Application of antigenic biomarkers for *Mycobacterium tuberculosis*

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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 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

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
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 surcattae, 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 16S 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 immunoabsorption 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 non-inflammatory bronchoalveolar fluid (Fenton and Vermueelen, 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 Vermueelen, 1996). These receptors bind to nonopsonized \( \text{Mtb} \) 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). \( \text{Mtb} \) 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 \textit{Mycobacteria} helps to identify novel biomarkers for diagnosis or treatment.

The cell envelope of \( \text{Mtb} \) 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 
\textit{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).

\textit{Mtb} has a peculiar cell wall core consisting of \textit{N}-glycosylated peptidoglycan linked to \textit{d}-arabino-\textit{d}-galactan (AG) via phosphoryl-\textit{N}-acetylglucosaminosyl-rhamnosyl linkage units. AG is esterified by \textit{α}-alkyl-\textit{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., 2013), has been used to develop novel antibiotic 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 \textit{N}-acetylglucosamine (GlcNAc) and \textit{N}-acetylmuramic acid (MurNAc) residues linked by \textit{β}-1>4 bonds (Egan et al., 2018). Catalytic enzymes and secretion products are released from the \textit{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 \textit{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 \textit{Mtb} survival in the host cells. ESAT-6 also interacts with host protein \textit{β}-2-microglobulin (\textit{β}2M), 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 dimyccocosate exporter), FbpA (diacetyl-glycerol 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 \textit{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 antimycobacterial 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 \( \text{Mtb} \) (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 \( \text{M. tuberculosis} \) 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 \( \text{Mtb} \) 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 polypeptide fusion proteins were designed with polypeptides, 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 \( \text{Mtb} \) 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
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 Mtb72f from Glaxo-Smith-Kline, that combines two Mtb immunogenic antigens—Mtb39a (Rv1196) and Mtb32a (Rv0125)—with the adjuvant ASO2a or ASO1b. 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 (Mtb39a, Mtb32a, 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

### 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      |
| Mtb39a (PPE18) and Mtb32a | M72                                                                                    | Weiner and Kaufmann, 2014; Kaufmann, 2020      |
| Rv2660                   | H56                                                                                    | Weiner and Kaufmann, 2014; Kaufmann, 2020      |
| Rv2608, Rv3619, Rv3620, and Rv1813 | Multistage DNA vaccine, A39                                                             | Weiner and Kaufmann, 2014; Kaufmann, 2020      |
| 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
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 documentary 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**

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

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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，一些生物标志物已经开始受到关注。研究最广泛的抗原有 CFU-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

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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，一些生物标志物已经开始受到关注。研究最广泛的抗原有 CFU-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。

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