection became easier (Marais et al., 2004). Evidence of pulmonary TB in chest radiographs varies, but usually radiographs show enlargement of hilar, mediastinal, or subcarinal lymph nodes and lung parenchymal changes with hilar lymphodenopathy with or without a focal parenchymal lesion. The most common findings are segmental hyperinflation then atelectasis, alveolar consolidation, interstitial densities, pleural effusion, and, rarely, a focal mass. Cavitation is rare in young children but is more common in adolescents, who may develop reactivation disease similar to that seen in adults (Marais. and Pai, 2007). High-resolution computed tomography is the most sensitive tool currently available to detect hilar adenopathy and/or early cavitation (Hesseling et al., 2002).

4.1.3. Diagnostic algorithms

These are point-scoring systems to make diagnostic classifications. Diagnostic algorithms were developed to deal with these diagnostic difficulties and provide the health care worker with a rational, stepwise tool to identify children in need of treatment. They are very helpful and very easy to use in countries with restricted technology, but only few of them are available especially in resource limited countries (Edwards et al., (2007). Although the natural history of tuberculosis (TB) in children follows a continuum, the American Thoracic Society (ATS) definition of stages is useful (Blumberg, et al., 2003). According to the ATS the stages are as follows:

Stage 1: Exposure has occurred, implying that the child has had recent contact with an adult who has contagious TB. The child has no physical signs or symptoms and has a negative tuberculin skin test (TST) result. Chest radiography does not reveal any changes at this stage. However, not all patients who are exposed become infected, and the TST result may not be positive for 3 months. Unfortunately, children younger than 5 years may develop disseminated TB in the form of miliary disease or TB meningitis before the TST result becomes positive. Thus, a very high index of suspicion is required when a young patient has a history of contact.

Stage 2: This second stage is heralded by a positive TST result. No signs and symptoms occur, although an incidental chest radiograph may reveal the primary complex.

Stage 3: In stage 3, TB disease occurs and is characterized by the appearance of signs and symptoms depending on the location of the disease. Radiographic abnormalities may also be seen.

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Stage 4: Stage 4 is defined as TB with no current disease. This implies that the patient has a history of previous episodes of TB or abnormal, stable radiographic findings with a significant reaction to the TST and negative bacteriologic studies. No clinical findings suggesting current disease are present.

Stage 5: TB is suspected, and the diagnosis is pending. Any patient with pneumonia, pleural effusion, or a cavitary or mass lesion in the lung that does not improve with standard anti-bacterial therapy should be evaluated for tuberculosis (TB). Also, patients with fever of unknown origin, failure to thrive, significant weight loss, or unexplained lymphadenopathy should be evaluated for TB (Marais et al., 2006).

4.1.4. Mycobacterial detection and isolation

Microbiological confirmation of TB in young children is not routinely attempted in many high burden settings due to the difficulty in obtaining samples and the poor performance of smear microscopy (Nicol and Zar, 2011). Diagnosis of TB still relies primarily on examination of Acid-Fast Bacilli- (AFB-) stained smears from clinical specimens in adults, however, children with pulmonary TB usually do not cough up voluntarily, either because they do not produce sputum or because it produces discomfort. When sputum samples cannot be obtained, gastric aspirate samples are used for detection and isolation of M. Tuberculosis. Most of the current TB diagnostic methods were developed over a century ago. In 1898, Neunier became the first person to culture stomach contents for the evidence of tuberculosis in children (Marais and Pai, 2007; Lalvani and Millington, 2007), so even with this method, fewer than 20% of children with TB have a positive AFB smear of sputum or gastric aspirate.

For many years, the collection of three consecutive early morning gastric lavages or gastric aspirate samples has been the accepted method for attempting microbiological confirmation even as the yield is very low and that in many populations cannot be performed due to the lack of infrastructure. In addition low pH is known to kill tuberculous bacilli, indicating that stomach pH may inhibit TB survival for subsequent culture (Marais. and Pai, 2007). More recently, a number of less invasive alternative methods have been proposed, including induced sputum (administration of an inhaled bronchodilator followed by nebulized hypertonic 3–5% saline and then collecting nasopharyngeal aspiration or expectoration of mucus from lower respiratory tract). In the nasopharyngeal aspiration, a cannula elicits a cough reflex and the sweet string test mentioned above (Nicol and Zar, 2011). One of the methods that can be used to collect samples for microbiological analysis is the string test. This is a non-invasive collection method and is reported to be well tolerated by children as young as 4 years (Chow et al., 2006). Inducing sputum after hypertonic saline nebulization has also been shown to be feasible for young children, although the most widely used procedure is still the early-morning gastric aspiration or lavage. However, all these procedures involve hospitalization, trained personnel, and attention to infection control.

All of these alternative ways of sampling have been made to increase yield because a positive culture is regarded as the "gold standard test" to establish a definitive diagnosis of TB in a symptomatic child (Hesseling et al., 2002). If culture is negative, diagnosis is made on the basis of a positive TST. With clinical and radiographic findings suggestive of TB, and history of

contact with an adult source case, the child may be diagnosed with positive TB based on symptomatology. This measure was taken because the yields in children are less than 50%. Zar et al., (2000), investigated whether sputum induction could be successfully performed in infants and young children with and without HIV and determined the utility of salbutamol-induced sputum compared to gastric lavage (GL) for the diagnosis of pulmonary tuberculosis. They concluded that sputum induction can be effectively performed and is well tolerated and safe even in infants and this induction is better than GL for the isolation of M. tuberculosis in both HIV-infected and uninfected infants and children.

Although culture on Lowenstein-Jensen medium is considered to be the gold standard, liquid culture systems (commercial and non commercial) offer the possibility of more rapid and more sensitive diagnosis of active TB and drug susceptibility but are not widely available in resource-poor settings (Brittle et al., 2009) compared mycobacterial yields and time to detection in pediatric clinical samples with use of mycobacterial growth-indicator tubes with those with use of solid Lowenstein-Jensen slants and found that the yield was substantially higher with use of mycobacterial growth-indicator tubes (11% compared to 1.6%). Furthermore, the mean time to detection could be reduced from 18.5 days to 12.4 days with use of a nutrient broth supplement; newer approaches, such as the colorimetric culture systems and phage-based tests are of interest, but limited data are available for children.

4.1.5. Smear microscopy

Advances have been done in the performance of smear microscopy for the rapid detection of MTB, for example, the concentration of specimens by centrifugation or the change of the staining of carbol fuchsin (Ziehl-Neelsen or Kinyoun) for a fluorescent dyes (auramine-rhodamine), which both increases sensitivity and reduces the time for screening (Bakir et al., 2008). However, even under optimal circumstances, the sensitivity of smear microscopy for the diagnosis of childhood TB remains less than 15%, except in older children with adult-like disease (Nicol and Zar, 2011).

4.1.6. Tuberculin skin test (TST)

This is one of the major classes of tests that are currently used to detect Latent TB. Tuberculin which is also called purified protein derivative or PPD is a standardised killed extract of cultured TB, which is injected into the skin to estimate an individual's immune response to TB. There are three methods of testing: the Mantoux test, the Heaf test and the Tine test but not all of them are currently available for use and some countries prefer one over the other. The Heaf test is no loner available because its continued manufacture was not economically viable.

The Tuberculin skin test, or Mantoux TST, is based on the detection of a cutaneous delayed-type hypersensitivity response to purified protein derivative, a poorly defined mixture of antigens present in M. tuberculosis, Mycobacterium bovis Bacille Calmette-Guerin (BCG) and several nontuberculous mycobacteria (Nicol et al., 2011). TST is the standard method for detecting infection by M. tuberculosis. The reaction is measured as millimeters of induration

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after 48 to 72 hours. This test was the only method available for the diagnosis of latent tuberculosis infection (LTBI) until very recently.

The Heaf test uses what is called a Heaf gun which uses disposable single-use heads, each head having six needles arranged in a circle. The device has standard heads and pediatric heads - the standard head being used on all patients aged 2 years and older while the pediatric head is for infants under the age of 2. For the standard head, its needles protrude 2 mm when the gun is actuated while for the pediatric heads, the needles protrude only 1 mm. Before application, the skin is cleaned with alcohol, then 100,000 units/ml (equivalent to about 0.1 ml) of tuberculin is evenly smeared on the skin and the gun applied to the skin and fired. The excess of the solution is then wiped off and a waterproof ink mark is drawn around the injection site as an indicator of the site of administration and the test read 2 to 7 days later. The results of the test are interpreted as follows:

Grade 0: no reaction, or induration of 3 or less puncture points;Grade 1: induration of four or more puncture points; Grade 2: induration of the six puncture points coalesce to form a circle; Grade 3: induration of 5 mm; or more and Grade 4: induration of 10 mm or more, or ulceration.

There is not much difference between the Heaf and Mantoux test, but the two tests can be related as follows: Heaf grade 0 and 1 approximately equivalent Mantoux less than 5 mm; Heaf grade 2 approximatley equivalent to Mantoux 5–14 mm and Heaf grade 3 & 4 being approximately equivalent to Mantoux 15 mm or greater, To avoid cases of false positives and false negatives, the tuberculin used for Heaf tests is 1000 times more concentrated than that used for Mantoux tests. In countries where both tests are used, use of the correct concentration avoids false positive and false negative results.

The recommended Tuberculin Skin Test (TST), which has now been standardised by the WHO to contain 0.1 ml of tuberculin (100 units/ ml), is the Mantoux test (CDC, 2010). The dosage of 0.1 ml containing 5 tuberculin units [TU] of purified protein derivative (PPD) should be injected intradermally into the volar aspect of the forearm using a 27-gauge needle. A detergent called Tween 80 to prevent loss of efficacy on contact and adsorption by glass stabilizes the PPD. A wheal should be raised and should measure approximately 6-10 mm in diameter. Skilled personnel should always read the test 48-72 hours after administration. Measure the amount of induration and not erythema. This should be measured transverse to the long axis of the forearm. Multiple puncture tests such as Tine test and Heaf test lack sensitivity and specificity and hence are not recommended in this situation (Marais et al., 2006).

Subcutaneous injection should be avoided because it results in false negative results. The site of administration is indicated by a water-proof ink mark drawn around the site of injection to serve as an indicator for the site. The reading, which is done two to seven days involves measuring area of induration transversely (left to right) across the forearm and recorded to the nearest millimetre. It should be borne in mind that the induration (dermal thickening causing the cutaneous surface to feel thicker and firmer) should not be confused with erythema (redness of the skin) caused by hyperemia of the capillaries in the lower layers of the skin.

If a patient who has previously had a negative tuberculin skin test develops a positive tuberculin skin test at a later date, tuberculin conversion is said to have occurred. When such

a reaction occurs, it provides strong evidence for significant exposure to TB. Different countries have different standards about the time interval between tests. The UK recommendation is that the two tests have to be done at least six weeks apart; while in the U.S. the recommendation is that the two tests can be done one week apart.

Another phenomenon associated with tuberculin skin test is what is called boosting, which occurs when people who have had some traces of infection with M. tuberculosis and/or previous exposure to BCG vaccination against tuberculosis, are given repeated tuberculin skin tests. In these cases, the first test revives or primes the immune response so that on repeat testing, the response is much stronger and the patient now appears to have a positive reaction. The second tuberculin skin test result is what is taken to be the correct one. Again, the guidelines on how to approach the phenomenon of boosting are different in different countries with the U.S. guidelines emphasising that, ignoring previous immunisation with BCG would lead to a person showing the phenomenon of boosting, being falsely described as a tuberculin converter. On the other hand, UK guidelines advocate two tuberculin skin tests one week apart, if boosting is suspected, taking the result of the second test as being the true result. The phenomenon of boosting can occur up to two years after the first Mantoux test.

According to the American Academy of Pediatrics (AAP) immediate skin testing is indicated for the following children: 1)Those who have been in contact with persons with active or suspected TB; 2) Immigrants from TB-endemic countries or children with travel histories to these countries; 3) Those who have radiographic or clinical findings suggestive of TB. 4) Children who are infected with human immunodeficiency virus (HIV) or those living in a household with persons infected with HIV and; 5) Incarcerated adolescents.

Testing at 2-year to 3-year intervals is indicated if the child has been exposed to high-risk individuals including those who are homeless, institutionalized adults who are infected with HIV, users of illicit drugs, residents of nursing homes, and incarcerated adolescents or adults. Testing when children are aged 4-6 years and 11-16 years is indicated for the following children:1) Children without risk factors residing in high-prevalence areas; 2) Children whose parents emigrated from regions of the world with a high prevalence of TB or who have continued potential exposure by travel to the endemic areas and/or household contact. Performing an initial TST before the initiation of immunosuppressive therapy is recommended in any patient (AAP, 1996).

Wit regard to administering the TST to previous recipients of the Bacille Calmette-Guérin (BCG) several problems are encountered when it comes to interpreting the results of the test. Immunization with BCG is not a contraindication to the TST but differentiating tuberculin reactions caused by vaccination with BCG versus reactions caused by infection with M tuberculosis is difficult. History of contact with a person with contagious TB or emigration from a country with a high prevalence of TB suggests that the positive results are due to infection with M tuberculosis. However, multiple BCG vaccinations may increase the likelihood that the positive TST result is due to the BCG vaccination. The positive reactivity caused by BCG vaccination generally wanes with the passage of time. With the administration of TST, this positive tuberculin reactivity may be boosted. However, previous BCG vaccination does

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not affect interpretation of a TST result for a person who is symptomatic or in whom TB is strongly suspected (Marais et al., 2006).

For the UK the guidelines for interpreting tuberculin skin tests are formulated according to the Heaf test. For patients who have had BCG previously, latent TB is diagnosed if the Heaf test is grade 3 or 4 and have no signs or symptoms of active; if the Heaf test is grade 0 or 1, then the test is repeated and, in patients who have not had BCG previously, latent TB isiagnosed if the Heaf test if grade 2, 3 or 4, and have no signs or symptoms of active TB. Repeat Heaf testing is not done in patients who have had BCG of the phenomenon of boosting.

The Centers for Disease Control and Prevention (CDC) and the AAP provided recommendations regarding the size of the induration created by the TST that is considered a positive result and indicative of disease [http://www.cdc.gov/tb/]. The TST is interpreted on the basis of 3 "cut points": 5 mm, 10 mm, and 15 mm. Induration of 5 mm or more is considered a positive TST result in the following children: 1) Children having close contact with known or suspected contagious cases of the disease, including those with household contacts with active TB whose treatment cannot be verified before exposure; 2) Children with immunosuppressive conditions (such as HIV) or children who are on immunosuppressive medications; 3) Children who have an abnormal chest radiograph finding consistent with active TB, previously active TB, or clinical evidence of the disease.

Induration of 10 mm or more is considered a positive TST result in the following children: 1) Children who are at a higher risk of dissemination of TB disease, including those younger than 5 years or those who are immunosuppressed because of conditions such as lymphoma, Hodgkin disease, diabetes mellitus, and malnutrition; 2) Children with increased exposure to the disease, including those who are exposed to adults in high-risk categories (such as homeless, HIV infected, users of illicit drugs, residents of nursing homes, incarcerated or institutionalized persons); 3) those who were born in or whose parents were born in high-prevalence areas of the world; and those with travel histories to high-prevalence areas of the world. Induration of 15 mm or more is considered a positive TST result in children aged 5 years or older without any risk factors for the disease.

False-positive reactions and false-negative results are common and can be due to various causes. False-positive reactions are often attributed to asymptomatic infection by environmental non-TB mycobacteria (due to cross-reactivity). False-negative results, on the other hand, may be due to vaccination with live-attenuated virus, anergy, immunosuppression, immune deficiency, or malnutrition. In cases of anergy, a lack of reaction by the body's defence mechanisms when it comes into contact with foreign substances, the tuberculin reaction will occur weakly, thus compromising the value of Mantoux testing. For example, anergy is present in AIDS, a disease which strongly depresses the immune system. Therefore, anergy testing is advised in cases where suspicion is warranted that it is present. However, routine anergy skin testing is not recommended. Other factors that may cause a false-negative result include improper administration (such as subcutaneous injection, injection of too little antigen), improper storage, and contamination. PPD has been recognized to have an initial false-negative rate of 29% (Marais et al., 2006).

With a TST, it is not possible to assert or deny the presence of TB, but it only indicates infection with a mycobacterium. In a child who has not been BCG-vaccinated, a TST has been defined as positive when the diameter of skin induration is greater than 10 mm, and in a BCG-vaccinated child, when the diameter of induration is greater than 15 mm. A negative TST does not exclude TB and some induration (5–14 mm) could be supportive if the clinical features and contact history are suggestive (Lewinsohn et al., 2004). Furthermore, the utility of this conventional test is hampered by technical and logistical problems: potential for false-positive and false-negative results; problems in administration and interpretation; difficulty in separating true infection from the effects of prior BCG vaccination, infection due to nontuberculous mycobacteria (Dogra et al., 2007). In children with debilitating or immunosuppressive illnesses, malnutrition, or viral (as HIV) and certain bacterial infections, the yield is unknown, but it is certainly higher than 10%. Moreover, false-positive reactions to TST are often attributed to asymptomatic infection by environmental nontuberculous mycobacteria (Nicol et al., 2011).

Given that the US guidelines recommend that previous BCG vaccination be ignored in the interpretation of tuberculin skin tests, false positives are possible. People who have previously had BCG, will falsely appear to be tuberculin converters and this may lead to treating more people than necessary, with the possible risk of those patients suffering adverse drug reactions. However, considering the fact that BCG vaccine is not 100% effective, and that it is less protective in adults than pediatric patients, not treating these patients could lead to a possible infection which tennds to justify the current US policy. The U.S. guidelines also allow for tuberculin skin testing in immunosuppressed patients whereas the UK guidelines recommend that tuberculin skin tests should not be used for such patients because it is unreliable

4.2. New approaches in TB diagnostics

4.2.1. Polymerase chain reaction (PCR)

Diagnostic PCR is a technique of in vitro DNA amplification that uses specific DNA sequences (oligonucleotides) as effective fishhooks for the DNA/cDNA of microorganisms. In theory, this technique can detect a single organism in a lot of specimens such as sputum, gastric aspirate, pleural fluid, cerebrospinal fluid, blood, and urine. Various PCR assays, mostly using the mycobacterial insertion element IS6110 as the DNA marker for M. tuberculosis-complex organisms, have a sensitivity and specificity greater than 90% for detecting pulmonary TB in adults. This is a rapid, sensitive, specific, and reasonable-cost (Montenegro et al., 2003) method for the detection of M. tuberculosis in clinical samples. The PCR may be used to (a) diagnose tuberculosis in difficult samples with negative microscopic examination, negative culture, or with scarce sample; (b) determine if the organisms in the sample are M. tuberculosis or atypical mycobacteria; (c) identify the presence of genetic variations like a mutations or deletions known to be associated with resistance to some antimycobacterial agents (Marais et al., 2005).

Studies in children have obtained better sensitivity by PCR than by culture. In 2001, Gomez-Pastrana et al., (2001) reported a comparison between sensitivity of culture and PCR showing higher sensitivity for the latter. PCR may have a special role in the diagnosis of extrapulmonary TB and pulmonary TB in children since sputum smears are usually unrevealing in these cases.

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However, these tests are not performed correctly in all clinical laboratories. The cost involved, the need for sophisticated equipment, the limitations in their specificity, the need to obtain multiple samples to optimize yield and scrupulous technique to avoid cross-contamination of specimens preclude the use of PCR techniques in many developing countries (Montenegro et al., 2003).The sensitivity of PCR of gastric lavage/bronchoalveolar lavage has been found to be 56.8% in children with clinically active disease. Authors conclude that nested PCR is a rapid and sensitive method for the early diagnosis of TB in children. Additionally, other unique sequences of M. tuberculosis have been suggested as diagnostic test for TB, because they are absent in M. africanum, M. microti, M. bovis, and M. bovis BCG (Liang et al., 2008).

4.2.2. In-house nucleic acid amplification (NAA) assays

These assays are highly dependent on the operator's skills. Performance is also influenced by the choice of target sequence and DNA extraction method. Interpretation of the performance of these assays in pediatric TB suspects is hindered by the lack of a sensitive and specific reference standard. When compared with culture, the sensitivity of NAA for the diagnosis of childhood TB is typically low (40–83%). However, it appears, at least from some reports, that NAA identified a group of children who are clinically diagnosed with TB but in whom mycobacterial culture is negative. This means that with a proper technique it could be done efficiently (Nicol and Zar, 2011).

4.2.3. Adenosine deaminase

Adult studies have shown increased levels of adenosine deaminase (ADA) in pleural TB and TB-caused meningitis, both paucibacillary forms of TB, and have advocated for its use in diagnosis. Due to this evidence, a serum ADA has already been evaluated in a childhood population with a very high sensitivity (100%) and specificity (90.7%) for pulmonary TB. This study demonstrated the great potential of this technique because it has significant difference in serum ADA levels between children with disease and infection. However, there were several weaknesses in the study design, including unclear case definition, exclusion of nontuberculous patients, and a relatively small TB patient population (20 with active disease) (Marais. and Pai, 2007).

In the case of extrapulmonary TB, ADA measurement can be helpful, but its sensitivity and specificity varies widely and has been lower than multiplex PCR using primers for IS6110, dnaJ, and hsp65. Specifically, a meta-analysis of 63 studies of ADA in tuberculous pleuritis reveals that the sensitivity of the test is of 0.92 (95% CI 0.90–0.93) and specificity of 0.90 (95% CI 0.89–0.91) (Lawn and Nicol, 2011).

4.2.4. Serology and antigen detection

In absence of good diagnostic method for tuberculosis, the interest in serodiagnosis has been increased (Marais et al., 2005). Serological tests vary in a number of features, including antigen composition (38 kDa, Ag 60, and lipoarabinomannan, LAM), antigen source (native or recombinant), chemical composition (protein or lipid), extent of antigen(s) purification, and

immunoglobulin detected. The majority are based on the enzyme-linked immunosorbent assay (ELISA) rapid versions and use various immunochromatographic formats, with lateral flow being the most popular.

A recent review of serological tests concluded that commercial antibody detection tests for extrapulmonary TB have no role in clinical care or case detection (Steingart et al., 2007). The search for novel biomarkers in blood or urine that can reliably distinguish active from latent TB in children with and without other co-infections remains an important global goal. Well-defined cohorts of paediatric patients in TB-endemic and non-endemic settings will be essential for initial screening and future validation of such potential markers. In the meantime, the diagnosis of TB in children in resource-poor countries continues to rely on practical algorithms, which lack standard symptom definitions and adequate validation (Marais et al., 2006). This poses an increased challenge in the context of HIV infection

Imaz et al., (2001) reported the importance of the recombinant 16-kDa antigen (re-Ag16) of M. tuberculosis in the serodiagnosis of tuberculosis (TB) in children measuring the values of IgA, IgM, and IgG and an increased mean antibody response to reAg16 was observed in contact children compared with nonmycobacterial disease patient with a 95% of specificity. A combining result of the IgG and IgA assays led to 43% positivity in children with active TB (Trilling. et al., 2011).

Mycobacterial antigen detection has been evaluated in adults, but rarely in children. Serology has found little place in the routine diagnosis of tuberculosis in children, even though it is rapid and does not require specimen from the site of disease. Sensitivity and specificity depend on the antigen used, gold standard for the diagnosis of tuberculosis, and the type of tubercular infection. Though most of these tests have high specificity, their sensitivity is poor because several factors can alter the results such as age, exposure to other mycobacteria, and BCG vaccination (Marais et al., 2005).

4.2.5. In vitro interferon-γ (IFN-γ) release assays (IGRAs) and antigen-testing

In addition to the traditional TST, which is known to lack both sensitivity and specificity, blood based assays have recently become available. These T-cell assays rely on stimulation of host blood cells with MTB specific antigens and measure production of IFN-γ. Numerous published studies compare the two available commercial assays, T Spot TB (Oxford Immunotec) and Quantiferon-Gold IT (Cellestis), with the TST for both detection of active disease and LTBI (Ferrara et al., 2006). T-cell assays have proven to be more specific than the TST, (Arend et al., 2007) but they are still unable to distinguish between active disease and LTBI. Interpretation therefore remains dependent on the clinical context. Some few studies have presented paediatric data but none have provided an assessment of age-related performance of these assays, and reservations remain regarding their performance in very young children and in immunocompromised populations, such as those with HIV (Clark et al., 2007).

There is still a lot of on going research aimed at establishing the proper role of gamma interferon tests and the guidelines ar still under constant review. The interferon-γ release assays (IGRAs) currently commercially available include QuantiFERON-TB Gold (QFT-G),

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Quanti FERON-TB Gold In-Tube and T-SPOT.TB. These tests are aimed at he body's response to specific TB antigens not present in other forms of mycobacteria and BCG (ESAT-6). The tests are not affected by prior BCG vaccination, and despite their being new, these are now becoming available globally and CDC recommends that QFT-G may be used in all circumstances in which the TST is currently used, including contact investigations, evaluation of recent immigrants, and sequential-testing surveillance programs for infection control such as those for health-care workers. Health Protection Agency (HPA) recommends the use of IGRA testing in health care workers, if available, in view of the importance of detecting latently infected staff that may go on to develop active disease and come into contact with immunocompromised patients and the logistical simplicity of IGRA testing.

4.2.6. GeneXpert MTB/RIF system

GeneXpert includes the development of integrated DNA extraction and amplification systems. This requires minimal manipulation of sample and operator training. It utilizes real-time PCR (rt-PCR) technology to both diagnose TB and detect rifampicin resistance. The test amplifies a region of the rpoB gene of M. tuberculosis. Mutations of this region give rise to 95% of rifampicin resistance. Resistant strains contain mutations localized within the 81 bp core region of the bacterial RNA polymerase rpoB gene, which encodes the active site of the enzyme. In addition, the rpoB core region is flanked by Mycobacterium tuberculosis-specific DNA sequences. Thus, it is possible to test for M. tuberculosis and for rifampicin resistance simultaneously. The simplicity for the user makes this an assay that could feasibly be widely implemented outside centralized laboratories and potentially impacts on TB control (Gordetsov et al., (2008). The Xpert system has some advantages over the cultivation, mainly in specificity and a shorter time to get results (Imaz. et al., 2001).

Recently, Nicol et al., (2011), reported the application of this method in 452 hospitalized children from South Africa, with or without HIV, with a median age of 19.4 months, and suspected of having TB. Two Xpert tests doubled the case detection rate compared with smear microscopy (76% versus 38%), identifying all smear-positive and 61% of smear-negative cases, the specificity was 98.8%. The sensitivities for smear-negative TB were 33.3% and 61.1% when testing one or two samples, respectively. The samplings were induced sputum and they detected three quarters of culture-confirmed tuberculosis with very high specificity; the yield of this method was twice that of smear microscopy. This could suggest the possibility of replacing the microscopy for this type of methodology which has greater sensitivity especially with a second sample (Rachow et al., 2011).

4.2.7. Gas sensor array electronic nose (electronic nose)

The potential to detect different Mycobacterium species in the headspaces of cultures and sputum samples is another innovative approach that is currently in development. The array uses 14 sensors to profile a "smell" by assessing the change in each sensor's electrical properties when exposed to a specific odour mixture. In an initial study using sputum samples from patients with culture-confirmed tuberculosis and those without tuberculosis, the E-Nose correctly predicted 89% of culture-positive patients with a specificity of 91% (Imaz. et al.,

2001). In a further development applying advanced data extraction and linear discriminant function analysis, obtained sensitivities were of 68% and 75%, and specificities of 75% and 67% for Rob and Walter electronic noses, respectively (Imaz. et al., 2001). Further applications of this test, including its potential value in the diagnosis of child tuberculosis, are needed.

4.3. Diagnosing congenital TB

Congenital TB is rare but symptoms typically develop during the second or third week of life and include poor feeding, poor weight gain, cough, lethargy, and irritability. Other symptoms include fever, ear discharge, and skin lesions. The principles in place are that for one to make a definitive diagnosis of congenital TB, the infant should have proven TB lesions and that it should have at least one of the following: 1) skin lesions during the first week of life, including papular lesions (ulcerated areas of the skin) or petechiae (bleeding into the skin); 2) documentation of TB infection of the placenta or the maternal genital tract; 3) presence of a primary complex in the liver and 4) the possibility of postnatal transmission should be ruled out. Signs of congenital TB include failure to thrive, icterus (jaundice or yellow skin), hepatosplenomegaly (enlargement of both the liver and spleen), tachypnea (rapid breathing), and lymphadenopathy (involving inflammation of lymphnodes) (Marais et al., 2006). Patients with asymptomatic infection have a positive tuberculin skin test (TST) result, but they do not have any clinical or radiographic manifestations. Children with asymptomatic infection may be identified on a routine healthy-child physical examination, or they may be identified subsequent to TB diagnosis in household or other contacts (for example, children who recently have immigrated or adopted children). Primary TB is characterized by the absence of any signs on clinical evaluation. As discussed above, these patients are identified by a positive TST result. Tuberculin hypersensitivity may be associated with erythema nodosum and phlyctenular conjunctivitis (Marais et al., 2006).

Endobronchial TB with lymphadenopathy, which is the disease with enlargement of lymph nodes, is the most common variety of pulmonary TB. Symptoms are the result of impingement on various structures by the enlarged lymph nodes. Enlargement of lymph nodes and persistent cough may result in signs suggestive of bronchial obstruction or hemi-diaphragmatic paralysis, whereas difficulty in swallowing may result from esophageal compression. Vocal cord paralysis may be suggested by hoarseness or difficulty breathing and may occur as a result of local nerve compression. Dysphagia (swallowing problems) due to esophageal compression may also be observed. Pleural effusions due to TB may also occur and usually occur in older children and are rarely associated with miliary disease. The typical history reveals an acute onset of fever, chest pain that increases in intensity on deep inspiration, and shortness of breath. Fever usually persists for 14-21 days. Signs include: tachypnea, respiratory distress, decreased breath sounds, and, occasionally, features of mediastinal shift (moving of the tissues and organs that comprise the mediastinum) (Marais et al., 2006).

Progression of the pulmonary parenchymal component of TB leads to enlargement of the caseous area (caseated = cheese-like necrotised tissue) and may lead to pneumonia, atelectasis (collapse of lung tissue), and air trapping. This is more likely to occur in young children than in adolescents. The child usually appears ill with symptoms of fever, cough, malaise, and

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weight loss. This condition presents with classic signs of pneumonia, including tachypnea, nasal flaring, grunting, dullness to percussion, egophony or egobronchophony (increased resonance of voice sounds, with a high-pitched bleating quality, heard especially over lung tissue compressed by pleural effusion); decreased breath sounds, and crackles (Marais et al., 2006). Reactivation of TB disease usually has a sub-acute presentation with weight loss, fever, cough, and, rarely, hemoptysis (coughing up of blood or bloody sputum from the lungs or airway). This condition typically occurs in older children and adolescent and is more common in patients who acquire TB at age 7 years and older. Physical examination results may be normal or may reveal post-tussive crackles (Marais et al., 2006).

4.4. Diagnosis of extrapulmonary TB

In this case the clinical picture is used to get an indication of the diagnosis. The diagnosis at any site should be confirmed by obtaining specimens for bacteriology wherever possible. This means that fluid aspirated or biopsies taken should be placed in a medium such as saline which will not kill the bacteria. Too often still biopsy specimens are placed in formalin so that bacteriological confirmation including sensitivity testing cannot be done. Miliary TB may manifest sub acutely with low-grade fever, malaise, weight loss, and fatigue. A rapid onset of fever and associated symptoms may also be observed. History of cough and respiratory distress may be obtained. Physical examination findings include lymphadenopathy, hepatosplenomegaly, and systemic signs including fever. Respiratory signs may evolve to include tachypnea, cyanosis, and respiratory distress. Other signs, which are subtle and should be carefully sought in the physical examination, include papular, necrotic, or purpuric lesions on the skin or choroidal tubercles in the retina (Marais et al., 2006).

Patients with lymphadenopathy (scrofula or deposits in subcutaneous lymphatic ganglia) may have a history of enlarged nodes. Fever, weight loss, fatigue, and malaise are usually absent or minimal. One of the most severe complications of TB is TB meningitis, which develops in 5-10% of children younger than 2 years; thereafter, the frequency drops to less than 1%. A very high index of suspicion is required to make a timely diagnosis because of the insidious onset of the disease. A sub-acute presentation usually occurs within 3-6 months after the initial infection. Nonspecific symptoms such as anorexia, weight loss, and fever may be present. After 1-2 weeks, patients may experience vomiting and seizures or alteration in the sensorium (the part of the cerebral cortex that receives and coordinates all the impulses sent to individual nerve centers which includes auditory, gustatory, olfactory, somatosensory and visual centers). Deterioration of mental status, coma, and death may occur despite prompt diagnosis and early intervention.

Three stages of TB meningitis have been identified. Stage 1 is defined by the absence of focal or generalized neurologic signs. Possibly, only nonspecific behavioral abnormalities are found. Stage 2 is characterized by the presence of nuchal rigidity (inability or discomfort during neck flexion), altered deep tendon reflexes, lethargy (abnormal lack of energy), and/or cranial nerve palsies. TB meningitis most often affects the sixth cranial nerve due to the pressure of the thick basilar inflammatory exudates on the cranial nerves or to hydrocephalus; this results in lateral rectus palsy. The third, fourth, and seventh cranial nerves may also be affected. Funduscopic

changes may include papilledema (swelling of the optic disc from increased intracranial pressure) and the presence of choroid tubercles (chroid plexus = vascular proliferation of the cerebral ventricles that serves to regulate intraventricular pressure by secretion or absorption of cerebrospinal fluid).which should be carefully sought. Stage 3, the final stage, comprises major neurologic defects, including coma, seizures, and abnormal movements such as choreoathetosis (irregular involuntary movements that may involve the face, neck, trunk, extremities, or respiratory muscles, giving an appearance of restlessness), paresis (slight or incomplete paralysis), paralysis of one or more extremities. In the terminal phase, decerebrate (elimination of cerebral brain function) or decorticate posturing, opisthotonus (a type of spasm in which the head and heels arch backward in extreme hyperextension and the body forms a reverse bow), and/or death may occur. Patients with tuberculomas or TB brain abscesses may present with focal neurologic signs. Spinal cord disease may result in the acute development of spinal block or a transverse myelitis–like syndrome (an abnormal condition characterized by inflammation of the spinal cord with associated motor or sensory dysfunction). A slowly ascending paralysis may develop over several months to years.

4.5. Treatment

4.5.1. General treatment overview

Each of the first-line drugs makes a specific contribution during different periods of drug action (assuming complete drug susceptibility and the absence of significant immune compromise). Period 1 lasts 2 to 3 days (van der Weert et al., 2006), during which time fast-growing extracellular bacilli, comprising the vast majority of the organism load, are killed, mainly by the excellent bactericidal activity of isoniazid (INH) (Kampmann et al., 2005). Period 2 lasts 4 to 8 weeks. Slower growing extracellular bacilli are killed (van der Weert et al., 2006) and the rate of killing is determined more by the physiological state of the bacilli and less by the bactericidal activity of the drug. During this period, the bactericidal activity of rifampin (RIF) is important and pyrazinamide (PZA) contributes by killing extracellular bacilli that persist in acidic areas of inflammation (van der Weert et al., 2006). Period 3 lasts 4 to 6 months. Persistent intracellular bacilli are eradicated mainly by RIF, although INH will continue to offer protection against the development of resistance and may assist with organism eradication, especially in fibrocaseous tissue with poor drug penetration. Host immunity plays an important role throughout, but is of particular importance to effect organism eradication and prevent disease relapse, as indicated by the high relapse rate in HIV-infected children.

Practical operational issues are extremely important for effective public health intervention. Operational issues include access to early and accurate diagnosis, the uninterrupted provision of quality-assured drugs and appropriate treatment regimens, as well as the establishment of systems to ensure good treatment adherence. Fixed-dose combinations should be used whenever possible to reduce the risk of drug resistance and to improve simplicity and adherence, but quality assurance is essential to ensure optimal bioavailability of all the constituent drugs (Dekker and Lotter, 2003). With proper implementation, the World Health Organization's directly observed therapy, short-course (DOTS) strategy addresses most of the

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important operational issues. However, the predominant emphasis of the DOTS strategy on sputum smear–positive disease excludes the vast majority of children. There is a desperate need to improve service delivery to children with tuberculosis, particularly in endemic areas with limited resources (Starke, 2002).

4.5.2. Preventive chemotherapy

Chemoprophylaxis refers to preventive treatment given after exposure (without proof of infection), whereas treatment of latent infection implies that infection (indicated by a positive TST) was documented. The term preventive chemotherapy is preferred because it is more inclusive and incorporates both chemoprophylaxis and treatment of latent infection. The TST is a fairly accurate measure of infection after exposure in immune-competent children, although TST conversion, which reflects a sufficiently strong delayed-type hypersensitivity response, may be delayed for up to 3 months (Marais et al., 2004). Therefore, household exposure, particularly involving high-risk children, should be treated as infection until the absence of infection can be convincingly demonstrated. In immune-competent children this can be done by repeating the TST 3 months after exposure ended (American Thoracic Society, 2000). In immunocompromised children the TST is not a sufficiently reliable test to exclude M. tuberculosis infection and children with documented exposure should receive preventive chemotherapy as if they are infected (Marais et al., 2006).

The reality on the ground is that most endemic areas do not have the capacity to follow current World Health Organization guidelines regarding the use of preventive chemotherapy in children, which advise active tracing and screening of all children less than 5 years old in household contact with a sputum smear–positive adult source case. This results mainly from the huge burden of adult tuberculosis and resource constraints that limit the ability to perform TST and chest X-ray screening tests. Because the TST and chest X-ray are regarded as prerequisite screening tests, screening of exposed children and the provision of preventive chemotherapy are not even attempted in most resource-constrained areas. Access to preventive chemotherapy in these settings may be improved by employing symptom-based screening, although the benefits and risks of such a simplified approach require further evaluation. A study from an endemic area indicated that symptom-based screening may identify those children who require further investigation to exclude active tuberculosis (Marais et al., 2006), thus allowing asymptomatic household contacts, especially those who are at high risk to progress to disease, immediate access to preventive therapy despite the inability to perform TST and chest X-ray–based screening (Marais et al., 2006).

Another consideration is that in some endemic areas the majority of disease transmission, particularly in children greater than 2 to 3 years of age, occurs outside the household (Verver et al., 2004). In endemic areas, narrowing the focus of contact tracing to those children who are at highest risk to progress to disease after exposure or infection (less than 3 years of age and/or immune compromised) will decrease the burden placed on already overstretched health care systems, while still ensuring access to preventive chemotherapy for the children who need it most (Van Zyl et al., 2006). In older (greater than 3 years of age), immune-competent children the risk of tuberculosis after exposure is low and disease progression is

usually indicated by the presence of persistent, slowly progressive symptoms. Therefore, passive case finding together with adequate diagnostic vigilance seems appropriate in this low-risk group.

In non endemic areas where resources permit and where the risk of future reinfection is low, it seems warranted to extend preventive chemotherapy to low-risk children as well, to eliminate the reservoir of latent infection within the community. INH monotherapy for 6 to 9 months is the best-studied chemoprophylactic regimen and it reduces the tuberculosis risk in exposed children by at least two-thirds; probably by more than 90% with good adherence. However, poor adherence is a major concern, particularly in endemic areas (Van Zyl et al., 2006).

In real life the effectiveness of a preventive chemotherapy regimen is determined first by its efficacy and second by adherence to the prescribed regimen. Because of documented poor adherence to 6–9 months of unsupervised INH monotherapy, consideration should be given to alternative preventive strategies with comparable efficacy but with improved adherence. Theoretically the addition of RIF has important advantages; RIF has strong sterilizing activity to eradicate latent bacilli and its addition will shorten the duration of treatment required (Mitchison, 2005). It will also improve efficacy in settings where INH mono-resistance is prevalent. The use of a 3-month INH and RIF regimen for preventive chemotherapy is well established and trials have shown equivalence to 6 to 9 months of INH alone, although the evidence is not as comprehensive as that for INH monotherapy (Ena and Valls, 2005).

PZA is another important sterilizing drug and in theory the combination of RIF and PZA represents the treatment of choice for latent infection. This combination has proven efficacy in animal but adverse reactions in adults have limited the initial enthusiasm (Priest, 2004). However, these adverse reactions have not been observed in children, in whom the three-drug combination of INH, RIF, and PZA is generally well tolerated (Marais et al., 2006). Adherence may be improved by shortening the duration of treatment, but consideration may also be given to the provision of supervised preventive therapy. Creative approaches will be required to achieve this, particularly in places where health care services are already overburdened. With curative treatment, intermittent (two or three times weekly) therapy during the continuation phase is as effective as daily therapy to achieve organism eradication, once the organism load has been sufficiently reduced (Al-Dossary et al., 2002). The same principle would apply to the treatment of latent infection, where the organism load is low. Targeting high-risk children for short-course, supervised intermittent preventive therapy seems achievable, but defining optimal preventive therapy regimens remains a fertile and important area for future research (Marais et al., 2006).

Vaccination with BCG is the most widely used preventive strategy, although its efficacy remains controversial and studies have shown that it contributes to this variable protection: variations in strain-specific immunogenicity, timing and technique of vaccine administration, genetic factors, the presence or absence of environmental mycobacteria, and the effect of multiple re-infection events as may occur in highly endemic areas. It is generally accepted that BCG vaccination offers significant protection against disseminated disease in young children (below 2 years), but that it offers little or no protection against adult-type tuberculosis.

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However, reports have documented significant protection against the development of adult-type tuberculosis when BCG was administered to TST-negative adolescents in locations with a low prevalence of environmental mycobacterial exposure (Bjarveit et al., 2003).

In addition, a report from Turkey indicated that contrary to the prevailing theory, BCG may also protect against M. tuberculosis infection as based on a positive enzyme-linked immunospot result. An even more controversial area is the risk versus benefit that BCG provides to HIV-infected children. There is a definite risk for HIV-infected infants to develop severe forms of BCG disease after neonatal BCG vaccination (Hesseling et al., 2006), but it remains poorly quantified. As the risk:benefit ratio has not been determined, the World Health Organization still advises BCG vaccination of asymptomatic HIV-exposed infants in tuberculosis endemic areas. Establishing the risk: benefit ratio of BCG vaccination in HIV-infected infants and the development of novel vaccines with improved efficacy and safety, remain major research challenges (Marais et al., 2006).

4.5.3. Curative treatment

The main variables that influence the success of chemotherapy, apart from primary drug resistance, are the bacterial load and the anatomic distribution of bacilli. Cavitary disease indicates a high bacterial load, as demonstrated by the frequency with which these patients are sputum smear–positive, which implies an increased risk for random drug resistance against individual drugs. Disseminated disease may signify penetration of bacilli into the central nervous system (CNS) (Van den Bosch et al., 2004) implying that adequate drug penetration across the blood–brain barrier is an important requirement for the treatment of disseminated disease (Marais et al., 2006).

From a public health perspective the challenge is to develop a pragmatic classification of childhood tuberculosis that incorporates the diverse spectrum of disease, but focuses primarily on treatment relevance. The main variables that influence the success of chemotherapy identify three groups of children with tuberculosis: (1) those with sputum smear–negative disease, (2) those with sputum smear–positive (often cavitary) disease and (3) those with disseminated disease. The discussion reflects current treatment guidelines for these three groups as well as the new regimens to consider on the basis of established treatment principles (Marais et al., 2006).

As a guide for individual patient classification and management five simple questions have been formulated: (1) Is the child exposed to or infected with M. tuberculosis? (2) Does the child have active tuberculosis? (3) If the child is exposed or infected, but does not have active tuberculosis, is preventive chemotherapy indicated? (4) If the child has active tuberculosis, what is the appropriate treatment regimen? (5) Are there any special circumstances such as HIV infection, retreatment, or exposure to a drug-resistant source case to consider? The underlying rationale is universally applicable irrespective of diagnostic or resource constraints; although areas with access to advanced technology may achieve improved levels of diagnostic certainty.

Sputum smear–negative disease is usually paucibacillary and therefore the risk of acquired drug resistance is low. Drug penetration into the anatomic sites involved is good and the success of three drugs (INH, RIF, and PZA) during the 2-month intensive phase, and of two drugs (INH and RIF) during the 4-month continuation phase, is well established. In the presence of extensive radiographic disease with or without cavitation, and/or suspicion of INH resistance, the use of ethambutol (EMB) in addition to the three drugs during the intensive phase should be contemplated. After completion of the intensive phase, successful organism eradication may be achieved with intermittent (two or three times weekly) therapy during the continuation phase (Al-Dossary et al., 2002). The efficacy of shorter treatment durations for HIV-uninfected immune-competent children with sputum smear–negative disease requires further evaluation, as a 4-month regimen of INH and RIF may be an acceptable therapy for some adults with sputum smear– and culture-negative tuberculosis.

Sputum smear–positive disease implies a high organism load and an increased risk for random drug resistance against individual drugs. Selecting drug-resistant mutants is a particular concern where INH mono-resistance is prevalent, as this increases the likelihood of selecting multidrug-resistant (MDR) organisms. The use of four drugs (INH, RIF, PZA, and EMB) during the 2-month intensive phase should reduce this risk. Once the organism load is sufficiently reduced, intermittent (two or three times weekly) therapy with INH and RIF during the 4-month continuation phase is sufficient to ensure organism eradication (Al-Dossary et al., 2002). However, caution should be exercised when initial treatment response has not been optimal and in HIV-infected patients. The use of long-acting rifamycins together with INH is discouraged (Rieder et al., 2001).

Disseminated disease is frequently associated with CNS involvement (Donald et al., 2005). It is therefore essential to consider the cerebrospinal fluid (CSF) penetration of drugs used in the treatment of disseminated disease. INH and PZA penetrate the CSF well. RIF and streptomycin penetrate the CSF poorly, but may achieve therapeutic levels in the presence of meningeal inflammation. The value of streptomycin is limited by poor CSF penetration and intramuscular administration. EMB hardly penetrates the CSF, even in the presence of meningeal inflammation, and has no demonstrated efficacy in the treatment of TBM. Ethionamide shows good CSF penetration and has been used successfully as a fourth drug in the treatment of TBM. The fact that RIF penetrates the CSF poorly in the absence of meningeal inflammation reduces its sterilization value and may warrant the inclusion of PZA during the continuation phase, to assist with CNS sterilization.

Several reports have illustrated the efficacy of short-course regimens in the treatment of TB meningitis, but the risk of CNS relapse is rarely reported. In two of these studies a relapse was documented despite the completion of 6 months of treatment with INH and RIF with an initial 2 months of PZA. Therefore, it seems prudent to include a fourth drug with good CNS penetration (such as ethionamide) for the treatment of disseminated disease, at least during the intensive phase, and to consider PZA for the full 6 months of treatment to reduce the risk of CNS relapse. CNS relapse is rare in the United States, where PZA is routinely discontinued after 2 months, but the total treatment duration is 9 to 12 months. Current fixed-dose combination tablets provide 4 to 6 mg of INH per kilogram. This dose may be suboptimal, particu-

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larly in settings where the majority of the bacterial population rapidly acetylates INH (Schaaf et al., 2005). In addition, the serum level achieved with a similar dose of INH per kilogram is lower in children than in adults, increasing the risk for suboptimal dosing in children (Schaaf et al., 2005). The majority of new INH resistance encountered in endemic areas is of an intermediate or low level, which underscores the importance of optimal INH dosing (Donald et al., 2004). A standard INH dose of 10 mg/kg seems appropriate in children, as even doses up to 20 mg/kg are well tolerated (Schaaf et al., 2005); children are less susceptible to the toxic effects of INH than are adults.

In general, adverse events are less common in children than in adults. The most severe adverse event is the development of hepatotoxicity, which can be caused by INH, RIF, PZA, or ethionamide. An elevation of liver enzymes (less than five times normal values) is not an indication to stop treatment, but the occurrence of liver tenderness, hepatomegaly, or jaundice should prompt the immediate stopping of all potentially hepatotoxic drugs. Jaundice is often preceded by a period of days or weeks of malaise and nausea. Hepatic reactions usually occur in the first weeks of therapy, but may happen at any time during the treatment period. Drug-related hepatic toxicity is usually caused by a single drug, but rarely a combination of drugs, which individually cause no problem, may cause hepatic toxicity. Children should be screened for other causes of hepatitis, as in many cases the anti-tuberculosis drugs are not the cause of liver function derangement. In South Africa, hepatitis A infection is frequently responsible for non–drug-related liver function derangement in children receiving anti-tuberculosis treatment. Potentially hepatotoxic drugs should be reintroduced only after liver functions have normalized. Non-hepatotoxic drugs should be used in the interim and expert opinion should be sought.

Ethambutol is usually not advised in children less than 7 years as visual acuity cannot be evaluated. However, its use may be warranted in children with hepatotoxicity, cavitary disease, or resistance to first-line drugs; it seems safe at recommended dosages. Ethionamide frequently causes vomiting, but this can usually be overcome by dividing the daily dose and by a slow increase up to the full dose during the first week or two of therapy. Recommended dosages for the various first- and second-line drugs are reflected in the publication by Marais et al., (2006) as indicated in the Table 1 below:

Despite significant symptomatic improvement radiographic disease resolution may take many months; persistent radiographic signs are not an indication to change treatment if there is clinical improvement. Paradoxical exacerbation of symptoms or signs may also occur after anti-tuberculosis therapy is initiated. This results from immune reconstitution with increased inflammation, particularly surrounding diseased lymph nodes or tuberculomas, that may follow nutritional rehabilitation (Marais et al., 2004), and/or antiretroviral therapy. The release of bacterial toxins after successful anti-tuberculosis treatment may also contribute.

Treatment should be continued unaltered, although the temporary addition of corticosteroids may be considered. Such adjunctive therapy may be helpful in a number of disease manifestations where the host inflammatory response contributes to disease pathology such as CNS involvement, severe lymph node compression of the airways, and pericardial effusion. There

		Maximum Dosage (mg/kg/dose)	
	Mode of Action	**Daily**	**Two or Three Times/wk**
First-line drugs			
Isoniazid	Bactericidal	10–15 (300 mg)	20–30 (900 mg)
Rifampin	Bactericidal and sterilizing	10–20 (600 mg)	10–20 (600 mg)
Pyrazinamide	Sterilizing	20–40 (2,000 mg)	50 (2,000 mg)
Ethambutol	Bacteriostatic	15–25 (1,200 mg)	30–50 (2,500 mg)
Second-line drugs			
Ethionamide or prothionamide	Bactericidal	15–20 (1,000 mg)	NA
Streptomycin	Bacteriostatic	20–40 (1,000 mg)	NA
Fluoroquinolones	Bactericidal		NA
Ciprofloxacin		20–40 (1,500 mg)	
Aminoglycosides	Bacteriostatic		NA
Kanamycin		15–30 (1,000 mg)	
Amikacin		15–30 (1,000 mg)	
Capreomycin		15–30 (1,000 mg)	
Cycloserine or terizidone	Bacteriostatic	10–20 (1,000 mg)	NA
Para-aminosalicylic acid	Bacteriostatic	200–300 (10 g)	NA

NA = not applicable. Source: Marais et al., (2006).

Table 1. First- And Second-Line Antituberculosis Drugs And Recommended Dosages In Children

is insufficient evidence to demonstrate whether steroids are effective in tuberculous pleural effusion.

4.5.4. Retreatment

Anti-tuberculosis treatment rarely fails in children and, if it does, every effort should be made to find the most likely cause. In settings where the prevalence of drug resistance is low the commonest cause is failure to properly take the medications, which can occur even during DOT, if supervision is not complete. It is important to remember that non-adherence has a differential diagnosis; there are psychologic, sociologic, religious, economic, and practical reasons why people are non-adherent and one must deal with all these issues for chemotherapy to be successful. With treatment interruption the child may be restarted on the original treatment regimen while ensuring adequate supervision, as the risk of developing drug resistance is small in children with paucibacillary disease. If an immune-competent child presents with a new episode of tuberculosis more than 6 months after completing treatment

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for a previous episode, then it most likely represents re-infection disease and standard first-line treatment is appropriate. In the case of genuine treatment failure (absence of clinical response to supervised treatment) drug susceptibility testing is of paramount importance. If an adult source case is identified with drug-resistant tuberculosis, the child should be treated according to the drug susceptibility pattern of the source case's strain (Marais et al., 2006).

4.5.5. Treatment of paediatric TB/HIV co-infection

The high risk of HIV-infected children to progress to disease after infection justifies the use of preventive chemotherapy in children who are latently infected. However, the difficult issue in endemic areas is how to deal with the ever-present risk of undocumented re-infection within the community. The prevention or reversal of severe immune compromise by using highly active antiretroviral therapy (HAART) should preclude the need for repeated or continuous preventive chemotherapy, although the risk for tuberculosis probably remains higher than in HIV-uninfected children. The cellular immune response assists with organism eradication and therefore it is not unexpected that disease relapse has been documented in HIV-infected children. The value of prolonging the treatment duration from 6 to 9 months, to ensure organism eradication in HIV-infected children, is under investigation. During a repeat episode both relapse and reinfection should be considered and every effort should be made to establish a culture-confirmed diagnosis and to do drug susceptibility testing (Marais et al., 2006).

When initiating treatment (curative treatment or RIF-containing preventive therapy) in HIV-infected children already receiving HAART or for whom HAART is contemplated, it should be appreciated that the rifamycins, especially RIF, and some of the nonnucleoside reverse transcriptase inhibitors and/or protease inhibitors may cause significant drug interactions. HIV-infected children may also develop particularly pronounced paradoxical reactions after the institution of HAART, because of immune reconstitution inflammatory syndrome. Recommendations on optimal drug combinations are frequently revised. The most recent recommendations can be obtained from the Centers for Disease Control and Prevention website, at [http://www.cdc.gov/nchstp/tb/].

Latest WHO recommendations advise starting antiretroviral therapy (ART) once anti-TB therapy (ATT) is established (after a period of 2-8 weeks) for all WHO clinical Stage Four HIV-infected children and Stage Three children with advanced or severe immunosuppression. For children in WHO clinical stage with mild or no immunosuppression, ART may be deferred until 6 months of ATT are completed (WHO, 2006). On-going prospective trials involving adults and children in TB/HIV endemic countries might provide future guidelines for the ideal timing of the initiation of anti-retroviral therapy (ART) in patients with HIV receiving TB therapy. There is already evidence from prospective trials that shows that high mortality is associated with TB in advanced stages of HIV-disease in children who do not receive ART promptly. Further research is required to improve our understanding of immune reconstitution disease (IRD) in children (Walters et al., 2006). Also, therapeutic drug monitoring (TDM), where available, should be undertaken when children are receiving concomitant ART and ATT. TDM data from ethnically similar children in resource-rich countries may in the future inform dosing recommendations in resource-poor settings where TDM is not available.

4.5.6. Treatment of extrapulmonary paeditric PTB

Treatment is as for pulmonary disease, with isoniazid, rifampicin, pyrazinamide and ethambutol for two months followed by isoniazid and rifampicin for four months, except for CNS disease when treatment should be continued for a full year. Steroids may be used in pericardial and meningeal disease. Surgery is usually unnecessary, especially where lymph glands and abscess are present, as long term discharging sinuses may result. Surgery is sometimes necessary in spinal TB where there is instability and may be needed to overcome strictures in genito-urinary or gastro-intestinal disease. Occasionally pericardectomy may be required when pericardial disease causes tamponade.

4.5.7. Treatment of latent paediatric TB infection

Treatment of LTBI, also known as chemoprophylaxis, is important to prevent future disease activation. The fact that over 50% of hospitalized children with culture-confirmed TB have a reported close TB contact and do not receive chemoprophylaxis, is an indication of the important missed opportunities using existing public health interventions. For the last 20 years the WHO guidelines recommended all children under 5 years in close contact with an infectious (usually smear positive) case receive 6 months isoniazid. Once active disease has been excluded, isoniazid monotherapy for 6-9 months has been proven to reduce the TB risk in exposed children by over 90% with good adherence. More recent studies suggest that 3 months of combined isoniazid and rifampicin are equally effective (Ena and Valls, 2005). In a recent study with very short follow-up, continuous isoniazid prophylaxis for HIV-infected children without documented evidence of latent infection, but living in an environment of high exposure, has also been shown to reduce overall morbidity and mortality from TB and other infections (Zar et al., 2007). Further trials in HIV-infected children receiving ART are ongoing.

Recommendations for chemoprophylaxis will continue to differ in TB-endemic and non-endemic settings, because of the perceived risk of exposure. Whilst most paediatricians in Europe and North America would advocate chemoprophylaxis for HIV infected, TB-exposed children only, this needs to be interpreted with caution if the exposure is potentially ongoing or recurrent, and the ability to distinguish LTBI from active disease is limited. In this context, many practitioners in TB-endemic settings are reluctant to place children on chemoprophylaxis because of the potential emergence of resistant strains, if indeed the child has active disease instead of LTBI.

4.5.8. Treatment of drug resistant paediatric TB

Acquisition of resistance rarely occurs in children due to the paucibacillary nature of their disease but overall, children may also be subject to less selection pressure from anti TB therapy. Thus most resistance in children is due to primary transmission of a resistant organism, and MDR /XDR-TB rates in children reflect community transmission rates. Diagnosis requires a high index of suspicion as the culture yield in children makes definitive microbiological confirmation difficult. Resistance should be suspected if an index case has known resistant TB; the child shows initial improvement on anti-TB therapy and then deteriorates; or there is no

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response to initial treatment. Acquired resistance is well described in HIV co-infected adults previously treated for TB, possibly due to malabsorption of anti-TB drugs (Wells, et al., 2007). The presence of acquired resistance in the paediatric population is reported and in particular children with TB/HIV co-infection should be closely monitored (Soeters et al., 2005).

Although the principles of DOTS Plus have been put forward for the management of MDR TB, at the moment, there is no consensus for any regimen or optimal treatment that should be used for persons with known exposure to MDR-TB. The recommendation by CDC is a combination of pyrazinamide and ethambutol, with either pyrazinamide or a fluoroquinolone and that immunocompetent contacts should be treated for 6 months while immunocompromised contacts should be treated for 12 months. Current guidelines recommend using at least four drugs to which the patient is naïve, including an injectable and a fluoroquinolone, in an initial phase of at least 6 months; followed by at least three of the most active and best tolerated drugs in a 12-18 month continuation phase.

Standardised regimens have been developed for settings where drug susceptibility testing is not available (WHO, 2006). Six classes of second-line drugs (SLDs) are available (WHO, 2003) but experience in children is limited for the majority and multi-centre paediatric trials are needed. Under optimum circumstances MDR-TB responds well to appropriate therapy. However delays in diagnosis and treatment, adherence issues, and a lack of child-friendly formulations and strategies for DOTS all frequently complicate management and contribute to a high morbidity and mortality (Drobac et al., 2006).

The WHO currently recommends avoidance of chemoprophylaxis in cases of contact with known MDR-TB and to observe for 2 years if clinically asymptomatic. Children with latent MDR-TB infection become the reservoir for future transmission following disease reactivation in adulthood, emphasizing the need to further research and improved management of MDR-TB infection in children, both at the clinical and operational level.

According to the European Centre for Disease Prevention and Control (ECDC) 2012 Guidelines, there are two valid options to consider for the management of MDR TB and XDR TB contacts; preventive treatment or follow-up by careful clinical observation. The purpose of preventive therapy is to prevent the progression of LTBI to TB disease in an individual who has been exposed to MDR/XDR TB. The concept of preventive therapy has been shown to be effective for LTBI after contact with drug-susceptible TB but corresponding evidence for preventive therapy of MDR TB and XDR TB contacts is very scarce. Although for children there are indications of a positive effect of preventive therapy, for other groups of contacts, the necessary body of evidence has yet to be generated, and there are ongoing studies to collect evidence in support of the use of preventive therapy in contacts of MDR TB cases.

There is currently no evidence available on the optimal follow-up time in contacts of MDR TB or XDR TB with regard to patient benefits and costs of the intervention. In young children under five years of age the majority (over 90%) of TB disease will develop within 12 months of infection. Infants and children under five years of age, immunocompromised individuals due to HIV infection or TNF-antagonist treatment are at increased risk of progression from LTBI to TB disease. These individuals as well as other

identified risk groups require special attention as part of the individual risk assessment (WHO, 2007; Salgado and Solovic et al., 2010).

The optimal duration of MDR-TB treatment in children is not known. World Health Organization guidelines recommend treatment until 18 months after the first negative culture (24 months in XDR-TB). As children often have paucibacillary disease, documenting a culture conversion is usually difficult. Thus, the same duration as in adults would apply. The duration of the intensive phase of treatment (when an injectable drug is given) should be at least 6 months. Surgical resection should be considered when the patient has localized lesions and has persistently positive smear or culture results inspite of aggressive chemotherapy (Shah, 2012).

4.5.9. BCG vaccination and HIV infection

Approaches for prevention of TB include prevention of infection (through immunization) or of progression from latent infection to disease (chemoprophylaxis). Bacille Calmette-Guérin (BCG) vaccine, a live attenuated vaccine derived from Mycobacterium bovis that was developed in the 1920s, is administered to children at birth in many countries. WHO guidelines recommend administration of BCG soon after birth to all infants in countries with a high TB prevalence. Current WHO guidelines advise that all children below 5 years of age, who are in close contact with a sputum smear-positive index patient, should be actively traced, screened for TB, and provided preventive chemotherapy after active TB has been excluded (Marais et al., 2004).

Although this is good policy, implementation is fraught with challenges, including difficulty in diagnosing latent TB in a highly BCG-vaccinated population, ruling out incipient active disease, and the lack of procedures for documentation and follow-up of contact screening and chemoprophylaxis in national programs. Because the majority of transmission in children below 3 years of age occurs in the household and they are also the group at highest risk of progression to disease after primary infection, this activity should be given higher priority in national infection-control programs. Moreover, active tracing and screening of household contacts at high risk would allow children with disease to receive a diagnosis earlier, thus reducing complications.

Furthermore, additional protection by revaccination with BCG has not been demonstrated (Rodrigues et al., 2005). To date, the efficacy of the BCG vaccination has not been determined in HIV infected individuals in whom the immune responses to BCG may be reduced, (Hesseli et al., 2007) although this is the subject of ongoing trials. Due to the risk of disseminated BCG disease which may rarely complicate use of this live vaccine in immunocompromised individuals, BCG vaccination is no longer recommended in children known to be HIV-infected (Hesseling et al., 2007). In practice, this has had little impact in HIV-endemic countries, where the HIV-status of the baby is rarely established at birth, the usual time of BCG vaccination.

A large trial in southern India that included over 350,000 participants aged above 1 year concluded that BCG vaccine did not offer protection against the development of adult pulmonary TB (WHO, 2006). However, BCG vaccine has been shown to be protective against

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disseminated forms of TB in young children, with a protective estimate ranging from 67%–79% against TB meningitis and 58%–87% against miliary disease. A theoretical model estimated that a universal BCG vaccine program would have a beneficial impact in settings with prevalence rates of greater than 30 sputum smear-positive cases/100,000 population (WHO, 2007). However, there is no evidence of any BCG-induced protective effect in HIV-infected children. On the contrary, studies have documented BCG-induced disseminated disease and adverse reactions. Therefore, the WHO recommendations have been revised, making HIV infection a contraindication for BCG vaccination, even in settings where TB is highly endemic. Strategies required for effective implementation of this policy change include high uptake of maternal HIV testing coupled with implementation of proven strategies to prevent mother-to-child HIV transmission, including maternal treatment with HAART and early virological diagnosis of HIV infection in infants, followed by treatment.

The revised recommendations present a dilemma for national programs. Although the benefits of BCG vaccine far outweigh the risk among HIV-uninfected children living in high areas with a high prevalence of TB, the risk is higher among HIV-infected infants with or without symptoms at the time of vaccination. National recommendations will need to consider a variety of factors, including the prevalence of TB in the population, the prevalence of HIV infection, the availability of HIV testing and facilities for prevention of mother-to-child transmission during pregnancy, the capacity to conduct follow-up of vaccinated children, and the availability of early infant diagnosis of HIV infection. Abandoning the use of BCG vaccine before newer vaccines become available may put millions of young children at risk of TB. There is an urgent need for operational research in TB endemic countries to determine the best way to manage this issue programmatically.

5. On-going research targeting paediatric TB

5.1. New vaccine pipelines

The global commitment of the WHO and the Stop TB (WHO, 2005) campaign has spurred on the efforts of the international research community to develop a more effective anti-TB vaccine by the year 2015. In view of the proven efficacy of existing BCG vaccine in preventing disseminated TB in children and reducing child mortality (Roth et al., 2006) two conceptually different strategies have been pursued: firstly, the development of 'priming vaccines', which, it is hoped, will replace BCG by providing better and longer protection; secondly, the design of 'booster vaccines' to boost pre-existing BCG-derived immunity. Novel vaccines currently under development all use a "booster-strategy" after priming with BCG in infancy (Doherty et al, 2007). As the current candidates are progressing through phase I and II trials, including studies in HIV-infected individuals and age-de-escalation, it is most likely that more than one vaccine will progress into phase III.

The most advanced vaccine candidate is MVA- 85A, currently in phase II under a prime-boost strategy with BCG. Four products are in phase I (72f, Hybrid 1, Aeras 402, rBCG-UreC-Hly), each stemming from PPPs. Many of the candidates are results from the EU FP6 projects, i.e.

TBVAC and Muvapred, where valuable progress has been achieved. Several other candidates are still in the pre-clinical phase. For example, mutation of virulence genes produced a TB strain potentially conferring greater protection with fewer side effects than BCG. In addition, an improved, recombinant BCG vaccine with a higher efficacy and a better safety profile moving into phase I clinical trials is a possible prospect.

New research is directed at the development of a multistage TB vaccine containing latency antigens, an attractive concept, which is actively being pursued (Andersen, 2007). Such a vaccine could be used as a booster vaccine with the goal of preventing new infections in those uninfected with MTB and to prevent reactivation in those with LTBI. Unfortunately, the lack of reliable correlates of protective immunity currently remains a major obstacle to predict vaccine efficacy in all TB vaccine trials for both adults and children.

6. Existing research gaps

6.1. Research needs

Tracing of MDR TB contacts is important to prevent TB disease and further transmission. Priority studies needed include those to identify the most effective contact-tracing procedures for close contacts and the most effective follow-up procedures in healthcare workers constantly exposed to MDR TB. As part of the management of MDR TB contacts, studies on specific groups are needed, for example on children below the age of five years, children with HIV infection and other immunocompromised states. In particular, studies are needed: 1) for treated contacts: (randomised) clinical trials: 2) to determine which drugs and which drug combinations and dosages are optimal for preventive therapy; 3) to determine the duration of preventive therapy; 4) to assess the effectiveness of preventive therapy in conjunction with antiretroviral treatment; 5) to assess the risk of development of new drug resistance in contacts receiving (inadequate and adequate) preventive therapy; 6) for untreated contacts, and healthcare workers constantly exposed to MDR TB: 7) to identify the optimal follow-up period for different groups of individuals; and 8) to identify the optimal frequency of testing for LTBI during the follow-up period.

In order to increase adherence to treatment of MDR/XDR TB contacts (and reduce the risk of development of new drug resistance in contacts), studies are needed: 1) to identify new drugs with less adverse events and to explore possible (positive and negative) interactions between combined drugs; 2) to identify biomarkers indicating the risk of progression from LTBI to TB disease and 3) to assess operational management to shorten preventive therapy. Since the provision of preventive therapy has economic and logistic implications at the national and community level, cost-effectiveness and cost-benefit studies are also needed. These studies are particularly valuable because they can help to inform the decision on intervention policies..

A substantial amount of funding has been injected into research on various aspects of TB but there are still many issues that require additional research especially in the area of childhood tuberculosis. The most salient ones include:1) accurately quantifying the global burden of

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childhood tuberculosis especially in the endemic areas; 2) improving the understanding of the disease interactions with the immune system and re-evaluating the role of BCG and the new vaccine candidates in protecting children and adults against TB; 3) defining the diagnostic contribution of novel T-cell–based assays in endemic and non-endemic areas especially with regard to diagnosis of paediatric tuberculosis; 4) identifying new ways of diagnosing childhood tuberculosis in HIV negative and in TB/HIV co-infection in children, particularly in resource-limited settings; 5) carrying out operational research aimed at improving the access of children in endemic areas to preventive therapy and treatment, using the existing DOTS/DOTS Plus frameworks; 6) evaluating the efficacy of new short-course intermittent preventive chemotherapy regimens especially those aimed at childhood TB; 7) exploring shorter durations of treatment in immune-competent children with smear-negative disease; 8) defining the optimal treatment regimen and treatment duration in children with TB/HIV co-infection; 9) monitoring the impact of MDR and XDR tuberculosis on children and evaluating regimens for effective MDR/XDR disease prevention and treatment; 10) developing and evaluating new drugs that may shorten the treatment duration and/or assist with the treatment of MDR/XDR disease and emphasizing case finding and reporting as some of the strategies to combat the escalation of XDR-TB [http://ec.europa.eu/research/research-eu].

6.2. Need for more specific diagnostic tests

In the field of diagnosis, there is an urgent need to replace sputum microscopy the current gold standard test, with more sensitive tests that are applicable at point of care. Despite the fact that the technique can only pick up 60% of cases, it has been in use for over a hundred years. Furthermore, sputum culture is not suitable for extrapulmonary TB and for paediatric TB since children can not produce sputum. On the other hand, the newer immunological based tests such as IGRAs are not well suited for use in TB/HIV co-infection and in high burden TB areas, where they cannot be accurately used to distinguish active from latent TB. Since the majority of the infected people never actually develop the disease, there is need to have a diagnostic tool which is able to distinguish latent from active disease and help to identify healthy individuals from diseased ones. Improved diagnostics are critical to TB care and control.

The need for serious investment in the critical areas especially in new TB diagnostic tools, drug susceptibility testing, and development of new biomarkers to enable health providers detect TB disease activity and to determine follow up treatment outcomes cannot be over emphasised. The fact that a number of new diagnostic tools are in the pipeline, including culture-based tests to identify M. tuberculosis and those used to determine drug resistance based on molecular assays and immune response is good news. However, there is still need to ensure that the new tests can be availed world-wide and be used at the point-of-care even in resource-poor settings, where there may be limited technical expertise and the necessary equipment. [http://ec.europa.eu/research/research-eu]

6.3. Newer biomarkers for TB disease activity, cure and relapse

There are three major reasons that can be used to justify the need for new TB biomarkers : 1) a diagnostic test which is able to differentiate between healthy individuals with a latent TB

infection and patients with active dsease is needed; 2) a prognostic test which can be able to predict the risk of latent TB becoming active needs to be established; 3) there is need for a diagnostic test that can be used to serve as a surrogate endpoint of disease which can be used for monitoring drug and vaccine trials in TB. It is envisaged that the basis for these novel diagnostic measures will be biomics, comprising metabolomic, proteomic and transcriptomic profiles in custom-made biosignature. Identification of non invasive biomarkers, especially the molecular assay of M. tuberculosi fragments in urine and the measurement of volatile biomarkers of volatile organic compounds generated by Mycobacteria TB in the breath or the oxidative stress resulting from infection is one step in the right direction. [http://ec.europa.eu/research/research-eu]

6.4. Need for post-exposure vaccines and those effective against all M. tuberculosis strains

The Bacille Calmette-Guerin (BCG) vaccine is currently the only vaccine in use against tuberculosis. The efficacy of this vaccine is limited to prevention of severe forms of tuberculosis among children and there are lots of problems in cases of TB/ HIV coinfection.The current vaccine candidates are being developed for pre-exposure administration but, considering the fact that one third of the world's population is already infected, there is a serious need for a post-exposure vaccine to prevent re-activation. Another shortcoming with the vaccines in the pipeline is that they can delay clinical TB but cannot achieve sterile eradication. There is need for combination vaccines that can combine the effects of booster vaccines with another generation of vaccines that can act to effect sterilisation at the post-exposure stage. Focus should also be put on the development of a vaccine that can afford protection against the wide range of M. tuberculosis strains, to ensure universal effectiveness.To avoid complications in clinical and epidemiological research, the evaluation of all the vaccines should also deal with confounding factors such as prior BCG vaccination or HIV status and there should be well worked out guidelines for use of these vaccines in children [http//www.ec.europa.eu/research/research-eu]

6.5. Development of new drugs

When it comes to the current status of clinical, diagnostic and therapeutic strategies, childhood TB has been grossly neglected and there is, therefore need for better and standardized treatment strategy. Although there is a reasonable number of candidates in the discovery and pre-clinical pipeline, there are still gaps between the different stages of TB drug development, and drugs specically targeting paediatric TB are still needed. Most of the drugs in the pipeline use the same mechanisms with the majority aimed at boosting efficacy or shortening the duration, with very few targeingt dormant stages of the bacillus and, therefore, not suitable for eradication of latent infections. Thus urgency for serious research into the development of new drugs and treatment regimens aimed at achieving this therapeutic objective cannot be over emphasised. Dealing with TB/HIV co-infection is another gap that needs serious attention: there is need to develop drugs that can prevent dormant mycobacteria from re-activating in HIV-positive individuals [http://ec.europa.eu/research/research-eu.

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7. Future prospects

7.1. Reducing the burden of childhood tuberculosis

TB in children presents a number of difficult challenges which will only be solved by a shift in research priorities. Advances in paediatric TB research will provide wider insights and opportunities for TB control. While development of a new vaccine to prevent TB should be the ultimate goal, development of better diagnostics represent one of the most important steps towards improving individual case management and also providing a more robust case definition for much needed drug and vaccine trials for studies on TB epidemiology and correlates of protective immunity in childhood. Data on the epidemiology of childhood TB may in turn help to inform public health policy by providing a window on current transmission and the effectiveness of control strategies and by identifying children with LTBI for chemoprophylaxis to limit the future propagation of the epidemic. Emphasis should be placed on reducing the vulnerability of the community because successful control of the tuberculosis epidemic is the most effective way to reduce the burden of childhood tuberculosis However, this will require a holistic approach with sustainable poverty alleviation as a key element [http://www. ec.europa.eu/research/research-eu].

7.2. Involvement of funders and industry

Important resources are required for the exploration of pathways leading to TB diagnostics-oriented basic science in pathogen biology, biomarker discovery, systems biology and point of care test development. The current priorities for TB vaccine development are: i) new vaccine candidates that achieve sterile eradication, and that can progress into phase II and phase III trials; ii) vaccine testing in a naïve stage on M. tuberculosis uninfected individuals; iii) vaccines which can achieve post-exposure prophylaxis to those who are already infected (currently 2 billion people); iv) strategies on how to get vaccines from the research bench to the bedside and into the community. Apart from the protective effect of novel vaccine candidates, priority should be given also to their delivery route, formulation (storage, shelf-life and distribution) and utility for HIV-infected individuals, particularly children.

7.3. Back to basic research

As we talk about nano technology and pin point delivery of drugs it is a shame that 130 years after the Robert Koch's discovery of M. Tuberculosis we still have huge gaps in our understanding of the biology, immunology and pathophysiology of the bacillus. We are, as yet, not able to explain fully the molecular, biochemical and immunological mechanisms that enable TB infection to go on for years and cause severe disease and death. With the availability of state-of-the-art molecular research tools such as functional genomics and metabolomics a paradigm shift towards empahsising basic research could help provide answers to some of the unanswered questions about M.tuberculosis in general, and paediatric TB in particular. The currently available knowledge has proved insufficient when it comes to the rational design of vaccines or other control tools and this has resulted in a lot of trial-and-error approaches. With

the HIV virus still elusive, defeating the dual alliance of TB/HIV co-infection has added another diamension in the already complicated war against the resistant strains of M. tuberculosis. Promoting basic research by providing the necessary resources and involving stakehoolders on the political front can provide solutions to some of the outstanding problems if not solving all of them. Where there is will, there is a way [http//ec.europa.eu/research/research-eu].

7.4. Concluding remarks

Recent developments for TB diagnostics seem promising fields due to the fact they are fast and minimally invasive, but they have drawbacks of not being validated in diverse populations and improved according to the patient's needs. Refinement of existing tools and development and testing of new tools are urgently required to improve diagnosis and treatment of TB in children. Higher global priority and funding will be required to improve on childhood nutrition and promote improvement in the socioeconomic and environmental condition of communities if we are to have a significant impact on TB transmission to children. [Expand +Clinical Infectious Diseasescid.oxfordjournals.org

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Chapter 19

Economic Evaluation of Diagnosis Tuberculosis in Hospital Setting

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Additional information is available at the end of the chapter

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1. Introduction

Tuberculosis (TB) is an ancient disease, but not a disease of the past. After disappearing from the world public health agenda in the 1960s and 1970s, TB returned in the early 1990s for several reasons, including the emergence of the HIV/AIDS pandemic and the increase in drug resistance. More than 100 years after the discovery of the tubercle bacillus by Robert Koch, what is the status of TB control worldwide? The evolution of global TB control policies, including DOTS (Directly Observed Therapy, Short course) and the Stop TB Strategy, and assess whether the challenges and obstacles faced by the public health community worldwide in developing and implementing this strategy can aid future action towards the elimination of TB.(Lienhardt, Glaziou et al. 2012) The report of the Commission on Macroeconomics and Health of the World Health Organization has emphasized that tuberculosis is the most common of the infectious diseases. Tuberculosis is one of the most important health problems in the world, causing 1.4 million deaths each year, in 2011. (WHO, 2010)

The most of TB cases (82%) was concentrated in 22 countries around the world. In the year of 2010, in Brazil were detected 81946 cases, with 5000 death (WHO, 2010).

In Rio Grande do Sul, a state in extreme south of Brazil, the incidence of TB in 2011 was 46,1 per 100.000, with 4947 new cases. Porto Alegre, capital of Rio Grande do Sul shows incidence of 116 in 2009.(Sul 2011; Brazil 2012)

Tuberculosis is the first cause of death in patients with AIDS in Brazil. Patients with co-infection HIV/TB have had in treatment of Tuberculosis probability of worst outcome.

Rio Grande do Sul, has had the major incidence of TB/HIV co-infection. The co-infection adversely affects the lives of individuals in both the biological and psychosocial aspects. (Neves, Canini et al. 2012)

Some factors can be considered as risk factors for co-infection of TB and HIV, as the impoverishment of the population, use of injecting drugs, the disruption of services on the epidemiology of TB control, the delay in the diagnosis of TB and increased risk of acquiring multi-drug resistant TB (MDRTB), essentially associated to the expansion of the disease in the world.(Kritski, Lapa e Silva et al. 1998). Multidrug-resistant tuberculosis (MDR-TB) is a major clinical challenge, particularly in patients with human immunodeficiency virus (HIV) co-infection.(Nathanson, Nunn et al. ; Farley, Ram et al. 2011; Arjomandzadegan, Titov et al. 2012; Jain, Dixit et al. 2012; Udwadia 2012)

For the above, in recent years became consensus that the epidemic of TB in developing countries demands the evaluation of broader approaches, described in the Plan STOP-TB/OMS control global TB 2006-2015.

Among them have been prioritizing the implementation of:

a) improvements in access to diagnostic system user health; b) culture for mycobacteria in every patient suspected of TB and HIV positive and all TB patients in retreatment; c) sensitivity test for suspected cases of resistant TB (retreatment cases, treatment failure, contact MDR-TB or have been treated at the Health Unit with a high rate TB-MDR/XDR); d) review and economic evaluation under routine conditions of deployment of new technologies (phenotypic or molecular, automated or not) for the early diagnosis of TB, resistant TB patients with paucibacillary TB, HIV-infected or suspected drug-resistant TB.

Early detection of tuberculosis (TB) is essential for infection control. Rapid clinical diagnosis is more challenging in patients who have co-morbidities, such as Human Immunodeficiency Virus (HIV) infection. Direct microscopy has low sensitivity and culture takes 3 to 6 weeks (Sharma, Mohan et al. 2005; WHO 2006). Diagnostic testing for tuberculosis has remained unchanged for nearly a century, but newer technologies hold promise for a revolution in tuberculosis diagnostics. Tests such as the nucleic acid amplification assays commercial and in house technologies allow more rapid and accurate diagnosis of pulmonary and extrapulmonary tuberculosis.(Rodrigues Vde, Queiroz Mello et al. 2002; Sanchez, Rossetti et al. 2006; Scherer, Sperhacke et al. 2007; Scherer, Sperhacke et al. 2011; Hida, Hisada et al. 2012). Xpert MTB/RIF (Xpert) is actually a promising new rapid diagnostic technology for tuberculosis (TB) which has characteristics that suggests large-scale roll-out.(Vassall, van Kampen et al. 2011). In developing countries, *in house* Polymerase Rhain reaction (PCR) based on amplifying the IS*6110* insertion element can be used for the amplification of *Mycobacterium tuberculosis* (MTB) DNA and offers the potential of a sensitive, specific and rapid diagnostic for ruling out or considering pulmonary tuberculosis (PTB) (Mehrotra, Metz et al. 2002; Sarmiento, Weigle et al. 2003; Schijman, Losso et al. 2004; Flores, Pai et al. 2005).

The appropriate and affordable use of any of these tests depends on the setting in which they are employed (Perkins 2000; Brodie and Schluger 2005). New tools for TB diagnosis, treatment and control are necessary, especially in health settings with a high prevalence of HIV/TB co-infection.

Although TB is one the greatest causes of mortality worldwide, its economic effects are not well known, especially in Brazil. Despite the fact that the families did not have to

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pay for medications and treatment, given that this service is offered by the State, the costs to families related to loss of income due to the disease were very high. The proportion of public service funds utilized for prevention is small. Greater investment in prevention campaigns not only might diminish the numbers of cases but also might lead to earlier diagnosis, thus reducing the costs associated with hospitalization. The lack of an integrated cost accounting system makes it impossible to visualize costs across the various sectors.(Costa, Santos et al. 2005)

To make rational decisions about the implementation of new tools in the medical routine, cost-effectiveness studies are essential(Mitarai, Kurashima et al. 2001; Kivihya-Ndugga, van Cleeff et al. 2003; Hazbon 2004).

A key step in cost-effectiveness analysis is to identify and value cost. The economic concept of opportunity cost is central to cost-effectiveness analysis. When a public health agency spends money to provide health care, this money is not available for housing, education, highway construction, or as a reduction in income taxes. When a health care organization spends money for bone transplantation, this money is not available for example for mammography outreach or something. When an elderly man spends time being vaccinated for influenza, this time is not available to play golf or to work. An overall conceptual goal in cost-effectiveness analysis is comprehensive identification of all costs of the intervention and its alternative, including all of the opportunity costs.

Contributors to cost must be identified before the costs can be valued. The terms used to describe the contributors to cost (e.g. direct costs, indirected costs, opportunity costs) are used in different ways in different textbooks and in published cost-effectiveness analysis.

The definition of cost terms is the opportunity cost is the value of resources in an alternative use, the direct cost is the value of all goods, services, and other resources consumed in the provision of an intervation or in dealing with the side effects or other current and future consequence linked to it and the productivity costs are the costs associated with lost or impaired ability to work or engage in leisure activities and lost economic productivity due to death attributable to the disease. These costs have been substituted for indirect costs. There are several categories of direct costs. The first category of total direct cost is direct health cost, this category include costs with tests, drugs, supplies, personnel, equipment, rent, depreciation, utilities, maintenance and support services. The second category of total direct cost is direct non- health care cost, these cost include for example the cost to patients to partake of the intervention e.g., transportation, child care, parking). The third category of total direct cost is the cost of informal caregiver time, this is the monetary value of the time of family members or volunteers who provide home care. The fourth category of total direct cost is the cost is the cost of the use of patient time. Such studies provide insight into the composition of different cost components, which may be the most important factor from the patient and the health service's perspectives. Recent studies have compared the cost effectiveness of news tools for diagnosis, treatment and control in Tuberculosis.(Amicosante, Ciccozzi et al. ; Kowada, Deshpande et al. ; Baltussen, Floyd et al. 2005; Barbieri, Wong et al. 2005; Bachmann 2006; Dwolatzky, Trengove et al. 2006; Kominski, Varon et al. 2007; Kowada,

Takahashi et al. 2008; Rosen, Taylor et al. 2010; Shi, Hodges et al. 2010; Vassall, van Kampen et al. 2011; Fitzpatrick and Floyd 2012; Lienhardt, Raviglione et al. 2012; Mandalakas, Hesseling et al. 2012). In a recent study we compared the cost-effectiveness of direct microscopy by Ziehl Neelsen staining (AFB smear) with *in house* polymerase chain reaction (PCR) and with culture on the first sputum specimen collection, including staff costs, using culture and clinical evaluation as the gold standard (Scherer, Sperhacke et al. 2009). In contrast to the cost-effectiveness analysis described by van Cleef et al. in a reference ambulatory clinic in Kenya, where only culture for mycobacteria was used as the gold standard (Roos, van Cleeff et al. 1998; van Cleeff, Kivihya-Ndugga et al. 2005). The cost-effectiveness of the AFB smear plus PCR dot-blot strategy described in recent study was similar to other strategies, when lower TB prevalence made PCR more expensive for diagnosis of PTB (Roos, van Cleeff et al. 1998; van Cleeff, Kivihya-Ndugga et al. 2005). (Scherer et. aL., 2009).

The mathematical models may be particularly useful for predicting the long term tendency of occurrence of the infection or disease. These models can simulate situations epidemiological and preventive or curative interventions beyond their theoretical impact in reducing the problem. Such predictive models properly formulated and fed with consistent data, may assist the processes of planning and management in public health. Currently several strategies have allowed the use of Multiple Logistic Regression (MLR) in the construction of predictive models. Models of decisions trees are also used for classification decision making or to provide a decision algorithm for the clinical management of infectious diseases.(Aguiar, Almeida et al. 2012)

For developing countries, the emergence of continuous technological innovation represents a double burden. The rapid diffusion of scientific and technical information that are observed now and monetary action multinational companies create a local demand for innovation by health professionals, the media and more informed portions of the population, which further strains the health care system.

Many factors limit the realization of a health technology assessment (HTA) analysis, as the lack of human resources, infrastructure or budget or due to lack of evidence or information costs.

Another obvious problem is that often decisions are based on scientific evidence coming from developed countries and often in settings where the incidence of disease differs effusively of Brazilian and Latin American scenario.

Given this scenario health managers are often between two objectives: they have to incorporate new and more costly technologies to improve the health of the population and at the same time are responsible for the financial sustainability and access equity of this in the system health.(Project 2005)

Beyond the suffering caused directly by the disease, TB is requiring significant portions of the public budget in developing countries. It is estimated that by 2015 they will be required investments around $12 billion for control of diseases such as AIDS, TB and Malaria. The increased costs involved in care and control of TB are due also to the increasing number of

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cases of resistant bacteria to different types of chemotherapy. (Polansky, Dymer et al. 1968; Garcia Rodriguez, Marino Callejo et al. 1994; Weis, Foresman et al. 1999; Gomes, Soares et al. 2003; Elamin, Ibrahim et al. 2008; Kik, Olthof et al. 2009; Steffen, Menzies et al. 2010; Vassall, Seme et al. 2010; Pereira, Barreto et al. 2012)

Costs of TB diagnosis and treatment may represent a significant burden for the poor and for the health system in resource-poor countries. Costs incurred by TB patients are high in Rio de Janeiro, especially for those under DOT. The DOT strategy doubles patients' costs and increases by fourfold the health system costs per completed treatment. The additional costs for DOT may be one of the contributing factors to the completion rates below the targeted 85% recommended by WHO (Steffen, Menzies et al. 2010).

Even in a country with a good health insurance system that covers medication and consultation costs, patients do have substantial extra expenditures. Furthermore, our patients lost on average 2.7 months of productive days. TB patients are economically vulnerable. (Kik, Olthof et al. 2009)

In Brazil, the real costs of TB are estimated or poorly known and the overall costs of TB are not perceived by governments, given the fragmentation in the involvement of the three governmental levels: local, state and national.

The purpose of this chapter is to describe the direct and indirect costs for diagnosis and treatment of Pulmonary Tuberculosis in patients infected or not by HIV, admitted to a Hospital Unit of Public Health.

2. Costs of health system of Brazil

In order to describe the costs of Health system of Brazil, we evaluate the costs directs of diagnosis and treatment of screening of 1000 hypothetical patients suspects of Pulmonary Tuberculosis in according with clinical and laboratory Brazilian recommendations for treatment (Tuberculose 2004; Conde, Melo et al. 2009).

The cost components for each clinical and laboratory procedures of screening included costs incurred by the patient, laboratory costs, drugs, consumables and equipment costs. The strategy for screening was the same recommended for Brazilian Public Health System.

Clinical, radiological and laboratory staff costs were calculated from the salary base of Rio Grande do Sul (State of Extreme South of Brazi) in 2011.

For each procedure, costs were attributed based on procedure costs of the Brazilian Public Health System.

Running costs (material costs were used for each 1000 tests evaluated) included all laboratory materials used in procedures.

All costs were expressed in US$, using an exchange rate of US$ 1= R$ 1,72 (REAIS), the average exchange rate from 2010 to 2011. In the treatment costs, those were evaluated related to

the treatment of inpatients and outpatients. To estimate the values that are spent by the public health system of Brazil with the monitoring and control of TB in a hospital and an outpatient unit, we simulated two different scenarios:

a. TB cases diagnosed in hospital wards (hospitalized patients)

b. TB cases diagnosed in outpatient environment (outpatients).

The number of days considered to calculate the costs related to the treatment of inpatients they were considered as the same days that were spent in laboratory procedure.

It was hypothesized that the time to detect *Mycobacterium tuberculosis* in sputum culture from patients with pulmonary tuberculosis may be a better indicator for the duration of time of hospitalization(Ritchie, Harrison et al. 2007).

The time to detect *M. tuberculosis* in the culture was 30 days in this study. This cohort is the same as previous published by our group [20]. This value was used as the standard at which release from isolation could be permitted (Scherer, Sperhacke et al. 2007)

The time spent on laboratory procedure to provide access to the result of the laboratory technique was assumed to be 30 days for AFB smear plus culture. The number of days considered to calculate costs was the same as those spent on laboratory procedure. The number of days considered to calculate the cost of patient travel costs was assumed to be 2 days for AFB smear plus culture.

Total treatment included clinical officer and hospital costs, assuming cost per pill, to be US$ 0.22, using 3 pills per day, during 180 days; hospital room costs, US$ 7/day; costs with salary of clinical staff and clinical consultation, US$ 2.52 per patient and clinical nursing consultation, US$ 2.52 per patient.

Assuming that, during the treatment (6 months), in ambulatory situation, 6 AFB smear test, 6 chest radiographs, 6 consult of nurse and 2 consult of clinical were performed, we used this parameters to estimate the costs of ambulatory following the Brazilian recommendations for treatment (Tuberculose 2004).

Assuming that, during the hospitalization (30 days), 4 AFB smear tests, 4 chest radiographs, 30 nurse and physician consultations were performed, we used these parameters to estimate the costs of inpatient assistance in hospital, following the Brazilian recommendations for treatment (Tuberculose 2004; Conde, Melo et al. 2009). Staff salaries for the physician, nurse and radiologist were considered to be US$ 11,163 per year, and for the chest radiograph technician, the salary was US$ 4,988 per year. The work days were considered 20 days for all staff.

The days of admission to the hospital were considered to be the same number of days spent on each laboratory procedure. All estimated costs reflect an estimative of the public health system of Brazil expenses with the monitoring and control of TB.

The costs were expressed per 1000 suspects, according to the specific bibliographic references for economic analyses, thus, allowing the best decision for investment to be made (Petitti 2000).

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Table 1A shows the costs at the health service level and Table 1B shows costs due to laboratory investment. The AFB smear plus Culture require (US$ 39,535) for equipment. Table 1C shows costs incurred by patients.

A. Health service costs

	Staff Number	Salaries of all staff per year (US$)	Staff Cost per day (US$)	Time spent until access to result (days)
AFB smear plus Culture	2	16,151	67	30

B. Laboratory costs

	Equipment (US$)	Annualization Years	Running costs per 1000 suspects (US$)	Running costs per examination (US$)
AFB smear plus Culturea	39,535	5	12,507	12.50

C. Estimated costs incurred by patients, including costs for travel, food and income loss[d]

	AFB smear plus Culture (US$) (outpatients and inpatients)
Travel	1,390
Food	10,000
Income Loss[c]	310,000
Total patients Cost	341,000

[a] Microscopic and Laminar Flow Cabinets[b]. Other equipments were not included.

[b] Income loss of patients was calculated from monthly salary base of Brazil (US$207) and was based on proportional days spent by patients until access to the result of each laboratory procedure. Patient costs were estimated using the average of two visits to the laboratory for AFB smear and culture procedures for outpatients; Travel cost was considered as US$ 1.4 (one bus ticket). Food was considered as US$ 10 per meal. Base salary in Brazil was considered (US$ 10 per day /20 days of the work). For inpatients was considered just income loss; Staff costs in the laboratory were based on proportional days spent on each laboratory procedure; Costs of consumables and equipment were provided by the program as well as by the manufacturer.

Table 1. Estimative of Costs in US$ in Tuberculosis Diagnosis in Brazil

We annualized the capital cost of the equipment for 5 years, according to the literature [25]. Building costs were not included. Opportunity costs were not applicable.

	AFB smear plus Culture
Laboratory Costs	
Labor Costs[a]	3,743
Investment costs	37
Running costs	3,700
Staff Costs per day	
Cost laboratory staff [b]	1,434
Cost of staff related to the treatment of patients[b]	2,791
Costs of chest radiograph staff related to the treatment of patients	404
Treatment Costs per day	
Costs of diagnostic service related to the treatment of no-hospitalized patients	2,771
Costs of diagnostic service related to the treatment of hospitalized patients	4,686
Treatment costs (hospitalized patients plus no- hospitalized patients)[c]	7,456
Income Loss	190,000
Total Patient costs	190,000
Total Health Service costs	9,479,033
Total Screening costs	9,668,815

[a] For each procedure, costs were attributed based on procedure costs of the Brazilian Public Health System (US$ 1,4 for AFB smear and US$ 1,9 for Culture) and from CDCT/FEPPS (US$ 11,7 for PCR dot-blot), assuming investment laboratory equipment for 5 years; [b]Staff salary was considered; for laboratory technician, US$2,860 per year; for Laboratory technologist, US$6,400 per year. Staff costs in the laboratory were based on proportional days spent on each laboratory procedure; Staff salary was considered for clinical physician, nurse and radiologist; US$6,400 per year; for the X-RAY technician, salary was US$2,860 per year. [c]The days of admission to the hospital were considered as the same as the days spent on each laboratory procedure. The time spent on each laboratory procedure until access to the result of the laboratory technique was assumed to be 30 days for AFB smear plus Culture. Total treatment included clinical officer and hospital costs, assuming US$ 0,22 cost per pill, using 3 pills for day, during 180 days; hospital room costs, US$ 4,16/day; costs of salary of staff clinical; clinical consultation cost, US$2,52 per patient; clinical nursing consultation, US $2,52 per patient. Assuming that during the treatment of inpatients (4 months) 4 ZN and 4 chest radiograph were performed, and during the treatment of no- hospitalized patients (6 months) 6 AFB smear and 6 chest radiograph were performed, following the Brazilian recommendations for treatment (Tuberculose 2004);

[d] Travel was considered 2 days for AFB smear plus Culture strategy. Food and income loss for AFB smear plus Culture strategy was considered 30 days

The health service costs analysis was based on processing 50 AFB smear slides and 14 cultures per day. AFB smear plus Culture was performed by two trained staff.

Running costs were calculated from investments required to examine 1000 smears.

Table 2. Total cost of screening for 1000 suspects. The total screening costs to AFB smear plus Culture were US$ 9,668.815.

The total cost (in US$) related to the treatment (no hospitalized patients) for AFB smear plus Culture was US$ 2,771. The cost related to the treatment of hospitalized patients, for AFB smear plus Culture strategy was US$ 4,686. The cost related to the treatment of (no hospitalized patients) and (hospitalized patients), for AFB smear plus Culture strategy was US$ 7,456.

However, in a context of advanced technologies for the diagnosis of tuberculosis, economic resources has always limited the incorporation and diffusion of new technologies produced and validated by the academy. It is a challenge for health systems worldwide, and in many cases, the cause of serious sustainability problems.(Taylor, Drummond et al. 2004; King, Griffin et al. 2006; Mason, Weatherly et al. 2007; Hughes, Tilson et al. 2009; Weatherly, Drummond et al. 2009; Shi, Hodges et al. 2010)

The decisions related to incorporation, acquisition, reimbursement or coverage of new technologies and those that determine the way in which they should be used are the most important in the health system and should be taken in general and the management of health services in particular.(Greenberg, Peterburg et al. 2005)

The health systems of different countries are diverse with respect to decisions about incorporating technologies and expectations of service users. Tough choices are faced by managers at all levels of the health system. This reality makes the TB every year, become more difficult for the system to provide the user with the most effective intervention theoretically available, depending on the pressures placed on the health system in relation to increased costs, the training of human resources, needs updating certification and regulatory instruments, and investment in physical infrastructure (Newhouse 1992)

Attempts to improve the acceptability of resource allocation decisions around new health technologies have spanned many years, fields and disciplines. Various theories of decision making have been tested and methods piloted, but, despite their availability, evidence of sustained uptake is limited. Since the challenge of determining which of many technologies to fund is one that healthcare systems have faced since their inception, an analysis of actual processes, criticisms confronted and approaches used to manage them may serve to guide the development of an 'evidence-informed' decision-making framework for improving the acceptability of decisions.(Stafinski, Menon et al. 2011)

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Chapter 20

Pulmonary Tuberculosis in Latin America: Patchwork Studies Reveal Inequalities in Its Control – The Cases of Chiapas (Mexico), Chine (Ecuador) and Lima (Peru)

Héctor Javier Sánchez-Pérez, Olivia Horna–Campos, Natalia Romero-Sandoval, Ezequiel Consiglio and Miguel Martín Mateo

Additional information is available at the end of the chapter

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1. Introduction

Tuberculosis (TB) has been present in Latin America since pre-historical times. Paleopathological studies have found signs of TB in mummies from many parts of the world. In fact, whenever human mummies have been found, signs of TB have been observed in bones, lungs or skin [1].

Although TB may be considered as nearly as old as humankind, the current epidemiological profile of this disease must not be considered as the natural or expected one, given the large numbers of prevalent and newly occurring cases. The main questions related with the persistence and rise of TB in many regions of Latin America, have to do with social processes and inequalities. In this sense, the different processes usually resulting in TB disease are directly related with social and economic behavior of human communities [1].

TB constitutes one of the most complex situations in the health field. This complexity both makes visible, and raises questions about the existing inequities in the political and socio-cultural structure and in class relations, as it is the result of the health-illness-care process. Among the main elements permitting operationalization of an analysis of this situation, we find social vulnerability and accessibility to a whole spectrum of health services (in geographical, cultural and economic terms), from opportunistic diagnosis to effective treatment (meaning cure).

In this sense, we understand as social vulnerability that set of economic, political, social and cultural conditions which determine that some individuals become infected by the TB bacillus while others do not, depending on the structural conditions which favor or hinder exposure to the disease,[1] as well as those differential aspects by which, among those infected, some get TB disease while others do not, and among those with TB disease some are cured (whether spontaneously or as a result of anti-TB treatment), others remain chronically ill (possibly with multi-drug resistance) while others die (generally those presenting the worst socioeconomic conditions and poorer health in general).

Nevertheless, TB prevention and control programmes are designed as if the disease behaved in a homogeneous way in all countries and regions, based almost solely on biological and medical factors, without taking into account socio-cultural, economic and political factors, such as poverty, malnutrition, health services accessibility and quality, as well as intra and inter-community political conflicts, among others.

This approach impedes acting on the particularities of marginal populations, which are precisely the ones presenting the highest rates of morbidity and mortality of this disease, manifesting various gradients of exposure and susceptibility. This leads governments to act on the basis of global estimates, even when their interpretation of these is limited and partial, because the differential exposures and true extent of TB are unknown.

Furthermore, this way of tackling TB does not reflect the reality of the different regions within a given country, because local or regional variations in rates of morbidity and mortality are disguised. Such variability could easily be quantified by, at the very least, providing the standard deviation corresponding to the global values of these rates for each country. Consequently, areas which should be given priority, paradoxically receive only limited resources for interventions.

In this sense, it is important to point out that TB, like HIV/AIDS, is one of the diseases for which estimations of impact in terms of incidence and prevalence are frequently based only on the registered cases. While it is true that published national and international figures often include estimates of sub-notification, they do not usually include gradients of the magnitude of the disease, or of the intra- or inter-regional under-notification rates, nor the differential rates between different population groups. According to several authors, calculation of the number of cases of TB disease is possible based on the expected evolution of cases of infection [2] or through linear regression modeling involving age-specific prevalence values across a range of differently aged populations [3]. Although this calculation technique for the frequency of TB and HIV status has been considered [4], there are currently no models in which population impact has been measured in terms of social factors.

In summary, in general terms, national and international policies to cope with TB ignore this reality, applying criteria of homogeneity in the calculation of objectives, materials, costs and logistics, among other aspects. While it is well known that marginal groups are the ones presenting the highest TB morbidity and mortality rates, their characterisation is not usually

1 According to World Health Organization calculations, one third to the human population is infected with TB bacillus

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considered in the design of programmes for their prevention and control, so that TB continues to cause high rates of disease, death and ever-rising health costs in these groups, something which represents a violation of their human rights a consequence of governments having been incapable of preventing this situation.

Furthermore, the effectiveness of programs of TB Prevention and Control has been questioned because of their complexity. In this sense, a therapeutic intervention such as the Directly Observed Therapy – Short Course (DOTS) strategy, and socioeconomic and structural factors have been topics of discussion with regard the possible impact of one and the other, due to the decline of TB observed prior the use of antibiotics, as well as the goals met at present by DOTS strategy. In this regard, a correlation has been documented in Latin America between the early diagnosis of smear positive TB cases and improved cure rates [5]. So, one of the main emphasis of strategies to reduce the transmission of *Mycobacterium tuberculosis*, should be the identification of active TB cases, particularly in deprived and highly exposed populations.

The Africa and Latin America Research Groups Network (*Grups de Recerca d'America i Africa LLatines* -GRAAL) has conducted studies in marginal populations which reveal the conditions of patients, as well as the extent of the disease, of multi-drug resistance (MDR) and of mortality in these populations, producing figures which differ widely from the official average values. The main mechanism for tackling these aspects has been through doctoral theses. In this chapter we give examples of research undertaken in three different contexts of high poverty and social exclusion in Mexico, Ecuador and Peru.

2. Patch 1: Chiapas, Mexico

Chiapas is one of the poorest states in Mexico, and has one of the highest rates of indigenous margination as well as an acute lack of health care resources. According to official government statistics, Chiapas ranks almost last among all Mexican states in terms of health and socioeconomic indicators [6]. It is precisely in Chiapas where, due to the conditions of social exclusion, poverty, malnutrition and high mortality from infectious contagious diseases, the Zapatista National Liberation Army (EZLN) initiated an armed rising against the Mexican government in 1994, which drew attention, both nationally and internationally, to the precarious living and health conditions of the indigenous and peasant populations, not only in Chiapas, but throughout the entire country.

Several studies have been carried out by our team in areas of high levels of poverty in Chiapas: Our first attempt to analyze the pulmonary tuberculosis (PTB) situation arose out of the discovery that in the only hospital (Comitán General Hospital, Ministry of Health) in the region of the border with Guatemala for patients not covered by insurance (the majority of whom are indigenous),[2] there was empirical evidence of a high prevalence of PTB cases. In 1994, active case finding of patients with chronic cough (15 days or more) was carried out among all patients aged over 14 years seeking care in the hospital for whatever reason [7]. In this study

2 In Chiapas, over 80% of population is not covered by social security [6]

a rate of 21 positive PTB smears per hundred patients was found (95% CI=15.5-26.6), and the main factors associated with PTB were age (35-44 years), occupation (engaged in agricultural) and weight loss. Through a logistic regression model, we found that the subgroup of chronic cough patients aged 35-44 years, agricultural workers and who had lost weight, had the greatest likelihood of being PTB positive (68.7% compared to the overall average of 21% in the studied patients).

In addition, we noted that in the case of men, patients came to the hospital from near, far and very far distant communities, but in the case of women, the majority of them only came from communities which were near or very near. So we decided to carry out other studies in the hospital's area of influence, with the aim of analyzing factors related with the high PTB prevalence among users of secondary level care, not only in terms of health system aspects, but of demographic and socioeconomic characteristics:

a) In 1997 active case-finding was carried out among all patients aged over 14 years seeking consultation in a random sample of seven primary care centers [8]. We found a PTB positivity rate of 11.1 (95% CI=6.6-17.2) per hundred patients studied. The factors associated with PTB were size and poverty level of the locality of residence. Of the coughers identified, 56% sought care for non-respiratory symptoms.

b) In 1998 active case-finding was carried out among those aged over 14 years who had a cough of 15 days or more of duration, in a convenience sample of 1,894 households in 32 communities chosen at random based on the level of poverty and on travel time to reach the nearest health services (< 1 hour, 1 hour and over). In this study we found a rate of 276.9 per 100,000 persons studied (95%CI: 161-443) and that the only factor associated with PTB was blood in sputum, probably due to the homogenous conditions of extreme poverty among the populations studied [9].

Additionally, we found that the sensitivity of the smear testing was slightly lower than 50% in the primary care centers and in communities, and that the proportion of patients with active PTB that was receiving treatment was only 50% in the primary care centers, and 10.5% in the studied communities [10]. Also, we found high rates of anti-TB treatment defaulting [11], and very high levels of PTB multidrug-resistance (MDR): 4.6% and 29.2% primary and secondary MDR-TB, respectively. In fact, 14% of all studied PTB patients had MDR.

According to the logistic regression model fitted, the main variables associated with MDR were: having received anti-TB treatment previously, cough of three years or more of duration and not being indigenous. This is the only occasion in all our studies, which the condition of being indigenous appeared as a protective factor [12].

In 2000-2001 our team, together with Right to Health Defense Group and Physicians for Human Rights, carried out a population-based study to assess health conditions, and access to health services in the conflict zone initiated in 1994 between the EZLN and the Mexican government [6]. We found that the most affected regions by the armed conflict have fared even worse than the rest of Chiapas State. We performed a household survey in the municipalities most affected by the armed conflict among three types of communities: opposition communities, pro-

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government communities, and divided communities, i.e. which contained both opposition and pro-government groups.

This investigation identified serious deficiencies in both detection and treatment of PTB. In the 46 studied communities (n=2,997 households), we detected 29 cases of PTB among the population aged over 14 years. This means a rate of PTB of 85.3 per 100,000 in the general population, and of 161.2 among those aged 15 and older, almost three times the rate reported for the entire state. In this sense, only 13 (45%) cases of the 29 detected, had been identified by health services and were being treated. Of these 13 cases, one had not received any anti-TB treatment and six had defaulted from anti-TB treatment.

We also carried out two evaluations of a cohort of patients aged over 14 years diagnosed with PTB from January 1998 to July 2005, and found poor survival among them. In the first follow-up (performed during 2004-2006), the principal factors associated with PTB mortality were: age (45 years and over, OR=1.3; 95% CI=0.98-1.3), 0-3 years of schooling (OR=3.3; 95% CI=1.1-4.4), not living in the main village of their municipality (OR=1.2; 95% CI=1.0-1.3), living in a rural community (OR=2.7; 95% CI=1.1-6.8), not having been treated in DOTS (OR=1.2; 95% CI=1.0-1.3) and having defaulted from treatment (OR=11.5; 95% CI=5.3-24.8) [13].

In the second follow-up (carried out in 2008-2009), the factors associated with PTB mortality were age (45 years and over) and anti-TB treatment duration of under six months. The median survival time of those patients aged 45 and over who died was 718 days (range 0 to 3,185), while the median survival time in the reference group consisting of patients aged 15-34 years, was 688 days (range 8-1,841). With regard to the duration of anti-TB treatment, the median survival time among patients with incomplete treatment was 261 days (range 0-1,658), whereas among those dying in the reference group (with treatment completed), the median survival time was 1,137 days (range 202-3,185) [14].

The mortality rate in the patients studied was 4.6 per 100 person-years. Of the 78 deaths from PTB documented in this study, 25% occurred during the first six months following diagnosis (in other words, during treatment), 38% by the end of the first year from the date of diagnosis, 53% had died by the end of the second year, and 72% after three years.

The most important features of these studies are shown in Table 1.

3. Patch 2: Chine, Ecuador

During the decade from 1997 to 2006, inequalities of wealth and human development were extremely marked in Ecuador. The indigenous population, such as that residing in the central Andean province of Cotopaxi, has the highest poverty rates, and has many of its basic needs unmet. In Ecuador, up until 2006, the TB Prevention and Control Program was based on passive case finding of patients with respiratory symptoms (health personnel would check whether a patient visiting a health center had a productive cough of more than 15 days of duration). In contrast to what happens in cities, in rural areas the organization and functioning of the program relies on the presence of basic rural health teams; this means that is not

uncommon for health personnel to be absent. This situation, among others, has resulted in TB notification being irregular. Although the average incidence reported is 65/100,000,[3] given the important level of under-reporting of TB cases, the true extent of the disease in Ecuador is unknown.

Setting	Frequency	Associated Factors
Chiapas, Mexico (only people 15 years of age and over)	Prevalence of PTB 21% in a hospital-based population with symptoms suggestive of TB	Association with age (35-44 years old), working in agricultural, and weight loss.
	Prevalence of PTB 11.1% among patients, consulting in primary health centers (PHC) with symptoms suggestive of TB	Association with poverty level
	Prevalence of PTB 277 per 100,000 persons studied in household surveys	Presence of blood in sputum; 50% of sensitivity in sputum test performed in PHC setting. High rate of defaulting treatment; and very high MDR-TB rates associated (by Logistic Regression) with previous PTB treatment, cough for more than 3 years and not being indigenous.
	Cohort of patients with PTB	Mortality was associated with poverty and deprivation characteristics and no access to DOTS.

Table 1. Pulmonary Tuberculosis (PTB) and associated factors observed within studies performed by GRAAL members in Mexico.

In Chine, an indigenous community of 653 inhabitants, in the parish of Angamarca, located in Cotopaxi Region, over 90% of its population have their basic needs unmet [15]. It is situated at an altitude of 3,500m above sea level and is two hours walk from the nearest health center, which during the period 2000 to 2004 was practically without staff. One of the co-authors of the present work (Natalia Romero) collaborated with the health team of this parish, during her period of rural medical training several years earlier. Following the diagnosis of one PTB case (the schoolmaster) in 2001, we conducted a study between 2001 and 2003, and found a prevalence rate of PTB-positive cases of 6.7% for the community as a whole [16].

On the basis of this single case, we saw the convenience of studying the total population of the community through a household survey (taking into account the experience obtained in Chiapas, México). The data collected was analyzed using the technique of multiple correspondence analyses, which allowed us to ascertain the risk and exposure factors in the community. All persons with chronic productive cough were asked to provide three sputum

3 World Health Organization. Ecuador: Health profile. Available at: http://www.who.int/countries/ecu/es/ (accessed 12 August 2012).

specimens. Given the degree of social and geographical exclusion of the community, PTB was diagnosed only by smear test.

Two hundred and two persons were identified with chronic cough (fifteen days or more), 173 of them, productive. Of 92 coughers in which it was possible analyze their sputum, 44 (48%) were PTB positive (representing 6.7% of the whole population and 11.3% of those aged 15 years and over). Among men, the highest prevalence was in the 35–44 age group (20.6%) and among women in the group aged ≥ 45 years (16.7%). Also, 27% of families had between one and four smear positive members. The factors associated with presence of PTB were: previous history of active TB (OR=6.0; 95% CI=2.9-12.3), haemoptysis (OR=3.8; 95% CI=1.5-10.0), and history of participating in seasonal migration (OR=2.44; 95% CI=0.91-6.54) [16].

With the intention of making some contribution to resolving the PTB problem, our team reached an agreement with the inhabitants of the community of Chine, in order to implement the DOTS strategy, while at the same time taking account of aspects of the community's world view. As consequence of this approach, we obtained a cure rate of 100%, confirmed by three negative smear-tests during the anti-TB treatment and cultures at the end of it (there were no defaults and no deaths) [17].

Although TB prevention and control programs encourage patients to visit health services and follow instructions, if they continue in their tendency to give little attention to socioeconomic, cultural and anthropological aspects, the results will be the same. How can better outcomes be expected if health services persist in acting as they always they do, including opening for restricted hours (from 8 am to 12 pm and from 2 to 6 pm)? In our intervention in Chine, symbolic referents, the religious dimension and rituals, as well as aspects of daily life (working hours, school, community and family calendar, seasonal migration, and traditional medical practices, among others) were taken into account.

The main results obtained in Ecuador, are shown in Table 2.

Chine, Cotopaxi, Ecuador	Prevalence of PTB 6.7% in an entire indigenous community	PTB was associated with prior history of PTB (OR= 6.0; CI 95%: 2.9-12.3), with haemoptysis (OR= 3.8; CI 95%: 1.5-10.0).
		Cure rate of 100% based on community consent for the performing of DOTS strategy

CI: Confidence interval; OR: Odds Ratio

Table 2. Pulmonary Tuberculosis (PTB) and associated factors observed within studies performed by GRAAL members in Ecuador.

4. Patch 3: Lima, Peru

One of the coauthors of the present work (Olivia Horna), as a nurse responsible for coordinating the application of DOTS, realized that the TB Program was relaxing various aspects of

the DOTS application, largely motivated by the economic conditions imposed in the area by the World Bank but also by recommendations from the Pan-American Health Organization itself: cessation of active case finding of coughers of 15 days or more on the grounds that the system was of low efficiency, and a switch to an ambulatory form of DOTS rather than in the patient's home, changes due to a shortage of funds to pay health technicians performing this function.

Given this situation combined with a feeling that the number of patients was rising at a higher rate than that calculated based on national rates, it was decided to perform a study to detect coughers of 15 days or more making use of the structure of the health system itself. The district of Ate-Vitarte was chosen to be targeted, since it consists mainly of lands occupied by migrants from the interior of the country, many forced off their lands due to violence between Peruvian armed forces and guerrilla movements from the interior, in particular Sendero Luminoso.

For this study, as in the cases of Chiapas, Mexico and of Chine, Ecuador, the health system approved and participated in order to guarantee anti-TB treatment and medical care for possible new cases identified by the study. Thus the same scheme was set up as employed previously in the outpatients department of Comitán hospital, whereby subjects recruited were people approaching the health system facilities in the area for medical care.

We interviewed 150 persons over 14 years of age who had productive chronic cough (fifteen days or more) seeking care in health services (primary care and hospitals). Of these, we obtained sputum samples from 142. The observed PTB prevalence rate was 12% [18], a figure very similar to that obtained in primary care centers in Chiapas (11%) [8]. None of the demographic or socioeconomic indicators analyzed were associated with PTB.

Of the variables studied, those found to be significantly associated with PTB, were: working away from home with respect those working at home (OR=6.99; 95% CI=0.89-54.61), persons commuting by minibuses compared with persons who used individual forms of transportation (OR=4.9; 95% CI=1.06-23.09), and commuting time one hour or more in a minibus (OR=3.35; 95% CI=1.12-10.1) [18].

Given the high prevalence of PTB in peripheral areas of Lima, as is the case of Ate-Vitarte District, and the results mentioned in the previous paragraph, we planned a study to determine whether the use of minibuses was associated with the spread of PTB. Commuting in these minibuses means that people travel in overcrowded situations with closed windows regardless of the weather, making trips of at least 30 minutes duration every day, in the company of TB patients going to a health center to receive DOTS treatment. Furthermore, if there is a strong association between using microbuses and the risk of infection, what would be expected among microbus drivers and fare-collectors that spend more than 8 hours per day in this environment?

Based on these precedents, we decided to carry out a study to assess infection by *Mycobacterium tuberculosis* and working conditions among workers of public transport [19]. In 2008 we performed a cross-sectional study with 104 workers from two public transport minibus companies of the Ate-Vitarte District. These minibus workers were interviewed and a tuber-

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culin skin test (TST) administered. An induration greater than or equal to 10 mm was considered positive.

From these 104 workers, TST results were obtained for 73 (70.2%), of whom 56 (77%) were positive. We found that positivity was associated with the time they had worked on minibuses (more than two years, OR=11.04; 95% CI=3.17-38.43), and with working more than 60 hours per week (OR=9.8; 95% CI=2.85-33.72). This exposure gradient, a result of the working hours and time employed in the transport sector, stresses the importance of workers' job conditions.

Furthermore, strict revision of clinical histories of active TB patients in the health centers associated to the health districts of these workers, showed that standardized incidence rates for transport sector workers were 2.7- 4.5 times higher than those in the total working-age male and global populations of the health micro-network studied. The associations between TB and being a transport worker, and between MDR-TB and being a transport worker are both strong (OR 3.06, 95%CI 2.2-4.2 and OR 3.14, 95%CI 1.1-9.1, respectively). These results indicate that the use of informal public transport is a risk factor for TB infection and an occupational risk in countries with characteristics similar to those in Peru [20].

A summary of the main results obtained in Lima, is presented in table 3.

Lima, Peru	Incidence of PTB calculated within general population based on PHC micro-net data	TB associated with transport occupation (OR=3.06; CI 95%: 2.2-4.2) and with MDR-TB (OR= 3.14; CI 95%: 1.1-1.9)
	Prevalence of PTB 12% among commuters in a suburban area of Lima	Use of informal transport system: working away from home (OR= 6,99; CI 95%: 0.89-54.61; PPR = 6.06); commuting in minibuses (OR= 44.9; CI 95%: 1.06-23.09; PPR=4.09) and commuting more than one hour (OR=3.35; CI 95%: 1.12-10.1; PPR= 2.07)
	Prevalence of Mycobacterium Tuberculosis infection through PPD test of 77% among minibus drivers in informal transport	High work-related exposure: more than two years in job (OR= 11.04; CI 95%: 3.17-38.43) and working more than 60 hours per week (OR= 9.8; CI 95%: 2.85-33.72).

CI: Confidence interval; OR: Odds Ratio; PPR: Positive Prevalence Ratio

Table 3. Pulmonary Tuberculosis (PTB) and associated factors observed within studies performed by GRAAL members in Peru

5. The patchwork: What do these findings mean? Are they useful?

TB, far from being under control, as was believed at the end of the decade of the 1990s, continues to cause many deaths, disability, and health expenditure; indeed, it has been recognized that the situation may be worsening due to an accumulation of structural condi-

tions favoring its appearance and development: increased poverty in important population nuclei (which are the most susceptible to the disease), migratory movements (whether due to economic, work, political or even environmental issues), higher incidence of other immunosuppressive diseases (mainly HIV/AIDS and diabetes), or weakening of health of certain individuals (such as due to malnutrition, and chronic pneumopathies), the increasingly more common appearance of forms of MDR, and the shortage of health resources to cope with TB, mainly in areas of greater socioeconomic exclusion. In this sense, the so-called developed countries have also felt the impacts of the disease, largely due the appearance of HIV/AIDS and to cases among immigrants who, whether legally or illegally, settle in foreign territories seeking to improve on the conditions which caused them to leave their places of origin.[4] These populations are generally speaking the most socioeconomically disadvantaged groups.

There is no doubt that TB is an outcome indicator of the socioeconomic, cultural and political structure of a population. TB is a historical reflection of the forms of social construction, particularly of the post-industrial revolution era experienced in capitalist countries. TB in this sense feeds, to a greater or lesser degree, depending on circumstances, on the social context in order to reproduce, and this fact finds expression, as documented in the present studies, in various gradients of exposure and susceptibility to the disease, in which the more socioeconomically disadvantaged groups are the ones most affected by the disease, but the ones which, paradoxically, usually receive least attention, whether in terms of prevention, diagnosis, or treatment, and hence cure rates are low.

Two million people die every year from TB, the majority of them in the "under-developed countries". The Global Plan to Stop TB 2006-2015 aims to treat 50 million people, save 14 million lives, and expand equitable access to quality diagnosis and treatment. According to this Global Plan, by 2010 it was expected "to be using diagnostic test that allow rapid, sensitive and inexpensive detection of active TB… and introduce the first new TB drug in 40 years. It also expects to see a new, safe, effective and affordable vaccine available by 2015" [21]. However, the World Health Organization, Pan-American Health Organization and Governments in general, establishing targets for TB control programs, take as their basis the reports they receive from the countries themselves, with the result that programs elaborated are eminently political, whose objectives and information basis constitute a kind of feedback system which rapidly departs from reality.

TB control programs thus planned are designed as though the social structure of the countries was homogeneous, and this impedes acting in such a way as to take account of the particularities of marginal populations, which are the ones presenting the highest rates of prevalence of this disease. The usual ways of working lead to government planning and actions being based on central estimates and tackling of global objectives. For example, the global medium-term goal for TB control is to halve TB prevalence and death rates by 2015 as compared to 1990 levels, and to achieve a reduction in its incidence, as part of the Millennium Development Goals (number six) [22,23].

4 The first report of a rise in cases of HIV/AIDS was published in 1991, affecting New York City, and the status of TB was changed to that of an AIDS-defining disease, although it has been calculated that currently 50% of new TB cases in the European Union occur among immigrants.

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This type of planning and programming of objectives apparently does not take account of the particular situations affecting the population, above all those aspects which are notably different from the global mean values. In fact the few population based studies available, some involving Latin American countries, likewise fail to treat marginal populations specially. For example, if it was not for international support, few governments would have sufficient resources to conduct national health surveys, which are usually carried out through household interviews, based on self-perceived morbidity, and which hardly ever include laboratory tests to identify diseased individuals (TB in our case). Generally, the level of disaggregation of surveys of this type goes no further than large geographical regions (north, south, east, etc), tending to disguise inter- and intra-regional heterogeneity, and the data are analyzed based on artificially created convenience categories, not based on observed patterns of disease or deaths.

In other words, it is usual that global policies emanating from the international agencies and institutions ignore the true situation, applying criteria of homogeneity in the calculation of targets, costs of equipment and supplies and staffing levels, among other aspects. Curiously, despite it being well known that social factors are related with TB, they are not taken into consideration in order to improve the quality of plans to control it.

In order to identify, analyze and ideally contribute alternative solutions to the problem of unmet needs of socioeconomically excluded populations, means working with samples which are not representative of the general population, but rather focused on these sub-populations, biased precisely due to their conditions. In this sense, our team has been employing the patchwork approach, involving studies focusing on marginal or susceptible populations, those with the worst socioeconomic and health conditions. In the case of TB, these circumstances (poverty, social vulnerability, and shortage of health facilities) are well recognized as one of the basic determinants of the presence and spread of the disease, but its characterization usually is not considered by health systems in their solution proposals [24].

For example, in marginalized rural areas, in the best case, active TB case finding is limited in practice, to identifying chronic coughers among users who seek health care. This results in at least three possible situations: a) there may be delays in the TB diagnosis (patients arrive in an advanced stage of the disease);[5] if the medical consultation is for reasons other than respiratory symptoms, TB may not even be detected;[6] and, c) that a certain proportion of patients do not use the health services (due to accessibility barriers which may be geographical, economic or cultural)[7] and hence are not even diagnosed [10].

5 Several studies have shown that groups living in conditions of greater socioeconomic margination present longer delays in seeking care for health problems [26,27].

6 In several studies we found that PTB status is not associated with the reason for visiting health services [10].

7 Political conflicts (belonging to a particular organization), religious conflicts (not belonging to the religion predominant in the community), administrative barriers (nearest clinic not the one assigned officially), and conflicts deriving from access to and utilization of natural resources (water, timber, etcetera) can mean that certain individuals are denied access to health services, or they are denied medical care or drugs [6].

In this context, women living in remote and marginalized regions, have a more pronounced lack of access to health services due to gender reasons: there are differences in the process of seeking medical care, and in the quality of the care received between women and men [25].

Furthermore, in Chiapas, Ecuador and Peru, as in many other regions of Latin America, TB cases notified to the information systems of the health sector, and from which incidence rates are estimated, correspond to cases detected in health services by acid-fast bacilli. We have documented that in rural and marginalized areas, its sensitivity is around 50%. This is an important aspect to consider because health system detection of TB cases is based on smear testing, meaning that in marginalized communities many cases are not detected. In consequence, the target of detecting at least 75% of cases is far from being reached, implying the presence of a not unappreciable risk of transmission of TB [9]. Indeed, the detection rate in hospitals studied in Chiapas is below this figure of 75%. The suboptimal case detection rate reflects an inadequate of quality medical care, probably health personnel are often overwhelmed by daily activities, as well as insufficiently trained, motivated, aware, and remunerated [10].

In order to increase detection rates, as it was demonstrated, the health system must take into account the considerable difficulties involved in obtaining and analyzing sputum samples in marginalized areas: cultural barriers (language spoken, world view) and economic barriers, as well as technical problems to be overcome in order to obtain adequate quantity and quality of sputum samples. It is therefore necessary to reduce the cultural and socioeconomic barriers between health care providers and people [10]. Not surprisingly, some results of our investigations show that apart from cultural barriers, there are also structural barriers [11].

In the same way, we have found that a very low proportion of patients eligible for anti-TB treatment effectively receive such treatment, and very high proportions of treatment failures and incomplete follow-up [10].

Two further aspects deserve special attention in the context of the studies conducted in Chiapas, Mexico, as well as in Peru: the problem of MDR, and the high mortality among patients diagnosed of PTB. Both indicators constitute expressions of the complete failure of the health system which, for whatever reason, did not manage to adequately treat these people, who consequently either died of TB, or were left as chronic MDR cases (which would lead to their death also, sooner or later).

In the case of MDR, it is well documented that the vast majority of cases of this type result from inappropriate treatment and follow up by the health system. According to official statistics, worldwide, the rates of MDR recorded in 2009 and 2010 were the highest ever, and trends in MDR rates are unclear in the majority of countries [28]. The observed rates of MDR in Chiapas suggest that in marginalized and excluded regions, it is a serious public health problem of alarming proportions. While the MDR rates calculated for the country as a whole are 1-3% for primary, and 20% for secondary MDR, in our studies these rates were 4.6% and 29%, respectively [12].

Although our results were made known to the health authorities, there are no signs to suggest that the TB situation has improved: the health system continues failing to diagnose cases

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appropriately and application of the DOTS strategy is very deficient: even if TB patients are diagnosed, in many cases they begin, but do not complete their anti-TB treatment. In this sense, we must emphasize the following aspects:

a. It is extremely difficult to perform culture analysis, in order to determine MDR status, in a patient with less than six months of treatment, due to poor quality sputum samples. The main obstacles to obtaining good quality sputum samples are: barriers in communication with indigenous people, distance of the communities from the centers where samples are processed, unsuitable transport conditions of samples (risk of exposure to sunlight or lack refrigeration), among others.

b. It is very plausible that in indigenous populations, due to their having less contact with health services, there are more undiagnosed TB cases and that, among non-indigenous patients, more TB cases are diagnosed but not necessarily treated adequately [12].

c. A patient confirmed with MDR condition, is practically impossible to treat, given the high cost of the secondary treatment, and because if the health system is incapable of guaranteeing the follow up of a patient sensitive to the four primary drugs during six months, it is probably even less able to follow up a MDR patient not only in terms of the time required (from 6 months to 1.5 or 2 years) but also in terms of level of patient care, due to the possible secondary effects of the "second line drugs" employed. In this sense, if a program cannot guarantee appropriate follow up and compliance with treatment among TB patients, it should not initiate their treatment, thus condemning them to a situation of no hope of cure, with all that this implies, not only for the patient, but also for his family, who apart from watching their family member suffer, are also exposed to the possibility of their catching the disease.

With regard to mortality due to TB, we have found unacceptably high rates. In addition, a considerable proportion of TB patients die without having received any medical care.[8] We found that 55% of patients whose death was related to TB, had died within two years of being diagnosed, possibly due to delays in diagnosis, and the poor quality of the follow-up in their anti-TB treatment. Whereas the life expectancy in Chiapas is 72.2 years [29], the average age of deceased patients was 47.4 years, representing an average of at least 24 potential years of life lost [13]. We believe that the accumulation of unfavorable living conditions such as malnutrition, poverty, as well as deficient and/or lack of health services, makes them an especially vulnerable group. According to official statistics, while in 2009 the PTB mortality for the country was 1.7/100,000 inhabitants in Chiapas it was 3.79 with the same denominator [14].

Our findings have provided evidence that in the area studied, patients being aged 45 years and over, not having completed the established six months of treatment, and not having been treated via the DOTS strategy, are all associated with a higher risk of the patient dying from PTB.

8 Eighteen percent of patients traced to their homes, in a study carried out in Chiapas, had died. Of the 40 deaths presumed to have been associated with PTB, 33 died without having received medical care [13]

Based on our findings, we can say that people from rural and indigenous communities suffer mistreatment by the health services [30], meaning, among other aspects, deficient application of the DOTS strategy (sometimes due to shortages in the supply of anti-TB drugs [31], or poor follow up), leading to higher mortality and increasing their chances of becoming MDR cases [12,13].

Another fact that reduces the chances of successfully carrying out patient follow up, is migratory movements. Migration in the region is mainly due to economic factors, but can also be for health reasons. Sometimes patients are registered by health services as urban patients when in fact they are not, or they give a false address in order to obtain the first consultation, but subsequently return to their rural communities or find another place to live without notifying the health services.

In addition, health services give little consideration to socio-cultural and anthropological aspects. For example, in indigenous medicine the process of health and illness involves their world view, their personal and community histories, in an atmosphere of trust in which supernatural intervention, transgression of social norms, culpability, or malice on the part of enemies, are all admissible possible causes of the disease [16,32,33]. In this sense, patients may seek care from traditional medicine practitioners, who attend them in accordance with their age-old diagnostic and therapeutic rituals.

On the other hand, the use of public transport is a risk factor of TB not only among users, but also among minibus drivers and fare-collectors, and hence may be considered an occupational disease in these workers, who work in conditions such that not only do they have precarious employment, with all its implications (temporary contract, no social security or medical insurance, among other aspects) but also their job places them in a position of greater vulnerability to TB, since if they don't work, they don't get paid, and hence it is very probable that many of them go to work despite their illness, if they are able to do so, only seeking care when the disease really makes it impossible for them to continue working. In this sense, a worker with active PTB is a source of infection not only for co-workers but also for passengers. In countries where TB is endemic with increased circulation of resistant mycobacteria, the situation could be even worse. In a situation of this kind, the health system should be implementing, at very least, home-based DOTS to avoid exposure as far as possible, as well as implementing specifically designed occupational health programs [19,20,34].[9]

Observation of the particular facts which determine the appearance of TB and its prognosis, shows that the diagnosis and treatment strategies employed by the health services are just that, strategies, rather than ends in themselves, something which, unfortunately, is frequently emphasized. If more clearly focused measures are not taken, TB will not disappear in marginalized areas, despite the fact that trends in the ecological indices suggest that TB is tending to decline in Latin American countries; rather, it will persist as a greater public health problem for years to come.

9 In fact, the authors believe that more attention ought to be given to the risk of infection, by any aerially transmitted disease that utilization of public transport represents, for both passengers and the workers.

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In our view, to remain with the idea that TB is decreasing in Latin America, tends to conceal the failure which the rise in MDR patients represents. In any case, countries are alarmed by the rise in MDR because of the cost of treatment and its inefficiency, not necessarily for the health and welfare of TB patients, particularly if they are poor, as our studies suggest.

One discussion point is clear. Objective-oriented programs which attribute to the entire country the same values of incidence and detection rates present a problem known as "trimmed estimation", meaning that when the established objectives are reached, case finding and detection are relaxed, or the resources to permit continuing with case finding and/or provision of treatment, are cut short.

If, together with these data we take into account cases in areas enjoying lower TB incidence, and if this total was the true number of existing cases, the result would necessarily be a systematic reduction in incidence rates, leading to false optimism, whose historical cost has been a relaxation of efforts to prevent and control TB worldwide during the decade of the 1980s of last century.

Imagine the simple case that we have an incidence rate of X, in a given country. This figure conditions the work plan for the coming year in terms of supplies, staff, anti-TB treatment drugs, etcetera. Later on, 70% of the reporting areas indicate a rate of 0.9X, another 15% of areas a rate of X, and the remaining 15% provide no data on rates, but their population is taken into account in the denominator. The final rate for this country, based on these hypothetical figures, would be 0.78X. In other words, not receiving reports from the areas with the worst conditions provokes an apparent reduction in the global rate.

Unfortunately, even without questioning the figures declared, we know that areas which do not report (or report, but at best with high levels of under-notification) are the ones with the poorest conditions, both in terms of the socioeconomic conditions of the population which theoretically must be cared for, and in terms of the lack of resources and other failures in the organization and functioning of the program. Thus, by apparently having fewer cases, the resources dedicated to the TB prevention and control program are also cut back, and this creates a vicious circle which is difficult to break, so that program outcomes may be false, i.e. underestimates of the numbers of cases.

There is therefore a clear need to promote studies specifically aiming to analyze the population groups most vulnerable to TB, and in this way ascertain more precisely their situation, even when they are not representative of what happens in a given country. Continuing to carry out representative population based studies can only yield the probably already known rate for the country as a whole, and the situation of marginal groups will not be reflected in such rate.

In this sense, the patchwork studies contribute very valuable elements which help to make more visible and understandable the situation of population groups which go unnoticed in the global rates utilized in public health. We would encourage potentiating studies which break with the classical schemes, and use methods appropriate for the analysis of samples considered too small by classical approaches, but without renouncing the maximum of scientific rigor, as demonstrated by the doctoral theses developed in projects conducted in the three settings we have dealt with, Chiapas (Mexico), Chine (Cotopaxi,

Ecuador), and Lima (Peru), and whose results have been published in journals of medium and high impact factor.

On the other hand, Ecuador and Peru present changes in their control program strategies: active case finding, incorporation of economic incentives, strategies to reduce stigma among patients, citizens' observatories and integration of TB research in academic circles, among other aspects. It will be fundamental to perform studies which evaluate possible effects of such changes.

As the studies have shown, a failure to introduce changes in the structure and functioning of TB prevention and control programs would have as a consequence that this disease will continue to severely affect the most marginalized sectors of society:

In the field of TB prevention, several authors recognize that effective efforts have not yet been fully considered, and that it is necessary to improve this issue, for example through better vaccines and better chemotherapy for preventive treatment [22].

In the field of TB diagnosis, efforts must be made to reinforce active case finding of coughers, as for example, incorporating other diagnostic tests which allow better detection of the disease (from the use of cultures, and conducting molecular tests, to the search for faster diagnostic methods, such as biosensors). Epidemiological surveillance systems rely on smear testing, failing to take into account that in marginalized and rural areas these tests with only around 50% sensitivity, leave large numbers of cases undetected by the health services, or who will only be captured in advanced stages of the disease [12-14]. It is not unusual to find, within a given Latin American country, that while highly developed regions have advanced technologies available for TB diagnosis, in others the only possibility for diagnosis is the smear test. Nor is it unusual in regions of this type to find that smear testing is done badly, both in numerical terms (hardly ever obtaining three samples from a given patient), and in terms of quality (for reasons attributable to the poor quality of samples, such as errors in collection, storage, transport, processing and reading of results) [10,35].

In regard to anti-TB treatment, while the DOTS strategy has achieved a certain level of effectiveness in curing patients and saving lives, the epidemiological impact has so far been less than predicted [22], perhaps among other reasons, because treatment programs do not find patients soon enough to significantly reduce transmission [5]. Thus it is necessary to ensure: a) training, awareness and supervision of health personnel about the importance of avoiding patients defaulting from treatment, as well as guaranteeing the appropriate supply of medication; b) when necessary to adapt the DOTS strategy, both socially and culturally, taking into account the community health agents, community world view, and implementing the scheme in the patient's homes, supporting them and their families economically during their treatment; and c) that patients and their family are accompanied during the six months of treatment, in order to cope with possible secondary effects and to overcome possible barriers (alcoholism, religion, gender issues, seasonal migration, etcetera) which make compliance with anti-TB treatment difficult.

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6. Conclusions: Tuberculosis as indicator of structural violence and violations of human rights

One of the main aspects we want to stress in the present work is the fact that analyzing TB through the measurement of global indicators conceals the situation of vulnerability to the disease suffered by socioeconomically disadvantaged population groups. The data obtained in the different studies we have presented show that there is a need for methodological approaches (such as that known as patchwork studies) which allow the measurement and analysis of the distribution of TB among different population groups.

It is well known that TB is one of the infectious diseases which has caused the most deaths among humans [36], above all among the socioeconomically most vulnerable groups [37]. These groups, apart from having higher risks of infection, developing active disease, and dying due to TB, are also the ones facing the greatest barriers (including information barriers) to access health services [38]. In this sense, and given that nowadays the medical resources to cure the disease are available, the fact that even today TB continues to cause deaths may be considered as an indicator of the violation of human rights in excluded and marginalized populations, as well as an indicator of "structural violence", given that it is precisely the social, economic, cultural and political structures which do not allow certain social groups to achieve their full potential, while other groups do so, due to the unequal distribution of power and available resources, placing some in conditions of social privilege and others in situations of social vulnerability [39].

The social context of TB is strongly related with social justice. The history of TB teaches us that the improvement of social conditions, work conditions ant the access to better quality food decreased its mortality in the pre-microbial stage [40].

Taking as a starting point that the appearance, development and distribution of TB is largely influenced by social determinants, and that public health achievements will depend on actions outside the health care sector [41], two forms of interventions are necessary: a) those reducing peoples' vulnerability, such as poor living and working conditions, and improving nutrition, among other aspects (such as structural and socioeconomic conditions), and b) by seeking alternatives that promote higher levels of prevention, diagnosis and cure of TB.

Two clear examples that help to visualize health related inequalities in respect to TB are firstly, the so-called "10/90 gap", in reference to the fact that only 10% of worldwide expenditure on health research and development is devoted to the problems primarily affect the poorest 90% of the world's population, and that 90% of worldwide expenditure is devoted to the problems that affect the richest 10% of the world's population.

The second example is the comparison between HIV-AIDS and TB. Whereas the first cases of HIV-AIDS were described during the decade of the 1980s of the last century, today it is one of the diseases which have received the most resources for its prevention and treatment, and notable advances have been achieved in these aspects, and in improving survival of patients. At the end of the last century, it was practically a death sentence, and yet today we have a series of drugs which increase both survival and quality of life of these patients.

In contrast, despite the fact that TB has been accompanying humans for thousands of years, and that the etiological agent was first described in 1882, it is the disease which has alone caused the greatest numbers of deaths in the adult population worldwide, and for which the resources currently dedicated are insufficient to lead us to expect, in the foreseeable short or medium term, the appearance of more effective measures for its control. Perhaps this is because it is considered a disease of the poor, and thus there is no incentive to "invest" in it? As some of our colleagues have pointed out: "even if the Global Plan to Stop TB is successfully implemented and results in the expected rate of reduction in incidence of about 6%, the global incidence rate in 2050 would still be of the order of 100 per million of inhabitants, i.e. about 100 times greater than the elimination target" [22].

From the viewpoint of international human rights law, by providing woefully substandard health services to marginalized populations, and failing to assure prevention of disease through appropriate public health measures, governments violate their obligations in human rights [6].

In this sense, high rates of TB constitute a reflection of the fact that certain populations face important obstacles in their exercise of the right to health, and other economic, social and cultural rights, due to the main social determinants of this disease being associated to social exclusion and poverty [31]. The presence of TB constitutes a violation of the right to the highest attainable standard of physical and mental health ("the right to health protection") which is inextricably related to the right to life and other human rights that allow an individual to live with dignity [6,42].

The International Covenant on Economic, Social and Cultural Rights (ICESCR) in its Article 12, Paragraph 2, sets out the steps states should take in order to fulfill the highest attainable standard of health, and includes "the prevention, treatment and control of epidemic, endemic, occupational and other diseases, as well as the creation of conditions which would assure to all medical service and medical attention in the event of sickness".

The General Comment issued by the Economic, Social and Cultural Rights Committee [43], establishes that "the underlying determinants of health, such as including adequate sanitation facilities, hospitals, clinics and other health related buildings, trained medical and professional personnel" have to be available in sufficient quantity with the States parties, and specifies that health facilities, goods and services must be available, accessible, acceptable and of adequate quality.

In this General Comment, accessibility has four overlapping dimensions [43, paragraph 12 (b)]:

First, the principle of non-discrimination,[10] on the grounds of sex (poorer quality of care among women than men), ethnic group (patients from indigenous communities receive poorer care), color, political filiation (belonging or not to the dominant political party in a region can affect access to care and to medication), religion (care may be denied to community members not belonging to the dominant religion), physical or mental disability, health status, sexual

10 Non-discrimination is a core principle for the full realization of the right to health, as for all human rights [6]

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orientation, among others. A violation occurs when there is the intention or effect of nullifying or impairing the equal enjoyment or exercise of the right to health [43, paragraph 18].

Second, physical accessibility, meaning that health facilities, goods and services must be within safe physical reach for all groups, specially the vulnerable or marginalized ones, such as ethnic and indigenous populations.

Third, economic accessibility requires that health facilities, goods and services must be affordable for all, including socially disadvantaged groups. It points out that poorer persons should not be disproportionately burdened with health expenses, and those individuals (e.g. peasants) who through not having access to cash face particular difficulties, need to be considered in governmental policy and practice.

Fourth, the right to seek, receive and impart information and ideas concerning health issues, which includes health information in indigenous languages.

With regard to acceptability, it is understood that "health facilities, goods and services must be respectful of medical ethics and culturally appropriate… [and] requires respect for traditional medicines and practices which have not been shown to be harmful to human health" [43, paragraph 12 (c)].

For adequate quality, it requires that health facilities, goods and services must be scientifically and medically appropriate and of good quality (skilled medical personnel, scientifically approved and unexpired drugs, and hospital equipment, among other aspects) [43, paragraph 12 (d)].

Unfortunately, the findings of our studies indicate that health care is not sufficiently available or accessible (in either quantity or quality) to more disadvantaged social groups, creating mistrust among them of the government health services, something that is reflected in the relatively high percentage of people that do not use these services, even for vaccinations. This situation is more marked when health services are not culturally adapted, when people perceive mistreatment on the basis of their ethnicity or social conditions and health personnel make disparaging remarks about their habits and demeanor [6].

Other indicators of violation of human rights in TB patients are:

- Failure to improve the level of health in a population. The fact that health indicators, in our case indicators of TB do not improve in a population, even though they do not worsen, constitutes a violation of human rights (specifically of the right to "Non-retrogression and Adequate Progress").
- The presence of inequalities in access to quality and coverage of health services (in the case of TB affecting aspects from prevention to cure).
- The lack of meaningful popular participation, in regard to the making of decisions which involve the design, organization and functioning of health services. It is common to find that local health services nominate a "health promoter", charged with various activities such as vaccination, routine pediatric checkups, etcetera, but this does not necessarily mean the community has a voice or participates in the definition of its own priorities, decision-making

in regard to planning of activities or elaboration and evaluation of health programs for the community. Veneklasen and colleagues have said [44]: "True rights-based participation requires programs that enable people to be active, informed and critical agents and citizens, rather than objects of charity". In this sense, the International Labor Organization, Convention 169 [45] stresses that health services shall, to the extent possible be, "planned and administered in co-operation with the peoples concerned and take into account their economic, geographical, social and cultural conditions as well as their traditional preventive care healing practices and medicines".

- The lack of accountability in health programs, which in addition are usually not evaluated. In this sense, the fact that a person is not treated appropriately by the health services, which in itself constitutes a violation of his right to health, should also imply a right to compensation by the State, which could take the form of restoration of his health, economic compensation, satisfaction or guarantees that the situation will not be repeated. On the other hand, if it is true that more resources are needed, they must be spent in such a way as to foster self-sufficiency and reduce inequities. Greater health care expenditure does not necessarily reduce inequalities [5].

- This dimension is also related to the enactment and enforcement of laws to provide sanctions for gender based violence or sexual abuse of women patients by health personnel, as well as people affected by mistrust. In addition, a legal framework must be adopted to operationalize the protection of patient human rights in the health services, establishing mechanisms for monitoring their compliance. The figure of a human rights ombudsman is a good example for monitoring, to investigate and sanction perpetrators in cases of abuse or malpractice and medical negligence claims [6].

- The lack of multi-sectorial strategies. Governmental programs should not compete among themselves, but rather be designed inter alia to promote health services, improve adequate dwelling conditions, education, work, and adequate nutrition. Until this happens, the plight of marginalized populations will persist. In the last instance, violations of the Economic, Social and Cultural Rights occur "when a state fails to satisfy a minimum core obligation to ensure the satisfaction of, at very least, minimum essential levels of the rights" [46].

- The lack of access to health information. Social participation and monitoring are impossible without access to information. This implies that governments should collect data on a disaggregated basis (by ethnicity, gender, socio-economic status, language, among other aspects) and this information, together with the methodologies used, must be readily available to the public. Of course, it also includes the right of TB patients to see their medical records, to give informed consent in all procedures, and to confidential management of their disease.

The performance of patchwork studies has allowed us to identify, evaluate and measure the situation of marginalized population groups in three different contexts (Chiapas, Mexico; Chine, Cotopaxi, Ecuador and Lima, Peru). Our findings revealed the poor quality of diagnosis and treatment of TB patients. Our data can be useful not only in the studied regions, but also in other countries with similar socioeconomic inequalities, if they are taken into account by

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the health authorities in order to provide all people (especially the more socially vulnerable groups) with: effective prevention programs, a reliable and timely diagnosis, adequate anti-TB treatment and follow-up, clear and appropriate information and counseling about TB (what it is, mechanisms of transmission and possibility of infecting others, etcetera).

In consequence it is necessary change the dysfunctional health system that contributes to the persistence and intensification of exclusion, voicelessness, and inequity, while simultaneously defaulting on its potential and obligation to fulfill human rights and contribute to the building of more equitable, egalitarian and democratic societies. The history of TB teaches us that the improvement of social justice led to increase the global health conditions and thus, it avoids the called "social diseases", including TB. The academic community has much to say and actively contribute in these aspects. The first step is to do research in order to make visible excluded people. To analyze, sensitize and lead to better socioeconomic conditions is an assignment for all of us.

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Data are rapidly accumulating from all over the world regarding the efficacy of standardized treatment regimens for drug-sensitive, drug-resistant TB and latent TB infection. While we are facing the menace of multi drug-resistant TB [MDR-TB], extensively drug-resistant tuberculosis [XDR≠ TB] has emerged threatening to undermine global efforts at TB control. Hence we have included chapters to cover all aspects of the diagnosis and management of MDR TB. This book will cover all these developments in great detail. With the widespread availability of internet globally various standard web resources available on TB have also been included so that the readers may get the comprehensive and updated guidelines from these resources. The changing clinical presentation of TB, advances in laboratory, imaging diagnostic modalities, therapeutic measures and emergence of MDR TB all suggest a pressing need to have a updated book on TB. Furthermore, while all physicians encounter the TB disease in their clinical practice, there have been a lot of controversies and misconceptions over various issues for the diagnosis and management of TB. Paucity of a well referenced, updated, standard book of TB has prompted us to undertake this venture sharing the clinical experience of global experts of TB. Our book contains chapters on epidemiology, immune-pathology, diagnosis, treatment and latest advances for TB, highlighting the global perspective of tuberculosis. World-wide resurgence of MDR TB indicates that the battle against this foe of mankind will continue in the coming years. TB still remains to be a research priority of paramount importance from medical, social and financial aspects and we have attempted to highlight all the aspects for the treatment of TB. We believe that this book will serve as a practical guide for the diagnosis and management of TB for practicing physicians (especially pulmonologists and internists) and all those who are involved in the management of TB.

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