Journal of Zhejiang University-SCIENCE B (Biomedicine and Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.jzus.zju.edu.cn; www.springerlink.com
E-mail: jzus@zju.edu.cn

Review:

Application of antigenic biomarkers for *Mycobacterium tuberculosis**

Elba RODRÍGUEZ-HERNÁNDEZ[§†1], Laura Itzel QUINTAS-GRANADOS[§2], Susana FLORES-VILLALVA[1], Jorge Germinal CANTÓ-ALARCÓN[3], Feliciano MILIÁN-SUAZO[3]

[1]*Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), Centro Nacional de Investigación Disciplinaria en Fisiología y Mejoramiento Animal, Km. 1 Carretera a Colón, Ajuchitlán Colón, 76280, Colón, Querétaro, México*

[2]*Universidad Mexiquense del Bicentenario, Unidad de Estudios Superiores de Tultitlán, Avenida Ex-Hacienda de Portales s/n, Villa Esmeralda, Tultitlán Estado de México, 54910, Tultitlán, México*

[3]*Universidad Autónoma de Querétaro, Facultad de Ciencias Naturales, Avenida de las Ciencias s/n, Juriquilla, Delegación Santa Rosa Jáuregui, 76230, Querétaro, México*

[†]E-mail: rodriguez.elba@inifap.gob.mx

Received June 16, 2020; Revision accepted Aug. 19, 2020; Crosschecked Oct. 13, 2020

Abstract: The study and characterization of biomolecules involved in the interaction between mycobacteria and their hosts are crucial to determine their roles in the invasion process and provide basic knowledge about the biology and pathogenesis of disease. Promising new biomarkers for diagnosis and immunotherapy have emerged recently. Mycobacterium is an ancient pathogen that has developed complex strategies for its persistence in the host and environment, likely based on the complexity of the network of interactions between the molecules involved in infection. Several biomarkers have received recent attention in the process of developing rapid and reliable detection techniques for tuberculosis. Among the most widely investigated antigens are CFP-10 (10-kDa culture filtrate protein), ESAT-6 (6-kDa early secretory antigenic target), Ag85A, Ag85B, CFP-7, and PPE18. Some of these antigens have been proposed as biomarkers to assess the key elements of the response to infection of both the pathogen and host. The design of novel and accurate diagnostic methods is essential for the control of tuberculosis worldwide. Presently, the diagnostic methods are based on the identification of molecules in the humoral response in infected individuals. Therefore, these tests depend on the capacity of the host to develop an immune response, which usually is heterogeneous. In the last 20 years, special attention has been given to the design of multiantigenic diagnostic methods to improve the levels of sensitivity and specificity. In this review, we summarize the state of the art in the study and use of mycobacterium biomolecules with the potential to support novel tuberculosis control strategies.

Key words: *Mycobacterium tuberculosis*; Recombinant antigen; Diagnostics; Biomarker
https://doi.org/10.1631/jzus.B2000325 **CLC number:** R52; R446.5

1 Introduction

Tuberculosis (TB) is an infectious disease caused by the bacillus *Mycobacterium tuberculosis* (*Mtb*), which can cause latent or active disease in humans. In 2018, about 10 million people had the disease, of whom 1.5 million died. *Mtb* is an obligate aerobic rod-shaped bacterium that contacts host cells and is subjected to the host immune response, resulting in latent TB infection (LTBI) or complete clearance of the bacteria. In LTBI, the *Mtb* reproduces inside the cellular host, developing a complex relationship that might progress to active TB infection (ATBI). Because TB is one of the top 10 causes of death from an infectious agent, prevention strategies and the

§ The two authors contributed equally to this work

* Project supported by the Consejo Nacional de Ciencia y Tecnología (CONACYT; No. 284118), México

ORCID: Elba RODRÍGUEZ-HERNÁNDEZ, https://orcid.org/0000-0002-9551-7111

development of better vaccines have been pursued (Kaufmann, 2020). Also, about 2000 million people worldwide have LTBI that might progress to ATBI at any time (Weiner and Kaufmann, 2014). Thus, the development of new treatments and vaccines is urgently needed (Kaufmann, 2020).

The most effective strategy, implemented by governments to control TB in humans, has been immunization with Bacille Calmette-Guérin (BCG) applied during childhood (Weiner and Kaufmann, 2014). This vaccine induces a robust Th1 response and promotes the development of mycobacterial-specific polyfunctional and cytotoxic T cells. Although it confers a variable and incomplete protection against pulmonary TB in humans, it is highly effective in preventing the severe forms of TB in childhood (Fine, 2001; Weiner and Kaufmann, 2014). The variability in the vaccine's efficacy is multifactorial, comprising genetic variation among vaccinated individuals, interference of the mycobacterial environment, and the use of different BCG strains (Weiner et al., 2013; Weiner and Kaufmann, 2014). Therefore, the goal is to develop a more efficient vaccine (Paylor, 2014).

The *Mtb* complex (MTC) includes *M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium pinnipedii*, *Mycobacterium avium*, *Mycobacterium canetti*, *Mycobacterium orygis*, *Mycobacterium caprae*, *Mycobacterium suricattlae*, *Mycobacterium mungi*, and *Mycobacterium microti*, which are genetically related bacteria that cause similar clinical symptoms (Sinha et al., 2016). The MTC species are genetically similar in nucleotide sequence, have identical sequences in their l6S ribosomal RNA (rRNA), and share >95% homology in their genomes (Forrellad et al., 2013; Banuls et al., 2015). There are differences among these species in their host tropism, phenotype, and pathogenicity (Forrellad et al., 2013; Banuls et al., 2015). Among the MTC species, *Mtb* is the most important clinically, due to its relevance as the main causative agent of TB in human beings. Thus, in this review, we focus on *Mtb*.

TB biomarkers are molecules or substances that can be found in the infected body when *Mtb* is present. They can be products of *Mtb* itself or of the body in response to infection caused by *Mtb*. Biomarkers play a crucial role in the detection, treatment, and control of TB. They are useful in diagnostic tests for the detection of ATBI, assisting in early diagnosis and facilitating surveillance during and after treatment. Therefore, the application of biomarkers provides an alternative to current methods that have low sensitivity and specificity. Antigen characterization leads to antibody- or antigen-directed assays that can improve the diagnosis or prognosis of TB. These approaches will improve detection strategies aimed at distinguishing between active and latent diseases and preventing dissemination of contagion, as well as informing decisions on appropriate treatments needed by patients. In this review, we discuss some of the most relevant *Mtb* antigenic biomarkers.

2 TB diagnosis strategies

Diagnosis allows TB control in a simple, quick, and affordable manner (Amanfu, 2006). However, about 36% of new TB cases are undiagnosed due to the limitations of current diagnostic methods (WHO, 2019). As an initial test for TB diagnosis, acid-fast bacillus (AFB) smear microscopy and culture are used (Monkongdee et al., 2009). *Mtb* isolation by culture is considered the "gold standard" for pulmonary TB and extrapulmonary TB (EPTB) (Bourassa, 2018). However, the sensitivity and specificity of these methods are limited, and they are time-consuming (Swaminathan and Rekha, 2010). Nonpulmonary TB is the most difficult to diagnose because of the lower mycobacteria concentration in samples (Delogu et al., 2013).

Culture is the most reliable test for TB diagnosis; however, the results are obtained between three and four weeks after sample collection due to the slow growth of *Mtb*. Moreover, the sensitivity and specificity of culture tests are limited (Swaminathan and Rekha, 2010). Microbiological methods are the most conventional diagnostic tests, but depend on sputum samples, which are difficult to obtain (WHO, 2019). The Ziehl-Neelsen stain detects the MTC, but is unreliable in detecting nontuberculous mycobacteria (Aitken et al., 2019). Patients with a suspicious diagnosis or previous positive diagnosis with chronic fever and cough for more than two weeks and weight loss are evaluated by chest radiography. When the test results are inconclusive, patients must be monitored with additional tests (Ryu, 2015). Because these tests

are not quantitative, the monitoring of treatment efficiency is limited (Monkongdee et al., 2009; Denkinger et al., 2013).

Molecular diagnosis tests based on polymerase chain reaction (PCR) using sputum samples have also been used for TB diagnosis. However, mycobacterial DNA is difficult to obtain in samples from asymptomatic patients and the tests have low sensitivity, leading to additional challenges in the diagnosis of EPTB. No "gold standard" test is available for LTBI diagnosis. Currently, LTBI is diagnosed indirectly by detecting specific antigens using the Mantoux tuberculin skin test or tuberculin skin test (TST) that uses a tuberculin-purified protein derivative, or by using the interferon-gamma release assay (IGRA) (Mack et al., 2009; Dheda et al., 2016). However, false-positive reactions with nontuberculous mycobacteria and BCG-vaccinated individuals are observed with TST (Huebner et al., 1993; Seddon et al., 2016). Some diagnostic tests do not distinguish between ATBI and LTBI. The TST, Ziehl-Neelsen stain assay, and IGRA are used for ATBI verification, while LTBI is confirmed by chest X-ray (Katial et al., 2001). Furthermore, the detection of specific genes, such as guanylate-binding protein 5 (*GBP5*), dual specificity phosphatase 3 (*DUSP3*), and Kruppel-like factor 2 (*KLF2*), can distinguish between ATBI and LTBI, but not EPTB (Sweeney et al., 2016).

Mycobacterial culture-filtering antigens have been proposed as excellent candidates to diagnose pulmonary TB (Manca et al., 1997; Silva et al., 2003). *Mtb*-specific peptide fragments, such as ESAT-6 (6-kDa early secretory antigenic target) and CFP-10 (10-kDa culture filtrate protein), are involved in mycobacterial virulence and are recognized by T cells during human infection (Forrellad et al., 2013; Liu et al., 2017). For TB diagnosis, specific peptide fragments are quantified from serum samples using antibody-labeled silicon nanoparticles and high-throughput mass spectrometry (NanoDisk-MS) with high specificity (87%–100%) (Liu et al., 2017). The *Mtb* cell wall has a thick envelope comprising lipoarabinomannan (LAM) that modulates the host immune response (Briken et al., 2004). Purified LAM material has been used for immunoassays using sputum, blood, and urine samples from ATBI with promising results (Sigal et al., 2018; Bulterys et al., 2019; Broger et al., 2019; Kawasaki et al., 2019; Brock et al., 2020). Despite the significant advances in TB diagnosis, a better diagnostic method that allows accurate identification among infected individuals, infected vaccinated patients, and healthy individuals is needed. Following the World Health Organization (WHO) call, scientific efforts are being directed to develop a rapid test based on biomarkers to detect ATBI (WHO, 2014) in a point-of-care (POC) format such as that based on the lateral flow urine LAM assay (LF-LAM) with high specificity and sensitivity, allowing immediate administration of treatment if required (Lawn et al., 2012, 2015; WHO, 2015; Shah et al., 2016).

The production of soluble recombinant molecules is necessary to validate their use in the different types of diagnostic tests to ensure high yields of mycobacterial antigens. Therefore, the cloning, expression, and purification of these antigens should be generally and ideally optimized in *Escherichia coli* as a host that will allow easy and low-cost handling. *Mtb*-secreted antigens mediate important biological functions through interaction with the cells they infect. An important aspect of their function is that these antigens contribute to the virulence and pathogenesis of the disease (Beatty and Russell, 2000; Pieters and Gatfield, 2002; Adlakha et al., 2012). Little is known about the actions or exact destination of mycobacterial antigens during infection. However, several extracellular proteins are known to be secreted in a growing culture and are vital molecules to contact host cells in the initial phase of infection in humans and animals (Pollock and Andersen, 1997).

Serological tests are an important resource for diagnosing most infectious diseases, as they are simple, fast, and often inexpensive. These tests are useful for detecting EPTB, which is difficult to detect by conventional methods. Antigens are used to detect circulating antibodies in these assays. Among the tests used are complement fixation, hemagglutination, radioimmunoassay, and enzyme immunoadsorption tests (Nurwidya et al., 2018). Some of these assays are commercially available, but their specificity and sensitivity are low.

3 Antigenic components involved in *Mtb* pathogenesis

Mtb H37Ra is an attenuated strain that is used as a model to elucidate virulence mechanisms. When

tubercle bacilli from the air reach the alveoli of an uninfected host, TB infection is initiated. The recognition between macrophages and the mycobacterium is carried out through direct interaction of surface components, such as the cholesterol-rich domains of the host cell plasma membrane (Gatfield and Pieters, 2000). A macrophage-inducible C-type lectin (CLEC4E), called mincle, is an important factor in the interaction between mycobacteria and macrophages, leading to granuloma formation (Feinberg et al., 2013). In the initial infection site, phagocytosis through the direct interaction of the macrophage with the mycobacterium is crucial for the lung host defense, because opsonization does not occur efficiently in noninflammatory bronchoalveolar fluid (Fenton and Vermeulen, 1996). At this point, alveolar macrophages phagocytize mycobacteria; however, if bacilli survive, they replicate and diffuse into nearby cells (Wolf et al., 2008). Macrophages have specific receptors for various opsonins that coat the mycobacteria. Through the opsonization of the mycobacterium with complement molecules, immunoglobulins, mannose-binding proteins, and surfactant factor A, recognition is made, and the mycobacterium enters the macrophage efficiently (Schlesinger et al., 1990; Fenton and Vermeulen, 1996). These receptors bind to nonopsonized *Mtb* or recognize opsonins on the bacilli surface. Among the receptors are C-type lectins such as the mannose receptor (MR), dendritic cell-specific intercellular adhesion molecule-3, grabbing nonintegrin (DC-SIGN) and dectin-1, complement receptors, surfactant protein receptors, scavenger receptors, glycosylphosphatidylinositol (GPI)-anchored receptors such as CD14, and Toll-like receptors (TLRs) (Ehlers and Daffé, 1998; Ernst, 1998; Means et al., 1999). Thereafter, at the primary infection site, neutrophils and lymphocytes form a cellular infiltrate encapsulating the bacilli (Ghon complex) (Ghon, 1923). *Mtb* can prevent phagosome maturation due to its interference with Rab-controlled membrane trafficking (Vergne et al., 2004). The Ghon complex maintains bacilli in a dormant, nonmetabolic active state for many years or a lifetime. Occasionally, for unknown reasons, mycobacteria start replicating inside this primary lesion, and this might progress to active infection (Bishai, 2000). However, in LTBI patients, bacilli persist in tissues and cells not associated with the Ghon complex (Hernández-Pando et al., 2000), suggesting that the site of primary infection is not always associated with ATBI. Many molecular factors, which might trigger an immune response, are involved in the bacterial pathogenesis and correspond to major cellular components.

In the mycobacteria–host interaction, the components of the capsule and those that are secreted by the mycobacterium are decisive for the invasion, replication, and survival of the bacillus in the new environment. The functional and structural components of the mycobacterial plasma membrane, as well as the cell envelope, are the main strategic targets for developing effective drugs and synthesizing candidate molecules for diagnosis and vaccines (Santos et al., 2017). Moreover, carbohydrates are the most abundant structural molecules in mycobacteria, and they are an important factor for immune evasion. However, carbohydrates are recognized by macrophages, T lymphocytes, and other host immune response cells (Schlesinger, 1993).

Mycobacteria comprise a plasma membrane, the peptidoglycan-arabinogalactan complex (AGP) asymmetric outer membrane or "mycomembrane," covalently bound to AGP through mycolic acids, and an outer capsule (Kalscheuer et al., 2019). The cell envelope is the key factor for the survival of the mycobacterium, and the lipids and polysaccharides comprising it have unique chemical structures. Bacilli have a thick wall and a cell membrane separated by a periplasmic space (Kalscheuer et al., 2019). When discussing mycobacterial composition, the molecules that activate the host's immune system are important; wall biomolecules play a major role in infection pathogenesis. Because effective drugs against TB target the synthesis of cell wall components (Delogu et al., 2013), the cellular composition of *Mycobacteria* helps to identify novel biomarkers for diagnosis or treatment.

The cell envelope of *Mtb* comprises the plasma membrane, cell wall core (a covalently linked mycolic acid, arabinogalactan, and peptidoglycan complex (MAPc)), and the outermost layer (polysaccharide-rich, capsule-like material) (Brennan and Nikaido, 1995). Structural and functional molecules from the plasma membrane and cellular envelope are targets for new anti-TB drugs (Santos et al., 2017).

The capsule is formed by polysaccharides and proteins, but also contains specific lipids that form the

outer leaflet and outer membrane whose inner leaflet has mycolic acid residues (Brennan, 2003). The resistance of *Mtb* to antibiotics and other therapeutic agents is due mainly to this asymmetric permeability barrier, which allows molecular traffic in both directions. Capsule proteins such as LpqH (lipoprotein) and PstS1 (phosphate-binding protein), Ag85B (secreted antigen) and capsule polysaccharides have been proposed as target molecules for vaccine development (Kalscheuer et al., 2019).

Mtb has a peculiar cell wall core consisting of *N*-glycosylated peptidoglycan linked to D-arabino-D-galactan (AG) via phosphoryl-*N*-acetylglucosaminosyl-rhamnosyl linkage units. AG is esterified by α-alkyl-β-hydroxy mycolic acids (Delogu et al., 2013). A thin layer of peptidoglycan is covalently linked to arabinogalactan and LAM, which are bound to mycolic acid, forming the AGP (Kalscheuer et al., 2019). AG strands and peptidoglycan are coiled and perpendicular to the plane of the plasma membrane (Dmitriev et al., 2000). The mycobacteria phosphatidylinositol phosphate synthase (PgsA1), which is involved in the synthesis of inositol-derived phospholipids that form the bacilli cell wall (Guerin et al., 2010), has been used to develop novel antibacterial drugs with promising results.

The major components of the wall are LAM, arabinogalactan, and other carbohydrates: mycolic acids, glycolipids, phenolic lipids, and peptidoglycan. Peptidoglycans in the first layer of the cell envelope allow rigidity and osmotic stability (Jankute et al., 2015). The cell wall is a porous membrane that surrounds the surface of the bacterium, allowing the transport of molecules into and out of the cell. It is a heteropolymer formed by short peptide chains and glycans that crosslink to produce a macromolecule with great elastic capacity. Also, the cell wall comprises *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues linked by β-1>4 bonds (Egan et al., 2018). Catalytic enzymes and secretion products are released from the *Mtb* cell wall, and many of these are virulence factors. Among the secretion products are CFPs, such as KatG (catalase-peroxidase), SodA (superoxide dismutase), HspX (alpha-crystallin), ESAT-6/CFP-10, and glutamine synthase. ESAT secretion systems (ESXs) are also important mycobacterial virulence factors (Abdallah et al., 2007). In *Mtb*, ESX1 secretes many antigens, such as ESAT-6 and CFP-10, which are highly immunogenic secreted proteins used in TB diagnosis. In culture, ESAT-6 and its chaperone CFP-10 are abundantly secreted highly immunogenic proteins that are involved in different mycobacterial virulence mechanisms (Dillon et al., 2000). CFP-10 and ESAT-6 form a 1:1 complex that specifically binds to the surface of macrophages and monocytes, suggesting a role in the host signaling mechanisms for the advantage of the mycobacteria that might lead to the modulation of host behavior (Renshaw et al., 2005). CFP-10 and ESAT-6 are encoded by pair-arranged genes in a single locus (RD1), which includes a large family of mycobacterial proteins (Renshaw et al., 2002). These genes are coordinately regulated, and despite lacking a signal sequence, both proteins are secreted (Pym et al., 2003). Together, these proteins modulate innate and adaptive immune responses. ESAT-6 inactivation results in an important reduction of virulence (Pym et al., 2002). Furthermore, ESAT-6 interacts with other proteins. When ESAT-6 interacts with TLR2, a decrease in interleukin-12 (IL-12) is observed, leading to the production of Th2 lymphocytes, which help *Mtb* survival in the host cells. ESAT-6 also interacts with host protein β-2-microglobulin (β2M), resulting in the downregulation of class 1-mediated antigen presentation (Sreejit et al., 2014). Also, several proteins of the mycobacterial secretory proteome include those between 35 and 45 kDa bound to host cells (Adlakha et al., 2012).

However, some of the virulence factors are not secretion products, but are constituents of the cell wall, such as ERP (exported repetitive protein), FadD26 (long-chain-fatty-acid–AMP ligase), FadD28, MmpL7 (phthiocerol dimycocerosate exporter), FbpA (diacylglycerol acyltransferase/mycolyltransferase Ag85A), MmaA4 (hydroxymycolate synthase), PcaA (cyclopropane mycolic acid synthase 3), OmpA (outer membrane protein A), HBHA (heparin-binding hemagglutinin), and LAM. HBHA is a mycobacterial adhesin (Delogu and Brennan, 1999) that, together with PE_PGRS (highly homologous gene families encoding complex proteins) (Iantomasi et al., 2012), is a surface *Mtb* constituent involved in the interaction of mycobacteria with the host cell and might be a possible target for diagnosis. Moreover, PhoP (possible two-component system response positive transcriptional regulator) involved in bacilli pathogenesis

(Ryndak et al., 2008) and enzymes that combat the reactive oxygen intermediates, such as SodA, SocC (superoxide dismutase [Cu–Zn]), KatG (catalase-peroxidase) and AhpC (alkyl hydroperoxide reductase C) (Smith, 2003), might be promising targets for vaccine design.

4 Omics approaches for identifying TB biomarkers

Genome sequencing of mycobacteria such as *Mtb* has made it possible to characterize and study the genes important in TB's pathogenesis, such as immunogenic antigens, which have been analyzed using various approaches (Cole et al., 1998; Garnier et al., 2003). The *Mtb* genome showed that almost 100 genes encode large proteins with a sequence highly homologous with Pro-Glu (PE), and many also contain a repetitive domain rich in Gly and Ala, referred to as the polymorphic GC-rich repetitive sequence (PGRS) region. These PE and PE_PGRS protein families are present in the cell walls of mycobacteria and might be important in controlling TB infection (Brennan and Delogu, 2002).

Studies have reported several mycobacterial secretory proteins of the proteome that are candidate molecules for studying interaction with the host cell. Adlakha et al. (2012) investigated the interactions between mycobacterial secretion proteins in a human alveolar cell line and demonstrated that various mycobacterial proteins between 35 and 45 kDa bind to the cells with high affinity, resulting in lysis, guiding the cells to release important cytokines of anti-mycobacterial immunity. The expression of virulence molecules in bacteria is regulated by various environmental and host factors. In the host–pathogen interaction, some genes are stimulated and expressed by the host during infection (Mekalanos, 1992; Heithoff et al., 1997). Therefore, the identification of antigens in infectious microorganisms is crucial because the interaction of antigens with molecules involved in the immune response, and their potential value can be studied to aid the design of recombinant vaccines and diagnostic methods (Vir et al., 2014).

The description of cell molecular mechanisms has allowed the development of techniques to manipulate macromolecules. It is now quite easy to synthesize heterologous proteins. Protein synthesis through genetic engineering allows a detailed study of the binding sites of many protein structures through molecular dynamics and modeling. To understand the dynamics of the host–mycobacteria interaction, "omics" was used to integrate large-scale experimental data. Transcriptomic studies of *Mycobacterium* allowed the construction of metabolic pathways based on genomic information (Boshoff and Lun, 2010). The transcriptional profile of *Mtb* in the macrophage infection phase provides information about regulatory and catalytic routes, helping to identify the roles of key components, such as WhiB3, which maintains redox homeostasis, the transcriptional regulator Rv3574, RshA-PknB-SigH involved in the regulatory pathway for stress adaptation, IdeR related to iron regulation, RelA, and PhoP (Rodriguez, 2006; Kendall et al., 2007; Park et al., 2008; Singh et al., 2009). Transcriptional analyses performed on different infected tissues demonstrated that *Mycobacteria* responded differently to external signals, making it difficult to understand the infection dynamics. Analysis of the *Mtb* H37Rv transcriptome in a lipid-rich environment using RNAseq revealed the expression of 368 genes involved in the response of the efflux systems, iron capture, and sulfur reduction (Aguilar-Ayala et al., 2017).

Comparative genomic studies have revealed genomic differences in *Mtb* lineages, particularly in virulence and drug resistance. For example, methylation in RNA expression is associated with the pathogenesis of *Mtb*. Sequence-based genomic sequence analysis of 22 isolates of *Mtb* demonstrated lineage-specific differential gene expression linked to single-nucleotide polymorphism (SNP)-based quantitative expression trait loci, including SNP promoter regions and transcription initiation sites (Gomez-Gonzalez et al., 2019).

Proteomic studies have increased our knowledge about possible proteins for diagnosis. Thus far, 418 proteins have been identified as serum protein biomarkers from patients with ATBI and LTBI using the LC-Orbitrap Elite platform and enzyme-linked immunosorbent assay (ELISA). Patients with ATBI display serum markers such as the complement system and those related to inflammation and modulation of the immune response, such as C-reactive protein, haptoglobin, alpha-1 acid glycoprotein, complement

component C9, neutrophilic defensin 1, and amyloid P component. Furthermore, in those patients, a decrease in proteins, such as apolipoprotein A, plasma kallikrein, and serotransferrin, was observed, suggesting the importance of lipid transport and iron assimilation in TB infection (Mateos et al., 2020). Proteomic and lipidomic studies have helped elucidate the molecular composition of the mycobacterial capsule. Among the lipoproteins found are LpqH and PstS1, as well as proteins of the Ag85 complex. The enzymatic activity of BlaC, Ald, Adh, KatG, GlnA1, PncA, and SodA has also been measured (Kalscheuer et al., 2019). Capsule polysaccharides have been proposed as target molecules for vaccine development, because they are located on the surface of the mycobacterium and differ from human glycans. The mycobacterial capsule prevents dehydration, helps adherence to the host cell, and provides resistance to the host's immunity.

Several different transcriptomic and proteomic studies have analyzed the differences between virulent strains of *Mtb* (Jena et al., 2013; Jhingan et al., 2016; Verma et al., 2017). These studies identified proteins that were differentially expressed among strains, and mutations that could be associated with the attenuation of virulence of the H37Ra strain. Differences are also mediated by phosphorylation in the bacterial secretion system VII, regulatory system, and fatty acid biosynthesis in the same strain compared with H37Rv. Through multi-omics approaches, nine genes present in the H37Rv strain were shown to be absent in the H37Ra strain. Two putative virulence factors belonging to the ESAT-6 type protein family stood out and 408 new proteins have been identified (Pinto et al., 2018).

5 *M. tuberculosis* antigenic biomarkers and their application to prevent TB

The accurate identification of mycobacterial infection is the first step in determining the appropriate therapeutic regimen for a patient. The diagnosis of pulmonary TB or EPTB is challenging. Therefore, innovative strategies are currently being developed using in vitro, in vivo, and in silico approaches to identify new immunogenic proteins of the mycobacterium. One of these strategies is the synthetic production and validation of highly immunogenic mycobacterial antigens which have different uses, such as in diagnosis or therapy, and to design recombinant vaccines (Table 1) (Meier et al., 2018; Zhang et al., 2018). The antigens available for experimental research are varied. However, none of these synthetic antigens are approved for mass use in the diagnosis of TB. Currently, TB is prevented using vaccines which could be enriched with recombinant immunogenic antigens. Some vaccines designed and tested in infants have revealed an excellent safety profile, but failed to promote protective efficacy in that population (Weiner and Kaufmann, 2014). The antigenic components of *Mtb* are complex and highly variable, and many are not well defined.

The use of protective antigens in TB diagnosis or treatment is a topic that is widely addressed. TB bacilli at the time of infection in the host synthesize proteins that can be ignored by the immune system. However, some others are dominant antigens that activate a response through antigen-specific T cells (Aagaard et al., 2009). There is an arsenal of antigens that stimulate the T-cell-mediated immune response; however, these antigens may have subdominant or masked epitopes, inactivating the function of the antigen (Comas et al., 2010). Currently, numerous biomarkers have been identified and proposed to diagnose or treat TB. In the following paragraphs, we review some of the most relevant biomarkers.

The effectiveness of recombinant TB antigens in the diagnosis of TB has been evaluated on a large scale using microarrays and ELISA. A study showed that 42 TB antigens of 103 analyzed induce a specific immune response in infected patients. Three new polyepitope fusion proteins were designed with polyepitopes, and the characterization of these antigens was carried out by multiantigen print immunoassay (MAPIA); 90% of the patients infected with TB had specific antibodies. These antigens could be used to detect infection (Ireton et al., 2010).

Most of the identified biomarkers are used to determine ATBIs. These biomarkers are *Mtb* products that are detected in blood, saliva, or urine samples from an infected individual. For example, Ag85 complex antigens have been identified in blood. Thus, proposed diagnostic tests based on a biomarker combination to detect TB could provide better surveillance and control of the disease. Different types of TB

Table 1 Recombinant tuberculosis (TB) antigens included in TB vaccines and diagnostics assay

Protein	Vaccine/assay candidate	Reference
Ag85A	MVA85A, Crucell Ad35, Ad5Ag85A, ChAdOx1.85A+MVA85A	Weiner and Kaufmann, 2014; Kaufmann, 2020
Ag85B	H1, H4, H56, Crucell Ad35	Weiner and Kaufmann, 2014; Kaufmann, 2020
Ag85B and ESAT-6	Hybrid 1 (H1), TB-FLU-04L	Sable et al., 2019; Kaufmann, 2020
ESAT-6	H1, H56	Weiner and Kaufmann, 2014; Kaufmann, 2020
CFP-7	H4, Crucell Ad35	Weiner and Kaufmann, 2014; Kaufmann, 2020
*Mtb*39a (PPE18) and *Mtb*32a	M72	Weiner and Kaufmann, 2014; Kaufmann, 2020
Rv2660	H56	Weiner and Kaufmann, 2014; Kaufmann, 2020
Rv2608, Rv3619, Rv3620, and Rv1813	ID93	Weiner and Kaufmann, 2014; Kaufmann, 2020
Ag85A, PPE, and pfkB	Multistage DNA vaccine, A39	Su et al., 2017
Ag85C, MPT5, and HspX	rBCG-CMX	da Costa et al., 2017
Ag85B and ESAT-6	Lipo-AE vaccine formulation with the PolyIC adjuvant	Diogo et al., 2019
ESAT-6, CFP-10, TB10.3, AlaDH, Ag85B, PstS1, and HspX	ELISPOT assay	Chiappini et al., 2012
38kD, ESAT-6, and CFP-10	Multiple-antigen detection assay	Dai et al., 2017
CFP-10 and ESAT-6	Electrochemical-based SPCE	Bakhori et al., 2020

ESAT-6, 6-kDa early secretory antigenic target; CFP, culture filtrate protein; rBCG, recombinant bacille Calmette-Guérin; ELISPOT, enzyme-linked immunospot assay; CMX, Ag85C-MPT51-HspX fusion protein; SPCE, screen-printed carbon electrode. For further details on *Mycobacterium tuberculosis* (*Mtb*) proteins, see the database: http://www.tbdb.org and http://tuberculist.epfl.ch

biomarkers have been identified, such as antibodies, cytokines, metabolic activity markers, volatile organic compounds, and mycobacterial antigens. Antigenic biomarkers are of special importance in the design of novel methods to diagnose TB.

New vaccination strategies are needed to control TB. Several studies have identified candidate molecules for recombinant vaccines, including Rv0287, Rv1174, Rv1196, and Rv3616c, which have been tested in vaccines (Mustafa et al., 2006). Genetically recombined modified vaccines have also been designed, such as *Mtb*72f from Glaxo-Smith-Kline, that combines two *Mtb* immunogenic antigens—*Mtb*39a (Rv1196) and *Mtb*32a (Rv0125)—with the adjuvant ASO2a or ASOIb. This combination was immunogenic with no side effects (Morandi et al., 2013). The CFP-21 antigen (Rv1984c) induces markedly high interferon-γ (IFN-γ) responses in patients with TB that can be expressed by recombinant techniques to test its usefulness in future vaccines. A recent study showed that CFP-10 and CFP-21 antigenic proteins encapsulated in chitosan nanoparticles (ChNs) have protective and therapeutic potential against *Mtb* in immunized mice (Pandey et al., 2020). A computational approach was used recently to predict the epitope affinity of HLA molecules for ten *Mtb* antigens (*Mtb*39a, *Mtb*32a, Ag85B, ESAT-6, TB10.4, Rv2660, Rv2608, Rv3619, Rv3620, and Rv1813) representing the M72, H1, H4, H56, and ID93 vaccines found in clinical trials. The results showed that the ID93 vaccine, comprising the Rv2608, Rv1813, Rv3619, and Rv3620 antigens, had the best potential to prevent ATBI and LTBI (Ong et al., 2020).

A current challenge in medicine is the validation and use of recombinant vaccines. Various recombinant vaccines have been investigated. da Costa et al. (2014) designed a recombinant BCG vaccine (rBCG) with the capacity to express the immune-dominant epitopes of Ag85C, MPT51, and HspX. The vaccine was stable for more than 20 d after vaccination. In mice, this vaccine produced an immune response specific to each of the recombinant proteins used. A vaccine was recently developed with Ag85B and ESAT-6 antigens encapsulated in liposomes. The lipo-AE vaccine formulation in combination with the PolyIC adjuvant induced a combined Th1/Th17-Th2

immune response to the Ag85B antigen, but a weak immune response to ESAT-6 (Diogo et al., 2019). Using recombinant technology represents a new platform for the validation of new vaccines against TB.

Cytomegalovirus (CMV)-based vectors capable of expressing multiple *Mtb* antigens are a good strategy for vaccine development. CMV vectors can be genetically altered to stimulate highly diverse $CD8^+$ T-cell responses that differ in their intended epitope, making it possible to uniquely tailor the $CD8^+$ T-cell response for each disease to maximize therapeutic or prophylactic protection. This vaccine has shown protection in preclinical studies, with sustained antigenic expression representing long-term immunity, an attractive strategy for a new TB vaccine (Früh and Picker, 2017; Liu et al., 2019). Another strategy is the use of messenger RNA (mRNA) as a vaccine vector. This is a simple technology that could express antigens with good performance and could be used against different pathogens, including *Mtb* (Lorenzi et al., 2010). Promising vaccines exist in preclinical studies, but better models are needed to standardize and predict vaccine efficacy in humans.

Among the most studied antigens of *Mycobacterium* are ESAT-6, CFP-10, TB10.3, TB10.4, MTSP11, MPT70, Ag85, and MPT83. Regarding the inactivity of *Mtb* bacilli, transcriptomic analyses have shown that exposure to nitric oxide induces their inactivity, and differential expression of a set of DosR/S regulon genes of the dormancy regulon has been demonstrated (Voskuil et al., 2003; Chiappini et al., 2012). These genes induce bacillus inactivation, a state in which there is no replication of the mycobacterium (Murphy and Brown, 2008; Rustad et al., 2008). Inactivation or dormancy of *Mtb* bacilli within the host is important for mycobacterial pathogenesis because it allows the bacillus to remain in the body without being actively attacked by the host's immune system.

The inactive bacillus resides in a granuloma under hypoxic conditions and with limited access to nutrients. The molecular mechanism through which the bacilli reactivate is unknown. Mycobacterial antigens, such as Ag85A, ESAT-6, and CFP-10, are expressed in different growth phases of the bacillus. ESAT-6 and CFP-10 are also expressed in the stationary phase. MDP1, Acr, and DosS antigens are upregulated from the stationary phase to the dormancy phase, suggesting that they may be present in the growth phase and in dormancy in individuals with previous TB. This knowledge could be used to identify antibodies against these antigens and determine whether a patient has an LTBI or ATBI (Belay et al., 2015).

6 Conclusions

We have reviewed progress in the study of *Mtb* immunogenic antigens and the potential use of recombinant antigens in TB control. Various antigens have been identified and characterized, and more are yet to be uncovered. These study targets have been evaluated using different platforms, and the rapidly accumulating data are being used to understand and integrate the information and apply it effectively in the control of human and animal TB. New live vaccines are currently being investigated and developed, as well as recombinant antigens and vector-based vaccines that express immunogenic *Mtb* antigens.

The massive synthesis of antigens or molecules involved in *Mtb* infection allows the study and validation of new biomarkers to diagnose ATBI or LTBI. New vaccine candidates have already emerged that target patients diagnosed with LTBI. Despite the advances, more experimental data, clinical studies, and system biology are required to integrate the information and decipher the network of interactions between the components of the host immune mechanism and mycobacterial antigens. In this challenge, multidisciplinary research is expected to provide the tools to develop new diagnostic methods, vaccines, and therapies against TB.

Biomarker technologies, such as recombinant diagnostics or vaccine antigens, have proven to be a valuable platform for producing large-scale tests, simplifying test validation, and accelerating entry into the market. In theory, the current methods of TB control reduce the number of infections in the population. In highly developed countries, the TB incidence is low and the disease is almost eradicated (Gordejo and Vermeersch, 2006). However, in developing countries, TB is still considered a serious problem and a potential global risk. Therefore, it is crucial to invest in the development of biomarker technology and the study of TB.

The *Mtb* genome was sequenced over 20 years ago, and most of the proteins encoded within its

genome have been explored in detail. Precise functions have been attributed to more than 40% of the proteins. Immunogenic antigens have great relevance. Through years of research and observation, it was observed that these antigens cause variations in the patterns of the host immune response. Due to their potential use as biomarkers, designing a multigene cocktail to increase test sensitivity and improve diagnosis is feasible. Advances in the characterization of the molecules involved in the pathogenesis of TB and understanding the complex network of interactions between mycobacterial antigens and the host immune system will allow the future improvement of disease prevention and control strategies. Many points still need to be addressed concerning this disease. We need to validate better models to predict the efficacy of TB testing in humans without violating established ethical and regulatory issues. TB will be eradicated only if optimal sanitary measures are strictly implemented, eliminating the precarious living conditions of populations worldwide.

The detection methods for *Mtb* do not always guarantee a reliable diagnosis. However, many studies have demonstrated the usefulness of mycobacterial biomarkers, with important implications for TB diagnosis and treatment. More rigorous validation studies of these biomarkers are required for them to become successful diagnostic tools or vaccines against the disease.

Contributors

Elba RODRÍGUEZ-HERNÁNDEZ and Laura Itzel QUINTAS-GRANADOS performed the documental research, wrote and edited the manuscript. Susana FLORES-VILLALVA, Jorge Germinal CANTÓ-ALARCÓN, and Feliciano MILIÁN-SUAZO revised the manuscript. All authors have read and approved the final manuscript and, therefore, take responsibility for it.

Compliance with ethics guidelines

Elba RODRÍGUEZ-HERNÁNDEZ, Laura Itzel QUINTAS-GRANADOS, Susana FLORES-VILLALVA, Jorge Germinal CANTÓ-ALARCÓN, and Feliciano MILIÁN-SUAZO declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Aagaard CS, Hoang TTKT, Vingsbo-Lundberg C, et al., 2009. Quality and vaccine efficacy of $CD4^+$ T cell responses directed to dominant and subdominant epitopes in ESAT-6 from *Mycobacterium tuberculosis*. *J Immunol*, 183(4): 2659-2668. https://doi.org/10.4049/jimmunol.0900947

Abdallah AM, van Pittius NCG, Champion PAD, et al., 2007. Type VII secretion—mycobacteria show the way. *Nat Rev Microbiol*, 5(11):883-891. https://doi.org/10.1038/nrmicro1773

Adlakha N, Vir P, Verma I, 2012. Effect of mycobacterial secretory proteins on the cellular integrity and cytokine profile of type II alveolar epithelial cells. *Lung India*, 29(4):313-318. https://doi.org/10.4103/0970-2113.102796

Aguilar-Ayala DA, Tilleman L, van Nieuwerburgh F, et al., 2017. The transcriptome of *Mycobacterium tuberculosis* in a lipid-rich dormancy model through RNAseq analysis. *Sci Rep*, 7:17665. https://doi.org/10.1038/s41598-017-17751-x

Aitken JM, Borody TJ, Agrawal G, 2019. A revaluation of the use of conventional Ziehl-Neelsen stain for detection of non-tuberculous mycobacteria. *N Z J Med Lab Sci*, 73(2): 85.

Amanfu W, 2006. The situation of tuberculosis and tuberculosis control in animals of economic interest. *Tuberculosis*, 86(3-4):330-335. https://doi.org/10.1016/j.tube.2006.01.007

Bakhori NM, Yusof NA, Abdullah J, et al., 2020. Surface enhanced CdSe/ZnS QD/SiNP electrochemical immunosensor for the detection of *Mycobacterium tuberculosis* by combination of CFP10-ESAT6 for better diagnostic specificity. *Materials (Basel)*, 13(1):149. https://doi.org/10.3390/ma13010149

Banuls AL, Sanou A, van Anh NT, et al., 2015. *Mycobacterium tuberculosis*: ecology and evolution of a human bacterium. *J Med Microbiol*, 64(11):1261-1269. https://doi.org/10.1099/jmm.0.000171

Beatty WL, Russell DG, 2000. Identification of mycobacterial surface proteins released into subcellular compartments of infected macrophages. *Infect Immun*, 68(12):6997-7002. https://doi.org/10.1128/IAI.68.12.6997-7002.2000

Belay M, Legesse M, Mihret A, et al., 2015. Pro- and anti-inflammatory cytokines against Rv2031 are elevated during latent tuberculosis: a study in cohorts of tuberculosis patients, household contacts and community controls in an endemic setting. *PLoS ONE*, 10(4):e0124134. https://doi.org/10.1371/journal.pone.0124134

Bishai WR, 2000. Rekindling old controversy on elusive lair of latent tuberculosis. *Lancet*, 356(9248):2113-2114. https://doi.org/10.1016/S0140-6736(00)03485-1

Boshoff HIM, Lun DS, 2010. Systems biology approaches to understanding mycobacterial survival mechanisms. *Drug Discov Today Dis Mech*, 7(1):e75-e82. https://doi.org/10.1016/j.ddmec.2010.09.008

Bourassa L, 2018. The NanoDisk-MS assay: a new frontier in biomarker-based tuberculosis diagnostics? *Clin Chem*, 64(5):763-765. https://doi.org/10.1373/clinchem.2017.285460

Brennan MJ, Delogu G, 2002. The PE multigene family: a 'molecular mantra' for mycobacteria. *Trends Microbiol*, 10(5):246-249. https://doi.org/10.1016/S0966-842X(02)02335-1

Brennan PJ, 2003. Structure, function, and biogenesis of the cell wall of *Mycobacterium tuberculosis*. *Tuberculosis*, 83(1-3):91-97. https://doi.org/10.1016/S1472-9792(02)00089-6

Brennan PJ, Nikaido H, 1995. The envelope of mycobacteria. *Ann Rev Biochem*, 64(1):29-63. https://doi.org/10.1146/annurev.bi.64.070195.000333

Briken V, Porcelli SA, Besra GS, et al., 2004. Mycobacterial lipoarabinomannan and related lipoglycans: from biogenesis to modulation of the immune response. *Mol Microbiol*, 53(2):391-403. https://doi.org/10.1111/j.1365-2958.2004.04183.x

Brock M, Hanlon D, Zhao MW, et al., 2020. Detection of mycobacterial lipoarabinomannan in serum for diagnosis of active tuberculosis. *Diagn Microbiol Infect Dis*, 96(2): 114937. https://doi.org/10.1016/j.diagmicrobio.2019.114937

Broger T, Tsionksy M, Mathew A, et al., 2019. Sensitive electrochemiluminescence (ECL) immunoassays for detecting lipoarabinomannan (LAM) and ESAT-6 in urine and serum from tuberculosis patients. *PLoS ONE*, 14(4): e0215443. https://doi.org/10.1371/journal.pone.0215443

Bulterys MA, Wagner B, Redard-Jacot M, et al., 2019. Point-of-Care Urine LAM tests for tuberculosis diagnosis: a mini-review. *J Clin Med*, 9:111. https://doi.org/10.20944/preprints201912.0002.v1

Chiappini E, Bella CD, Bonsignori F, et al., 2012. Potential role of *M. tuberculosis* specific IFN-γ and IL-2 ELISPOT assays in discriminating children with active or latent tuberculosis. *PLoS ONE*, 7(9):e46041. https://doi.org/10.1371/journal.pone.0046041

Cole ST, Brosch R, Parkhill J, et al., 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature*, 393(6685):537-544. https://doi.org/10.1038/31159

Comas I, Chakravartti J, Small PM, et al., 2010. Human T cell epitopes of *Mycobacterium tuberculosis* are evolutionarily hyperconserved. *Nat Genet*, 42(6):498-503. https://doi.org/10.1038/ng.590

da Costa AC, de Oliveira Costa-Júnior A, de Oliveira FM, et al., 2014. A new recombinant BCG vaccine induces specific Th17 and Th1 effector cells with higher protective efficacy against tuberculosis. *PLoS ONE*, 9(11):e112848. https://doi.org/10.1371/journal.pone.0112848

da Costa AC, de Resende DP, de Santos BDP, et al., 2017. Modulation of macrophage responses by CMX, a fusion protein composed of Ag85C, MPT51, and HspX from *Mycobacterium tuberculosis*. *Front Microbiol*, 8:623. https://doi.org/10.3389/fmicb.2017.00623

Dai ZH, Liu ZQ, Xiu BS, et al., 2017. A multiple-antigen detection assay for tuberculosis diagnosis based on broadly reactive polyclonal antibodies. *Iran J Basic Med Sci*, 20(4):360-367. https://doi.org/10.22038/IJBMS.2017.8575

Delogu G, Brennan MJ, 1999. Functional domains present in the mycobacterial hemagglutinin, HBHA. *J Bacteriol*, 181(24):7464-7469. https://doi.org/10.1128/JB.181.24.7464-7469.1999

Delogu G, Sali M, Fadda G, 2013. The biology of *Mycobacterium tuberculosis* infection. *Mediterr J Hematol Infect Dis*, 5(1):e2013070. https://doi.org/10.4084/mjhid.2013.070

Denkinger CM, Pai M, Patel M, et al., 2013. Gamma interferon release assay for monitoring of treatment response for active tuberculosis: an explosion in the spaghetti factory. *J Clin Microbiol*, 51(2):607-610. https://doi.org/10.1128/JCM.02278-12

Dheda K, Barry CE, Maartens G, 2016. Tuberculosis. *Lancet*, 387(10024):1211-1226. https://doi.org/10.1016/S0140-6736(15)00151-8

Dillon DC, Alderson MR, Day CH, et al., 2000. Molecular and immunological characterization of *Mycobacterium tuberculosis* CFP-10, an immunodiagnostic antigen missing in *Mycobacterium bovis* BCG. *J Clin Microbiol*, 38(9): 3285-3290. https://doi.org/10.1128/JCM.38.9.3285-3290.2000

Diogo GR, Hart P, Copland A, et al., 2019. Immunization with *Mycobacterium tuberculosis* antigens encapsulated in phosphatidylserine liposomes improves protection afforded by BCG. *Front Immunol*, 10:1349. https://doi.org/10.3389/fimmu.2019.01349

Dmitriev BA, Ehlers S, Rietschel ET, et al., 2000. Molecular mechanics of the mycobacterial cell wall: from horizontal layers to vertical scaffolds. *Int J Med Microbiol*, 290(3): 251-258. https://doi.org/10.1016/S1438-4221(00)80122-8

Egan AJF, Maya-Martinez R, Ayala I, et al., 2018. Induced conformational changes activate the peptidoglycan synthase PBP1B. *Mol Microbiol*, 110(3):335-356. https://doi.org/10.1111/mmi.14082

Ehlers MRW, Daffé M, 1998. Interactions between *Mycobacterium tuberculosis* and host cells: are mycobacterial sugars the key? *Trends Microbiol*, 6(8):328-335. https://doi.org/10.1016/S0966-842X(98)01301-8

Ernst JD, 1998. Macrophage receptors for *Mycobacterium tuberculosis*. *Infect Immun*, 66(4):1277-1281. https://doi.org/10.1128/IAI.66.4.1277-1281.1998

Feinberg H, Jégouzo SAF, Rowntree TJW, et al., 2013. Mechanism for recognition of an unusual mycobacterial glycolipid by the macrophage receptor mincle. *J Biol Chem*, 288(40):28457-28465. https://doi.org/10.1074/jbc.M113.497149

Fenton MJ, Vermeulen MW, 1996. Immunopathology of tuberculosis: roles of macrophages and monocytes. *Infect Immun*, 64(3):683-690. https://doi.org/10.1128/IAI.64.3.683-690.1996

Fine PEM, 2001. BCG: the challenge continues. *Scand J Infect*

Dis, 33(1):58-60.
https://doi.org/10.1080/003655401753382576

Forrellad MA, Klepp LI, Gioffré A, et al., 2013. Virulence factors of the *Mycobacterium tuberculosis* complex. *Virulence*, 4(1):3-66.
https://doi.org/10.4161/viru.22329

Früh K, Picker L, 2017. CD8+ T cell programming by cytomegalovirus vectors: applications in prophylactic and therapeutic vaccination. *Curr Opin Immunol*, 47:52-56.
https://doi.org/10.1016/j.coi.2017.06.010

Garnier T, Eiglmeier K, Camus JC, et al., 2003. The complete genome sequence of *Mycobacterium bovis*. *Proc Natl Acad Sci USA*, 100(13):7877-7882.
https://doi.org/10.1073/pnas.1130426100

Gatfield J, Pieters J, 2000. Essential role for cholesterol in entry of mycobacteria into macrophages. *Science*, 288(5471):1647-1651.
https://doi.org/10.1126/science.288.5471.1647

Ghon A, 1923. The primary complex in human tuberculosis and its significance. *Am Rev Tuberc*, 7(5):314-317.

Gomez-Gonzalez PJ, Andreu N, Phelan JE, et al., 2019. An integrated whole genome analysis of *Mycobacterium tuberculosis* reveals insights into relationship between its genome, transcriptome and methylome. *Sci Rep*, 9:5204.
https://doi.org/10.1038/s41598-019-41692-2

Gordejo FJR, Vermeersch JP, 2006. Towards eradication of bovine tuberculosis in the European Union. *Vet Microbiol*, 112(2-4):101-109.
https://doi.org/10.1016/j.vetmic.2005.11.034

Guerin ME, Korduláková J, Alzari PM, et al., 2010. Molecular basis of phosphatidyl-*myo*-inositol mannoside biosynthesis and regulation in mycobacteria. *J Biol Chem*, 285(44):33577-33583.
https://doi.org/10.1074/jbc.R110.168328

Heithoff DM, Conner CP, Hanna PC, et al., 1997. Bacterial infection as assessed by *in vivo* gene expression. *Proc Natl Acad Sci*, 94(3):934-939.
https://doi.org/10.1073/pnas.94.3.934

Hernández-Pando R, Jeyanathan M, Mengistu G, et al., 2000. Persistence of DNA from *Mycobacterium tuberculosis* in superficially normal lung tissue during latent infection. *Lancet*, 356(9248):2133-2138.
https://doi.org/10.1016/S0140-6736(00)03493-0

Huebner RE, Schein MF, Bass JB Jr, 1993. The tuberculin skin test. *Clin Infect Dis*, 17(6):968-975.
https://doi.org/10.1093/clinids/17.6.968

Iantomasi R, Sali M, Cascioferro A, et al., 2012. PE_PGRS30 is required for the full virulence of *Mycobacterium tuberculosis*. *Cell Microbiol*, 14(3):356-367.
https://doi.org/10.1111/j.1462-5822.2011.01721.x

Ireton GC, Greenwald R, Liang H, et al., 2010. Identification of *Mycobacterium tuberculosis* antigens of high serodiagnostic value. *Clin Vaccine Immunol*, 17(10):1539-1547.
https://doi.org/10.1128/CVI.00198-10

Jankute M, Cox JAG, Harrison J, et al., 2015. Assembly of the mycobacterial cell wall. *Ann Rev Microbiol*, 69:405-423.
https://doi.org/10.1146/annurev-micro-091014-104121

Jena L, Kashikar S, Kumar S, et al., 2013. Comparative proteomic analysis of *Mycobacterium tuberculosis* strain H37Rv versus H37Ra. *Int J Mycobacteriol*, 2(4):220-226.
https://doi.org/10.1016/j.ijmyco.2013.10.004

Jhingan GD, Kumari S, Jamwal SV, et al., 2016. Comparative proteomic analyses of avirulent, virulent, and clinical strains of *Mycobacterium tuberculosis* identify strain-specific patterns. *J Biol Chem*, 291(27):14257-14273.
https://doi.org/10.1074/jbc.M115.666123

Kalscheuer R, Palacios A, Anso I, et al., 2019. The *Mycobacterium tuberculosis* capsule: a cell structure with key implications in pathogenesis. *Biochem J*, 476(14):1995-2016.
https://doi.org/10.1042/BCJ20190324

Katial RK, Hershey J, Purohit-Seth T, et al., 2001. Cell-mediated immune response to tuberculosis antigens: comparison of skin testing and measurement of in vitro gamma interferon production in whole-blood culture. *Clin Diagn Lab Immunol*, 8(2):339-345.
https://doi.org/10.1128/CDLI.8.2.339-345.2001

Kaufmann SHE, 2020. Vaccination against tuberculosis: revamping BCG by molecular genetics guided by immunology. *Front Immunol*, 11:316.
https://doi.org/10.3389/fimmu.2020.00316

Kawasaki M, Echiverri C, Raymond L, et al., 2019. Lipoarabinomannan in sputum to detect bacterial load and treatment response in patients with pulmonary tuberculosis: analytic validation and evaluation in two cohorts. *PLoS Med*, 16(4):e1002780.
https://doi.org/10.1371/journal.pmed.1002780

Kendall SL, Withers M, Soffair CN, et al., 2007. A highly conserved transcriptional repressor controls a large regulon involved in lipid degradation in *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*. *Mol Microbiol*, 65(3):684-699.
https://doi.org/10.1111/j.1365-2958.2007.05827.x

Lawn SD, Kerkhoff AD, Vogt M, et al., 2012. Diagnostic accuracy of a low-cost, urine antigen, point-of-care screening assay for HIV-associated pulmonary tuberculosis before antiretroviral therapy: a descriptive study. *Lancet Infect Dis*, 12(3):201-209.
https://doi.org/10.1016/S1473-3099(11)70251-1

Lawn SD, Kerkhoff AD, Nicol MP, et al., 2015. Underestimation of the true specificity of the urine lipoarabinomannan point-of-care diagnostic assay for HIV-associated tuberculosis. *J Acquir Immune Defic Syndr*, 69(4):e144-e146.
https://doi.org/10.1097/QAI.0000000000000672

Liu C, Zhao Z, Fan J, et al., 2017. Quantification of circulating *Mycobacterium tuberculosis* antigen peptides allows rapid diagnosis of active disease and treatment monitoring. *Proc Natl Acad Sci USA*, 114(15):3969-3974.
https://doi.org/10.1073/pnas.1621360114

Liu J, Jaijyan DK, Tang QY, et al., 2019. Promising cytomegalovirus-based vaccine vector induces robust $CD8^+$

T-cell response. *Int J Mol Sci*, 20(18):4457. https://doi.org/10.3390/ijms20184457

Lorenzi JC, Trombone AP, Rocha CD, et al., 2010. Intranasal vaccination with messenger RNA as a new approach in gene therapy: use against tuberculosis. *BMC Biotechnol*, 10:77. https://doi.org/10.1186/1472-6750-10-77

Mack U, Migliori GB, Sester M, et al., 2009. LTBI: latent tuberculosis infection or lasting immune responses to *M. tuberculosis*? A TBNET consensus statement. *Eur Respir J*, 33(5):956-973. https://doi.org/10.1183/09031936.00120908

Manca C, Lyashchenko K, Wiker HG, et al., 1997. Molecular cloning, purification, and serological characterization of MPT63, a novel antigen secreted by *Mycobacterium tuberculosis*. *Infect Immun*, 65(1):16-23. https://doi.org/10.1128/IAI.65.1.16-23.1997

Mateos J, Estévez O, González-Fernández Á, et al., 2020. Serum proteomics of active tuberculosis patients and contacts reveals unique processes activated during *Mycobacterium tuberculosis* infection. *Sci Rep*, 10:3844. https://doi.org/10.1038/s41598-020-60753-5

Means TK, Wang SY, Lien E, et al., 1999. Human Toll-like receptors mediate cellular activation by *Mycobacterium tuberculosis*. *J Immunol*, 163(7):3920-3927.

Meier NR, Jacobsen M, Ottenhoff THM, et al., 2018. A systematic review on novel *Mycobacterium tuberculosis* antigens and their discriminatory potential for the diagnosis of latent and active tuberculosis. *Front Immunol*, 9:2476. https://doi.org/10.3389/fimmu.2018.02476

Mekalanos JJ, 1992. Environmental signals controlling expression of virulence determinants in bacteria. *J Bacteriol*, 174(1):1-7. https://doi.org/10.1128/jb.174.1.1-7.1992

Monkongdee P, McCarthy KD, Cain KP, et al., 2009. Yield of acid-fast smear and mycobacterial culture for tuberculosis diagnosis in people with human immunodeficiency virus. *Am J Respir Crit Care Med*, 180(9):903-908. https://doi.org/10.1164/rccm.200905-0692OC

Morandi M, Sali M, Manganelli R, et al., 2013. Exploiting the mycobacterial cell wall to design improved vaccines against tuberculosis. *J Infect Dev Ctries*, 7(3):169-181. https://doi.org/10.3855/jidc.3114

Murphy DJ, Brown JR, 2008. Novel drug target strategies against *Mycobacterium tuberculosis*. *Curr Opin Microbiol*, 11(5):422-427. https://doi.org/10.1016/j.mib.2008.08.001

Mustafa AS, Skeiky YA, Al-Attiyah R, et al., 2006. Immunogenicity of *Mycobacterium tuberculosis* antigens in *Mycobacterium bovis* BCG-vaccinated and *M. bovis*-infected cattle. *Infect Immun*, 74(8):4566-4572. https://doi.org/10.1128/IAI.01660-05

Nurwidya F, Handayani D, Burhan E, et al., 2018. Molecular diagnosis of tuberculosis. *Chonnam Med J*, 54(1):1-9. https://doi.org/10.4068/cmj.2018.54.1.1

Ong E, He YQ, Yang ZH, 2020. Epitope promiscuity and population coverage of *Mycobacterium tuberculosis* protein antigens in current subunit vaccines under development. *Infect Genet Evol*, 80:104186. https://doi.org/10.1016/j.meegid.2020.104186

Pandey RP, Kumar S, Ahmad S, et al., 2020. Use Chou's 5-steps rule to evaluate protective efficacy induced by antigenic proteins of *Mycobacterium tuberculosis* encapsulated in chitosan nanoparticles. *Life Sci*, 256:117961. https://doi.org/10.1016/j.lfs.2020.117961

Park ST, Kang CM, Husson RN, 2008. Regulation of the SigH stress response regulon by an essential protein kinase in *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA*, 105(35):13105-13110. https://doi.org/10.1073/pnas.0801143105

Paylor R, 2014. Bovine tuberculosis. *In*: Mouser P (Ed.), Michigan State University Veterinary Student, Graduate Student. Michigan State University, p.1-3.

Pieters J, Gatfield J, 2002. Hijacking the host: survival of pathogenic mycobacteria inside macrophages. *Trends Microbiol*, 10(3):142-146. https://doi.org/10.1016/S0966-842X(02)02305-3

Pinto SM, Verma R, Advani J, et al., 2018. Integrated multi-omic analysis of *Mycobacterium tuberculosis* H37Ra redefines virulence attributes. *Front Microbiol*, 9:1314. https://doi.org/10.3389/fmicb.2018.01314

Pollock JM, Andersen P, 1997. Predominant recognition of the ESAT-6 protein in the first phase of interferon with *Mycobacterium bovis* in cattle. *Infect Immun*, 65(7):2587-2592. https://doi.org/10.1128/IAI.65.7.2587-2592.1997

Pym AS, Brodin P, Brosch R, et al., 2002. Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines *Mycobacterium bovis* BCG and *Mycobacterium microti*. *Mol Microbiol*, 46(3):709-717. https://doi.org/10.1046/j.1365-2958.2002.03237.x

Pym AS, Brodin P, Majlessi L, et al., 2003. Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. *Nat Med*, 9(5):533-539. https://doi.org/10.1038/nm859

Renshaw PS, Panagiotidou P, Whelan A, et al., 2002. Conclusive evidence that the major T-cell antigens of the *Mycobacterium tuberculosis* complex ESAT-6 and CFP-10 form a tight, 1:1 complex and characterization of the structural properties of ESAT-6, CFP-10, and the ESAT-6·CFP-10 complex: implications for pathogenesis and virulence. *J Biol Chem*, 277(24):21598-21603. https://doi.org/10.1074/jbc.M201625200

Renshaw PS, Lightbody KL, Veverka V, et al., 2005. Structure and function of the complex formed by the tuberculosis virulence factors CFP-10 and ESAT-6. *EMBO J*, 24(14):2491-2498. https://doi.org/10.1038/sj.emboj.7600732

Rodriguez GM, 2006. Control of iron metabolism in *Mycobacterium tuberculosis*. *Trends Microbiol*, 14(7):320-327. https://doi.org/10.1016/j.tim.2006.05.006

Rustad TR, Harrell MI, Liao RL, et al., 2008. The enduring

hypoxic response of *Mycobacterium tuberculosis*. *PLoS ONE*, 3(1):e1502. https://doi.org/10.1371/journal.pone.0001502

Ryndak M, Wang SS, Smith I, 2008. PhoP, a key player in *Mycobacterium tuberculosis* virulence. *Trends Microbiol*, 16(11):528-534. https://doi.org/10.1016/j.tim.2008.08.006

Ryu YJ, 2015. Diagnosis of pulmonary tuberculosis: recent advances and diagnostic algorithms. *Tuber Respir Dis*, 78(2):64-71. https://doi.org/10.4046/trd.2015.78.2.64

Sable SB, Posey JE, Scriba TJ, 2019. Tuberculosis vaccine development: progress in clinical evaluation. *Clin Microbiol Rev*, 33(1):e00100-19. https://doi.org/10.1128/CMR.00100-19

Santos R, Ursu O, Gaulton A, et al., 2017. A comprehensive map of molecular drug targets. *Nat Rev Drug Dis*, 16(1): 19-34. https://doi.org/10.1038/nrd.2016.230

Schlesinger LS, 1993. Macrophage phagocytosis of virulent but not attenuated strains of *Mycobacterium tuberculosis* is mediated by mannose receptors in addition to complement receptors. *J Immunol*, 150(7):2920-2930.

Schlesinger LS, Bellinger-Kawahara CG, Payne NR, et al., 1990. Phagocytosis of *Mycobacterium tuberculosis* is mediated by human monocyte complement receptors and complement component C3. *J Immunol*, 144(7):2771-2780.

Seddon JA, Paton J, Nademi Z, et al., 2016. The impact of BCG vaccination on tuberculin skin test responses in children is age dependent: evidence to be considered when screening children for tuberculosis infection. *Thorax*, 71(10):932-939. https://doi.org/10.1136/thoraxjnl-2015-207687

Shah M, Hanrahan C, Wang ZY, et al., 2016. Lateral flow urine lipoarabinomannan assay for detecting active tuberculosis in HIV-positive adults. *Cochrane Database Syst Rev*, 2016(5):CD011420. https://doi.org/10.1002/14651858.CD011420.pub2

Sigal GB, Pinter A, Lowary TL, et al., 2018. A novel sensitive immunoassay targeting the 5-methylthio-D-xylofuranose–lipoarabinomannan epitope meets the WHO's performance target for tuberculosis diagnosis. *J Clin Microbiol*, 56(12):e01338-18. https://doi.org/10.1128/JCM.01338-18

Silva VMC, Kanaujia G, Gennaro ML, et al., 2003. Factors associated with humoral response to ESAT-6, 38 kDa and 14 kDa in patients with a spectrum of tuberculosis. *Int J Tuber Lung Dis*, 7(5):478-484.

Singh A, Crossman DK, Mai D, et al., 2009. *Mycobacterium tuberculosis* WhiB3 maintains redox homeostasis by regulating virulence lipid anabolism to modulate macrophage response. *PLoS Pathog*, 5(8):e1000545. https://doi.org/10.1371/journal.ppat.1000545

Sinha P, Gupta A, Prakash P, et al., 2016. Differentiation of *Mycobacterium tuberculosis* complex from non-tubercular mycobacteria by nested multiplex PCR targeting *IS6110*, *MTP40* and *32kD alpha antigen* encoding gene fragments. *BMC Infect Dis*, 16:123. https://doi.org/10.1186/s12879-016-1450-1

Smith I, 2003. *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. *Clin Microbiol Rev*, 16(3):463-496. https://doi.org/10.1128/CMR.16.3.463-496.2003

Sreejit G, Ahmed A, Parveen N, et al., 2014. The ESAT-6 protein of *Mycobacterium tuberculosis* interacts with beta-2-microglobulin (β2M) affecting antigen presentation function of macrophage. *PLoS Pathog*, 10(10): e1004446. https://doi.org/10.1371/journal.ppat.1004446

Su HB, Zhu SL, Zhu L, et al., 2017. *Mycobacterium tuberculosis* latent antigen Rv2029c from the multistage DNA vaccine A39 drives TH1 responses via TLR-mediated macrophage activation. *Front Microbiol*, 8:2266. https://doi.org/10.3389/fmicb.2017.02266

Swaminathan S, Rekha B, 2010. Pediatric tuberculosis: global overview and challenges. *Clin Infect Dis*, 50(S3):S184-S194. https://doi.org/10.1086/651490

Sweeney TE, Braviak L, Tato CM, et al., 2016. Genome-wide expression for diagnosis of pulmonary tuberculosis: a multicohort analysis. *Lancet Respir Med*, 4(3):213-224. https://doi.org/10.1016/S2213-2600(16)00048-5

Vergne I, Chua J, Singh SB, et al., 2004. Cell biology of *Mycobacterium tuberculosis* phagosome. *Annu Rev Cell Dev Biol*, 20:367-394. https://doi.org/10.1146/annurev.cellbio.20.010403.114015

Verma R, Pinto SM, Patil AH, et al., 2017. Quantitative proteomic and phosphoproteomic analysis of H37Ra and H37Rv strains of *Mycobacterium tuberculosis*. *J Proteome Res*, 16(4):1632-1645. https://doi.org/10.1021/acs.jproteome.6b00983

Vir P, Gupta D, Agarwal R, et al., 2014. Interaction of alveolar epithelial cells with CFP21, a mycobacterial cutinase-like enzyme. *Mol Cell Biochem*, 396(1-2):187-199. https://doi.org/10.1007/s11010-014-2154-8

Voskuil MI, Schnappinger D, Visconti KC, et al., 2003. Inhibition of respiration by nitric oxide induces a *Mycobacterium tuberculosis* dormancy program. *J Exp Med*, 198(5):705-713. https://doi.org/10.1084/jem.20030205

Weiner J III, Kaufmann SHE, 2014. Recent advances towards tuberculosis control: vaccines and biomarkers. *J Int Med*, 275(5):467-480. https://doi.org/10.1111/joim.12212

Weiner J, Maertzdorf J, Kaufmann SHE, 2013. The dual role of biomarkers for understanding basic principles and devising novel intervention strategies in tuberculosis. *Ann New York Acad Sci*, 1283(1):22-29. https://doi.org/10.1111/j.1749-6632.2012.06802.x

WHO (World Health Organization), 2014. High-priority target product profiles for new tuberculosis diagnostics: report of a consensus meeting. Geneva, Switzerland, WHO.

https://apps.who.int/iris/handle/10665/135617
WHO, 2015. The use of lateral flow urine lipoarabinomannan assay (LF-LAM) for the diagnosis and screening of active tuberculosis in people living with HIV: policy guidance. WHO. https://apps.who.int/iris/handle/10665/193633
WHO, 2019. Global tuberculosis report 2019. WHO. https://apps.who.int/iris/handle/10665/329368
Wolf AJ, Desvignes L, Linas B, et al., 2008. Initiation of the adaptive immune response to *Mycobacterium tuberculosis* depends on antigen production in the local lymph node, not the lungs. *J Exp Med*, 205(1):105-115. https://doi.org/10.1084/jem.20071367
Zhang W, Shu QB, Zhao Z, et al., 2018. Antigen 85B peptidomic analysis allows species-specific mycobacterial identification. *Clin Proteomics*, 15(1):1. https://doi.org/10.1186/s12014-017-9177-6

中文概要

题　目：抗原性生物标志物在结核分枝杆菌中的应用

概　要：研究和鉴定分枝杆菌与其宿主间相互作用的生物分子，对于确定分枝杆菌在入侵过程中的作用至关重要，并为相关疾病生物学和发病机制提供基础知识。分枝杆菌是一种古老的病原体，它为自己在宿主和环境中生存发展了复杂的策略，这可能与其参与感染分子之间相互作用网络的复杂性有关。在结核病检测技术的发展过程中，一些生物标记物已经开始受到关注。研究最广泛的抗原有 CFP-10、ESAT-6、Ag85A、Ag85B、CFP-7 和 PPE18。其中一些抗原已被作为生物标记来评估病原体和宿主感染反应。设计新颖而准确的诊断方法对控制全世界的结核病至关重要。目前，诊断方法是基于对感染个体体液反应分子的识别。因此，这些测试依赖于宿主产生免疫反应的能力，而免疫反应通常是异质的。在过去的 20 年里，人们特别重视多抗原诊断方法的设计，以提高敏感性和特异性水平。本文综述了结核分枝杆菌生物分子的研究和应用现状，以期为结核控制提供新的支持。

关键词：结核分枝杆菌；重组抗原；诊断；生物标志物