ELSEVIER

The availability of validated biomarkers will accelerate the crusade against TB in terms of rapid diagnosis and better therapeutic management as well as rational drug and vaccine discovery.

The quest for biomarkers in tuberculosis

Shreemanta K. Parida and Stefan H.E. Kaufmann

Max Planck Institute for Infection Biology, Department of Immunology, Charitéplatz 1, D-10117 Berlin, Germany

No new vaccine has been licensed for tuberculosis (TB) for more than three-quarters of a century, and no new drug has been licensed for half a century. One major drawback has been the attrition caused by the lack of a reliable biological indicator (biomarker) to predict toxicity and efficacy early in the development pipeline. This review portrays the landscape of biomarker discovery for TB in the context of drug and vaccine development using emerging global biomics platforms. The time is ripe to move from single markers for correlates of protection to a biosignature comprising a well-defined set of robust indicators in TB that can accelerate rapid screening and early selection of potential drug and vaccine candidates.

Shreemanta K. Parida, MD, PhD is at the Max Planck Institute for Infection Biology as Program Coordinator of the Grand Challenges in Global Health consortium on "Biomarkers of Protective Immunity against TB in the context of HIV/AIDS in Africa", supported by Bill & Melinda Gates Foundation and led by Prof Stefan H E Kaufmann. He is a physician scientist with broad spectrum of expertise on clinical trials with vaccine, immunopathology, molecular pathogenesis, molecular immunology, epidemiology and vaccinology and experiences in field of infectious diseases with particular focus on tuberculosis in the last decade. He has been instrumental in building a cohesive and functional team with 15 partners including 7 African, 5 European and 3 US partners across geographical, cultural, social and working diversities. He is committed in translating research from the bench to the clinics at the peripheral level to combat the global tuberculosis emergency in the developing world.

Stefan H.E. Kaufmann Founding director and director of the Department of Immunology of the Max Planck Institute for Infection Biology in Berlin. Professor for microbiology and immunology at the Charité, Humboldt University, Berlin, and honorary professor at the University Clinics Benjamin Franklin, Free University Berlin. Past president and honorary member of the German Society for Immunology. President of the European Federation of Immunological Societies. Vice President of the International Union of Immunological Societies (IUIS). Born 1948 in Ludwigshafen am Rhein. Studied biology at the Johannes Gutenberg University of Mainz, 1977 PhD. Scientific interests: immunity to bacterial pathogens with emphasis on tuberculosis and rational vaccine design. Numerous scientific awards. Doctor Honoris Causa from Université de la Mediterranée, Aix-Marseille II. More than 600 publications mostly in high-ranking journals. Highly cited immunologist (ISI Thomson).

Introduction

In a recent editorial in *Drug Discovery Today*, S.J. Projan stated: 'As the spectre of pan-resistant strains of bacteria has become a clinical reality, the pipeline of new antibacterial drugs capable of treating such infections is virtually bone dry' [1]. There are many reasons for this failure, mostly related to callousness – both in industry and in academia – in the past quarter of a century. In academia, basic research on potential targets for novel classes of antibacterial drugs was not considered sufficiently glamorous. In industry, such drugs were poor candidates for a high return on investment and were unlikely to become blockbusters. At the same time, bacteria developed resistance to antimicrobial agents in use, favored by their rapid replication times and high mutation rates. Hence, today we are no longer coping simply with single-drug-resistant strains but are coping increasingly with multidrug-resistant (MDR) and even extensively drug-resistant (XDR) strains, which are hard to treat or virtually untreatable, respectively, with available countermeasures.

TB – the disease

Mycobacterium tuberculosis (*Mtb*), the causative agent of tuberculosis (TB), kills nearly two million people annually and has been a major health threat for centuries [2]. Although *Mtb* replicates at a slower rate than most bacteria, the current treatment scheme of four drugs over six months renders this pathogen particularly prone to developing resistance. It is no surprise that in numerous regions, 10–20% of all TB cases are of the MDR type (resistant to Isoniazid [INH]

Corresponding author:. Parida, S.K. (parida@mpiib-berlin.mpg.de)

1359-6446/06/$ - see front matter doi:10.1016/j.drudis.2009.10.005

and Rifampin) and that by now, XDR-TB, which is resistant to virtually all the effective anti-TB drugs, has been reported in 55 countries across the globe [3].

Although the 1980s showed a steady decrease in TB cases, at least in industrialized nations, the hope of eradicating TB was shattered by the emerging HIV/AIDS crisis. Today, HIV/AIDS is the driving force of increasing incidences of TB in Sub-Saharan Africa and TB is the prime cause of death in HIV-infected individuals in this region: one-eighth of the two million deaths caused by *Mtb* occur in individuals coinfected with HIV and *Mtb*. TB is transmitted through aerosols, generated by infected droplets shed through coughing of patients with active pulmonary TB. The economic burden of TB owing to morbidity and mortality is estimated as USD 12 billion annually; the estimated total cost of curing all cases is USD 2 billion [4]. Research into TB has been seriously under-funded and there were many lost opportunities to develop drugs, diagnostics and vaccines in the last quarter of the past century [5].

More than half a million new MDR-TB cases occur annually as a result of inadequate treatment and subsequent transmission. XDR-TB, a subset of MDR-TB with statistically significant worse outcome that is resistant to virtually all the effective anti-TB drugs (i.e. MDR-TB plus resistance to any fluoroquinolone and any one of the second-line anti-TB injectable drugs: amikacin, kanamycin or capreomycin), currently affects 40,000 individuals, two-thirds of whom succumb to the disease. Yet only 3% of cases of MDR-TB are being treated according to WHO standards. Furthermore, people living with HIV/AIDS are at increased risk of dying if increased by either MDR- or XDR-TB.

Every year, 40 million individuals become infected with *Mtb* and near to 10 million fall ill. It has been estimated that up to two billion individuals are currently infected with *Mtb*, only 10% of whom will develop active disease during their lifetime. In the remaining 90% of individuals, infection remains under immune control, which, however, fails to achieve sterile eradication [6]. These individuals are clinically healthy and free of TB disease. They have what is called latent infection and harbor *Mtb* in a dormant state (e.g. at lower metabolic activity and presumably in nonreplicating persistence) (Box 1). This condition prevails as long as the immune system operates efficaciously. Once it weakens, dormant *Mtb* bacilli resuscitate to metabolically active ones and cause active TB disease. This phenomenon is termed 'reactivation TB' or 'secondary TB'.

Mtb is mainly contained within solid granulomas that primarily comprise macrophages and T lymphocytes with the involvement of cytokines and chemokines, as well as interplay of numerous concurrent mechanisms [7]. A great deal has still to be learned about the complex cascades of dynamic events occurring during the pathogenesis of TB before we can define biomarkers that indicate different stages of disease and distinguish protective immunity, disease susceptibility and pathology. Box 1 summarizes distinctive and shared features of the latent and active stages in TB.

BOX 1

Latent infection versus active disease

During the TB disease process, granulomas in the lung become caseous and liquefaction enables bacterial spreading through the alveoli to the environment and dissemination to other organs. The patient becomes contagious, and extrapulmonary TB can develop in other organs. Even though TB primarily affects the lungs (~80% of all cases), it can invade virtually every organ. The most frequent and most hazardous type of extrapulmonary TB is meningeal TB, which occurs in 20–30% of all TB cases, either alone or in combination with pulmonary TB. In highly immunocompromised patients, dissemination results in miliary TB affecting multiple organs and *Mtb* can bypass the latent stage, directly causing active TB. Extrapulmonary TB has been on the rise in the HIV era, posing a greater medical problem in terms of both diagnosis and management [2].

Current evidence suggests that in reactivation TB, different types of lesions coexist: productive granulomas, which control *Mtb* in a dormant state, and caseous granulomas, in which metabolically active *Mtb* flourish. Given the coexistence of dormant and metabolically active *Mtb* in active TB disease, the immune response might be directed against both latency-associated and actively secreted antigens. Currently used drugs attack metabolically active organisms only, leaving dormant *Mtb* unaffected. Thus, resuscitation of dormant bacteria and/or replication of a minority of persisters replenish the diseased lung after eradication of metabolically active *Mtb*. This necessitates the need for prolonged drug treatment over many months.

In TB, biomarkers will be most useful for

- diagnosis of patients with TB,
- staging or classification of TB,
- TB prognosis
- TB drug and vaccine trials

Current treatment of TB

Today, more than 20 drugs are available for the treatment of TB. Many of them, however, have considerable side effects. The current drug treatment regime comprises an initial intensive phase with a cocktail of four drugs (Rifampin, INH, Ethambutol and Pyrazinamide) for two months, followed by a continuation phase with two drugs (Rifampin and INH) for four to seven months, depending on the severity of disease and response to therapy. Such a demanding treatment is poised for poor patient compliance. To achieve complete compliance, the WHO has introduced the directly observed treatment short-course (DOTS) program as the internationally recommended strategy for TB control. The DOTS program is based on sustained political and financial commitment; diagnosis by quality-ensured sputum-smear microscopy; standardized short-course anti-TB treatment given under direct and supportive observation; a regular, uninterrupted supply of high-quality anti-TB drugs; and standardized recording and reporting. DOTS has proven its value for nonresistant TB strains depending on rapid diagnosis but not for either MDR- or XDR-TB (see below). The Global Alliance for TB drug development (http://www.tballiance.org/new/portfolio.php/), a public–private partnership enterprise supported by the Bill & Melinda Gates Foundation (BMGF), has been instrumental in developing a portfolio with the most diverse TB drug pipeline in history [8].

HIV–TB liaison

In HIV-infected individuals, *Mtb* infection transforms into active disease more rapidly and more frequently than in healthy individuals because of impaired immune control. MDR-TB and XDR-TB are highly lethal in people coinfected with HIV. Drug-resistant TB, therefore, is a major threat to the effectiveness of both TB treatment and antiretroviral treatment (ART) programs.

Many countries in Sub-Saharan Africa are scaling up highly active antiretroviral therapy (HAART), using one of the first-line regimens. Current HAART and TB drug treatment continue to be a therapeutic challenge in terms of adverse effects, drug–drug interactions and immune reconstitution inflammatory syndrome (IRIS) [9,10]. Incidences of IRIS are on the rise. It develops as a consequence of the lowered HIV burden through ART or HAART leading to a strong restoration of the T-cell response, frequently of T cells specific for *Mtb* antigens. This often promotes an outbreak or exacerbates existing TB. Often, AIDS and TB are treated as separate diseases, even when they occur in a single patient. This unhealthy separation should be discontinued, particularly because IRIS is not the only complication of drug therapy of TB and AIDS. Drug interactions and shared drug toxicities further add to this dilemma. Novel combination therapies that minimize side effects in HIV–*Mtb* coinfected patients are needed [11,12].

TB diagnosis

More than 125 years ago, the etiology of TB was revealed by the German physician and scientist Robert Koch (1843–1910). In his groundbreaking presentation on March 24, 1882, Koch described how he stained the acid-fast bacilli (AFB) that cause TB. Identification of AFB in sputum remains the most widely used diagnostic test for active TB. In general, three sputum samples are tested for AFB by microscopy, which is laborious and time-consuming. Recently, cheaper light-emitting diode microscopes have been introduced to replace conventional high-cost fluorescent microscopes [13], and auramin staining is now used instead of the classical Fuchsin red (Ziehl-Neelsen) staining. The particular challenge for TB is to detect very few bacteria in specimens rapidly and cost-effectively, enabling quick diagnosis and treatment of all active cases.

In 1890, Robert Koch described his attempts to develop a vaccine for the treatment of TB. Although he failed, the methodology he used provided the basis for the tuberculin skin test (TST) that is still used today. This test measures the delayed-type hypersensitivity reaction mediated by T cells (mostly CD4 T cells of Th1 type) to intradermally inoculated tuberculin (crude antigen mixtures of >200 proteins from *Mtb* and a positive test indicates previous exposure to *Mtb*), without being able to distinguish between latent infection and active TB disease. This test also gives a positive result in live attenuated *Mycobacterium bovis* Bacille Calmette-Guérin (BCG)-vaccinated individuals because of cross-reactive antigens. The TST has the major disadvantages of poor specificity (false-positive tests caused by prior vaccination or previous exposure to environmental mycobacteria) and poor sensitivity (false-negative tests in children and immunocompromised individuals). Generally, clinical radiography and TST are routinely used for the diagnosis of TB. Culture of sputum for *Mtb* is specific confirmatory test for pulmonary TB, done either by solid media (Lowenstein-Jensen) or by the more sensitive Mycobacteria Growth Indicator Tube liquid culture system (Beckton Dickinson) using a fluorescence detection method.

It is probable that the different genetic programs in the pathogen are also reflected by differential antigen expression (i.e. dormancy antigens during latency and actively secreted antigens during active TB). Currently, immune responses to actively secreted antigens such as Antigen 85, ESAT-6 and CFP-10 are widely used in laboratory tests to determine TB infection and aid in the diagnosis process. Less is known about dormancy antigens; however, a cluster of ~48 antigens controlled by the DosR regulon have been shown to be expressed by dormant *Mtb* and, hence, probably indicate latent infection [14]. Recently, the Grand Challenges in Global Health (GCGH) consortium on TB biomarkers, supported by BMGF and coordinated by the authors, have shown interferon-gamma (IFNγ) response in whole-blood assays to several novel DosR regulon-encoded *Mtb* proteins in latently *Mtb*-infected subjects in three different African populations [15].

A new generation of IFNγ-release assays using specific antigens of ESAT-6 and CFP-10 is able to differentiate infection from BCG vaccination but still lack the specificity to distinguish disease from infection [16]. The two most widely used tests are QuantiFERON from Cellestis, Australia and the T-spot assay from Oxford Immunotech, UK. Quantiferon is based on a whole-blood stimulation assay and is also approved by the FDA as an *in vitro* test. T-SPOT uses an enzyme-linked immunosorbent spot (ELISPOT) assay of peripheral blood mononuclear cells and has obtained premarket approval from the FDA. Whether any of these tests can replace the TST as the diagnostic test of choice remains to be seen. Antibody-mediated or PCR-based kits have come to markets in different parts of the world without proper validation. Moreover, there are several new diagnostics in development pipelines using different technologies [17,18]. A recent report using a packaged DNA chip assay combined with an image analysis system for the detection of *Mtb* complex is promising, with ~84% sensitivity and ~94% specificity [19].

In the current epidemic of HIV/AIDS, incidences of extrapulmonary TB have increased considerably, and these cases cannot be diagnosed using sputum smear or culture for the detection of *Mtb*. Extrapulmonary TB often requires invasive procedures to obtain diagnostic specimens from the site of the lesion, such as fine needle aspiration cytology in the case of TB lymphadenitis. It has been claimed that up to 50% of HIV-positive TB patients have negative smear on direct microscopy and positive on culture. Biomarkers could improve the diagnosis of extrapulmonary TB and TB in HIV-infected individuals.

Vaccination against TB

BCG is widely and successfully used as a vaccine at birth to prevent severe forms of the disease, such as miliary TB and TB meningitis, in children. Yet the vaccine fails to protect against the most prevalent disease form of today – adult pulmonary TB. Although BCG has been proven safe in immunocompetent individuals, it bears a risk for immunocompromised individuals, which is why the WHO no longer recommends BCG vaccination of children known to be HIV positive.

In an attempt to develop a better vaccine for TB, strategies comprising heterologous prime-boost regimes have progressed in the development pipelines. Because BCG has been safe with beneficial effects during childhood, emphasis has been placed on boosting its effects through the addition of a booster vaccine with either viral-vectored vaccines or subunit vaccines (protein formulations in adjuvant) [20]. Five such candidates are in different stages of clinical trials: MVA85A is in Phase II clinical trial in the UK, Gambia and South Africa; Mtb72f (a fusion protein licensed by

BOX 2

Terminologies used in the context of biomarkers

A clinically useful biomarker needs to fulfill three important criteria:

- provide accurate, repeated measurements at reasonable cost and with a short turnaround time;
- provide information not available from clinical assessment;
- assist in medical decision making.

Surrogate marker should have the following characteristics:

- provide close link to disease pathogenesis;
- predict a long-term outcome in the absence of specific therapy;
- reveal changes early in the course of treatment with predictive value;
- capture the full effect of treatment on the disease process;
- be independent of the type of mechanism underlying treatment.

GlaxoSmithKline) is in early Phase II trial; and fusion of Ag85B-ESAT-6 (H1) from Statens Serum Institut, fusion of Ag85B and TB10.4 (H4) from SSI, and an Adeno-based vaccine from Crucell–Aeras are in Phase I trials [20]. There are also efforts to replace the BCG vaccine with an improved BCG vaccine. Two vaccines are currently in clinical trials – the first is a recombinant BCG over-expressing Ag85B [21], and the second is recombinant BCG with the insertion of listeriolysin gene from *Listeria monocytogenes*, which elicits broader and more potent T-cell responses [22]. Aeras Global TB Vaccine Foundation (http://www.aeras.org/home/home.php/) has been active in developing a series of additional strategies and permutations to push the TB vaccine agenda forward to realization.

Definitions

It is crucial to clearly define the terminologies used in the context of biomarkers [7] (Box 2). A **biological marker**, or **biomarker**, is a characteristic that is objectively measured and evaluated as an indicator of a physiological or pathological process or pharmacological response(s) to a therapeutic intervention [23]. A profile of combined biomarkers is called a biosignature (Figures 1 and 2).

Correlate is a frequent but not definitive marker of risk for a disease (e.g. cholesterol in heart disease). Correlate of protection is a measurable sign of host response to an infectious agent indicating resistance or susceptibility to developing disease.

Biomarkers in clinical trials are valuable for monitoring disease outcome. Ideally, they would replace the **clinical endpoint**, which is defined as a characteristic or variable that reflects the final outcome of the disease in terms of function, effect, progress, recovery, survival or death. Thus, a clinical endpoint is the characteristic reflection of a patient's health status.

Very few biomarkers exist that fulfill these requirements [24], none of them for use in TB. Biomarkers fulfilling these criteria could serve as **surrogates of disease**. A surrogate is a biomarker or biosignature that is statistically associated with and believed to be pathophysiologically related to the clinical outcome. A correlate does not imply disease or protection against disease, whereas a surrogate does [25]. A **surrogate endpoint** can substitute for a clinical endpoint [26]. It should predict clinical benefit, harm, or lack of benefit or harm and ideally characterizes efficacy of an intervention before disease as clinical endpoint.

Monitoring of treatment responses and relapses versus recovery

Regulatory requirements demand clinically meaningful endpoints for licensing new drugs. In the case of TB, we do not have any surrogate endpoints for relapse representing patients who fail to respond to multidrug therapy (MDT), sputum conversion from culture positive to culture negative for *Mtb* within two months and patients with recurrent disease within two years of therapy. A conventional drug trial needs at least five years of follow up of a large cohort of patients to assess the risk of relapse, as the occurrence is approximately 5% of all cases treated.

A two-month culture conversion has been used as surrogate marker for long-term outcome in drug trials. Time to sputum-culture conversion for *Mtb* or rate of decline of colony counts (read out of effectiveness of the drugs in killing and/or eliminating *Mtb*) in quantitative sputum cultures during the initial phase of MDT might be a better alternative to the currently used practice. The existence of two distinct bacterial populations with actively multiplying and persisting dormant *Mtb* has to be considered in the interpretation of these results.

Biomarkers to accelerate clinical trials

Composite datasets are required to compare and validate biomarkers that correlate *ex vivo* results with *in vivo* effects and to link observations made in *in vitro* model systems. This necessitates systematic prospective studies unraveling the complexity of the immune response against *Mtb* with the interplay of additional biomarkers for a reliable correlate of protection. Biosignatures using single or multiple platforms are probably more appropriate for TB than a single biomarker [27]. Experts envisage a potential for the development of epidemiological methods over next decades. These suggested methods emphasized the need for the extensive use of biomarkers, the greater standardization of data analysis and reporting methods, and an improvement of the interplay between observational studies and randomized controlled trials. It has been argued that a phased approach to epidemiologic hypothesis evaluation – with hypotheses that are promising in observational studies subjected to controlled trials with well-defined intermediate outcomes – is needed. It is also being argued that a multidisciplinary, coordinated network of scientists and clinicians interested in disease risk estimation and prevention will be needed for epidemiologic research to fulfill its potential over the coming decades [28]. Overall, the high prevalence of TB in resource-poor settings prohibits widespread use of any high-tech measure in countries suffering most from TB.

Drug trials

Biomarkers play a critical part in drug discovery by reducing the attrition of drug development and, hence, the overall R&D cost of drug development. In this area, biomarkers can be categorized into three categories: measuring the delivery of drugs to targets; understanding and predicting pathophysiological mechanisms; and the assessment of clinical effects, on the basis of contribution to the clinical plan. Evaluation of these three major characteristics early on in the development process facilitates the prioritization of rational drug discovery resources by enabling early proof-of-concept studies for novel therapeutic targets.

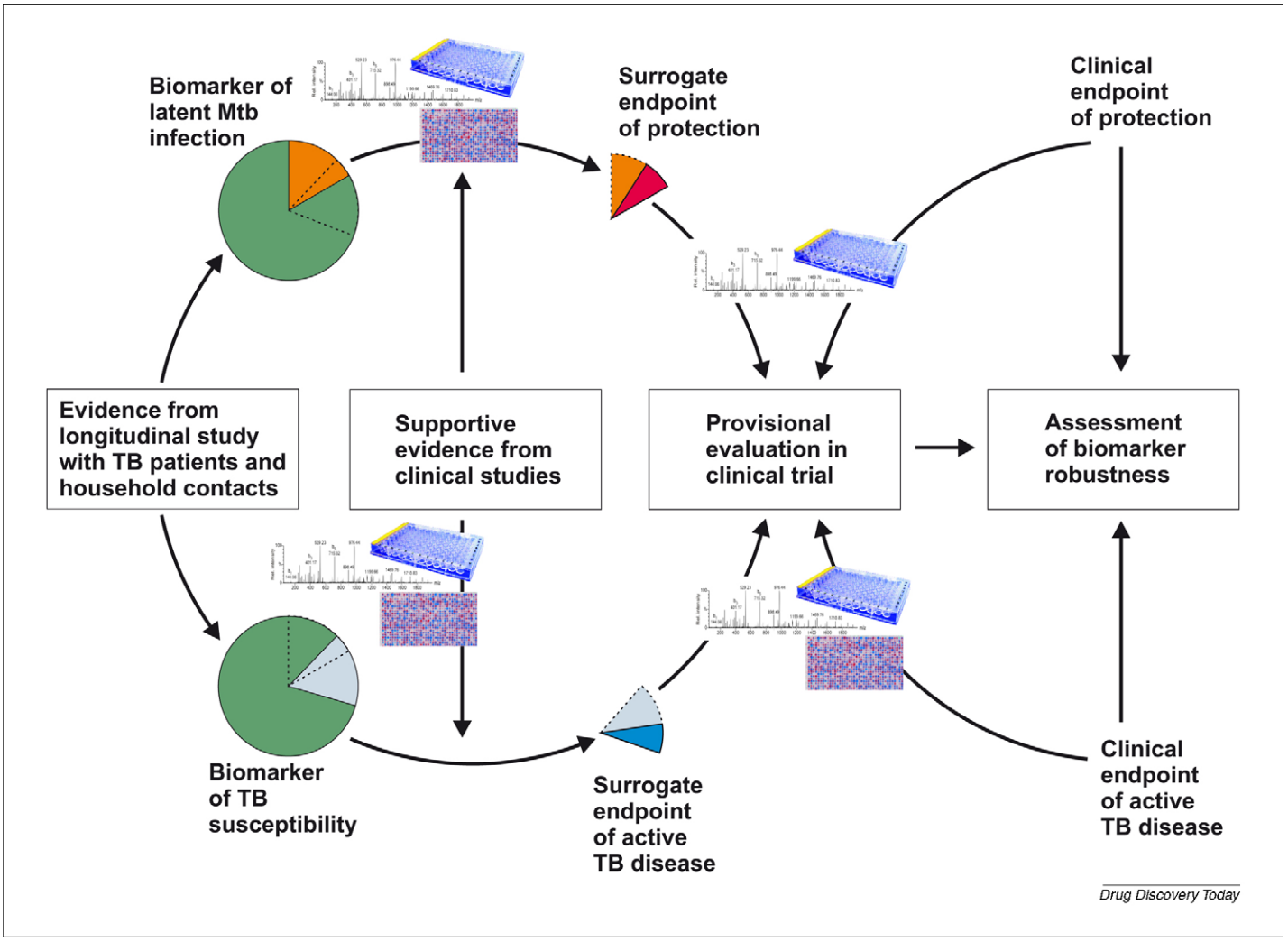

FIGURE 1

The iterative process of biomarker discovery. Depiction of processes involved in biomarker discovery: evidence from case-contact studies indicates sets of biomarkers of latent *Mycobacterium tuberculosis* (*Mtb*) infection and disease susceptibility. These sets of biomarkers might be distinct or might overlap, as indicated in the pie. Evidence from clinical observational studies needs to be corroborated with observations from clinical practice before being validated independently in clinical trials for robustness and reliability before widespread use.

Biomarkers to predict drug interactions or drug–immune response interactions, dose–response relationships and toxicity would accelerate the drug R&D pipeline in determining these parameters early to reduce cost and attrition, in particular in the areas of ART for HIV/AIDS and TB drugs [29] and combination therapy for AIDS and TB co-infections.

Transcriptomics has been widely used to discover genes that are associated with specific diseases, to classify diseases, to identify drug targets and to annotate gene functions [30,31]. Biomarkers predictive of drug toxicity or efficacy would strongly reduce attrition and thereby the R&D pipeline. Biomarkers predictive of mechanisms underlying success or failure of intervention would aid structure–activity relationships. Biomarkers based on transcriptomics profiling would facilitate pharmacokinetic–pharmacodynamic evaluation, toxicology determination, drug candidate selection and patient stratification, as well as being decisive for the identification of new indications for existing drugs or for early diagnostic or predisposition tests.

The changes at the global gene expression level should be linked with biochemical and signaling pathways. The clustering of genome-wide gene expression analysis at the molecular level revealed three distinct subtypes of breast cancer with impact on treatment [32]. The basic understanding of the disease gained through transcriptomics has led to individualized treatments according to patient and tumor factors with effective adjuvant therapy in improving survival [33,34]. This might have implications for the management of complicated MDR- or XDR-TB cases.

How to measure biomarkers

Although biomarkers can be studied in any tissue or body fluid (including urine, saliva, sputum and breath), peripheral blood is the most widely used source in clinical practice. Genes, transcripts, proteins, lipids and metabolites can all be measured in blood for biomarker studies. Informational complexity increases from genome to transcriptome to proteome (Figure 2). A high-throughput

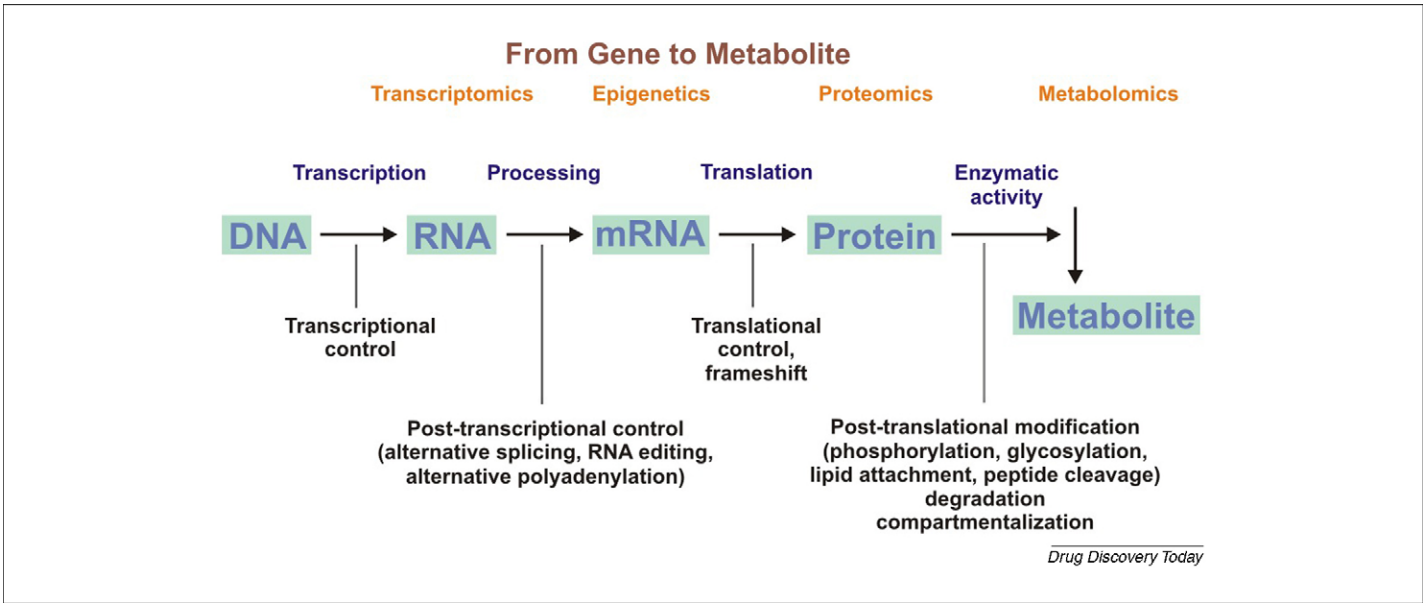

FIGURE 2

The plethora of biomarkers. Different biomics platforms, as well as compilation of molecules and processes, analyzed as indicators of biological phenomena.

proteome chip has been developed using protein microarrays to assess antigen-specific humoral immune responses [35]. Using this technique, the whole *Mtb* proteome on a chip has been developed and is being pursued for product development as a diagnostic biomarker by Foundations for Innovative New Diagnostics. This technique is also being exploited as a multiplex system for simultaneous serological detection of multiple infectious agents (PathogenChip) [36]. Peptide microarrays with peptides of *Mtb* proteome are also being developed as biomarkers for TB, assessing pattern recognition based on *Mtb*-specific humoral immune response [37]. Other biological materials such as urine, sputum, breath and tissue specimens (biopsies or aspirates) can also be used for biomarker measurements [7]. Whole-blood bacteriocidal assay has been proposed as a marker for relapse to assess sterilizing activities of new drug candidates [38]. Two months' sputum-culture conversion is another potential microbiological surrogate marker best studied in retrospective analysis of large controlled trials [39]. Positive sputum culture after two months of treatment was found to be associated with greater risk of relapse [40].

Transcriptomics

Transcriptome analysis evaluates global changes in gene expression profiles in a cell, tissue, organ or whole organism, providing a dynamic link between the genome and the proteome. Peripheral blood leukocytes are the most feasible tissue source in clinical assessment and have been studied for differential gene expression between disease with or without drug treatment. This enables the investigation of the pharmacodynamic effects of drugs at the genomic level to predict efficacy, side effects of drugs and, especially, clinical outcomes, as well as of the prophylactic efficacy of new vaccine candidates. This approach has been used successfully in the field of oncology and is currently a major focus in infectious diseases.

Validity and results of gene expression studies rely heavily on the appropriate choice of study groups [41]. Candidate biomarkers were assessed for optimal study group discrimination using a linear discriminant analysis that could cluster TB patients and *Mtb*-infected healthy subjects of Caucasian origin using a group of genes comprising lactoferrin, CD64 and the Ras-associated GTPase 33A as a minimal biosignature [42]. Thus, proof-of-principle has been achieved for a custom-made transcriptomic platform comprising a small number of selected differentially expressed genes in TB. Heterogeneity in the cellular composition of tissue specimens frequently confounds data analysis in microarray studies, and deconfounding of transcriptome analyses for cellular heterogeneity greatly improves interpretability and, hence, validity of transcriptome profiling results [43].

In a similar study conducted by another group that focused on the transcriptomic profiling of relapse cases of TB (comparing those with cured TB with control groups of active TB and latently infected healthy subjects) of South African origin, distinct patterns of gene expression were exhibited [44]. The gene-transcript profiles of patients with recurrent TB were more similar to those of patients with active TB than to those of the cured or latently TB-infected healthy subjects. Discriminant analysis of a training dataset showed that profiling nine genes was sufficient to classify the subjects into the four groups [44]. A simple test based on gene expression patterns could be used as a biosignature of cure while identifying patients who were at risk of relapse.

Attempts were made to emulate different stages of disease *in vitro* using parameters that affect the growth of *Mtb* in the host – such as nutrient status, phagosomal pH, oxygen availability and immunologic defense – to dissect the signals that are responsible for controlling subsets of genes and to understand the virulence factors [30]. Linking the genes to biochemical and signaling pathways and assessing these associations in the context of human disease would harness our understanding of the biochemical pathways and biological processes to develop novel therapeutics.

Proteomics

The proteome was originally described as functional output of the genome [45]. Proteomics evaluates global changes in the full complement of proteins expressed by a tissue or cell type at a point in time. Alterations in protein abundance, function and structure can indicate pathological abnormalities even before the onset of clinical symptoms and, hence, have the potential to be diagnostic or prognostic biomarkers [46]. Proteomics has been performed using mixtures of cells, such as peripheral blood leukocytes, selected cells (such as T cells), intracellular fractions or body fluids (such as serum or plasma). Biological cleavage products and artifacts from sample processing, protein isoforms, post-translational modifications, and ratios of intact to truncated forms further add to the challenges [47].

A proteome approach, combining high-resolution two-dimensional electrophoresis with MS, was used to compare the cellular protein composition of two virulent strains of *Mtb* with two attenuated strains of *Mycobacterium bovis* BCG to identify unique proteins expressed by these strains [48,49]. The Human Proteome Project is going to study each of the probably 21,000 proteins derived from a single gene [50]. There is a great potential for an integrated proteomics analysis of *Mtb* and human to find novel targets for drug discovery programs.

Surface-enhanced laser desorption-ionization time-of-flight (SELDI-TOF) techniques have been applied to identify serum biomarkers from patients with advanced *Mtb* infections based on a pattern-based diagnosis through simultaneous detection of multiple peptide and protein signals in a single mass spectrum [51]. Improved algorithmic interpretation of the resulting peptide masses from SELDI-TOF with concurrent use of tandem MS for amino acid sequencing led to the identification of four biomarkers as biosignatures for the diagnosis of TB with ~80% accuracy. Systematic study of mass spectral serum patterns from patients at different stages of disease would establish the evidence of the specificities to validate protein biomarkers. There have been concerns over the issue of raised protease activity after clotting in serum and the relative merits of plasma over serum. There are ongoing efforts to harmonize the operating procedures for serum and plasma proteomics [52].

Proteome analysis of anti-TB drug responses in a *Mycobacterium smegmatis* model system revealed that translation, cell-cycle control and energy production were downregulated with drug treatment. By contrast, systems related to the drugs' targets, such as lipid, amino acid and nucleotide metabolism, revealed specific protein expression changes associated with a particular drug treatment [53]. This *in vitro* approach can be used to elucidate novel targets for new drug developments.

Coupling two-dimensional gel electrophoresis and MS with laser capture microdissection enabled analysis of the protein expression profiles in specific cancer cell types in individual disease processes with regards to cancer development, progression and effects of treatment [54]. This technique has not yet reached the stage of genome-wide representation of all proteins present in a tissue but has indicated a feasible way to find differentially expressed proteins in target tissues. Recent developments in mass spectrometric detection followed by proper statistics and bioinformatics will enable the analysis of the proteome in as few as one hundred cells [55]. This approach has potential for studying tuberculous lesions to explore the differential expression of proteins in the major cell types involved in the granuloma.

Metabolomics

Metabolomics evaluates global changes in the profiles of the small-molecule metabolites, which are unique chemical fingerprints of the cellular processes [56]. The metabolome refers to the complete set of small-molecule metabolites (such as metabolic and catabolic intermediates, hormones and other signaling molecules, and secondary metabolites, as well as small structural lipids) found within a biological sample, such as a single organism [56–59]. During active disease and probably during latent infection, *Mtb* perturbs biochemical networks and induces changes in the types and amounts of small molecules present in the host. These changes (metabolic signatures) can be recorded in a time-dependent manner over the course of disease [57].

The first proof-of-principle study on metabolomics in TB, conducted by the current authors in collaboration with the industrial partner Metabolon Inc. (based in Durham, NC, USA) and colleagues at Stellenbosch University in South Africa, has yielded promising results that highlight its potential exploitation for biomarker discovery. Serum samples were analyzed from age- and gender-matched individuals with newly diagnosed pulmonary TB and latently infected (TST-positive) and clinically healthy contacts from an endemic region of South Africa. Metabolomics can provide biosignatures to distinguish states of TB infection, to monitor patients during chemotherapy and to predict potential nonresponders to effectively manage these groups of patients with other drug regimens, as well as providing potential targets for drug candidates in development. Using an unbiased metabolomic profiling approach, a recent study has identified the metabolite Sarcosine, an *N*-methyl derivative of the amino transferase that was highly increased during prostate cancer progression to metastasis and can be detected noninvasively in urine [60].

Four specific compounds (methyl phenylacetate, methyl *p*-anisate, methyl nicotinate and *o*-phenylanisole) from *Mtb* cultures grown *in vitro* were identified as distinctive volatile markers. These compounds are detectable before the visual appearance of colonies, are potentially useful as the basis of a noninvasive diagnostic test for TB and have characteristic odors [61]. Recently, methyl nicotinate has been detected in the breath of smear-positive TB patients at statistically significant levels compared with healthy (smear-negative) subjects [62]. This is a new field in the context of human diseases and needs to be further exploited.

Immune markers

Potential correlates of protective immunity in TB were studied by determining the antigen-specific immune response, initially with purified native secreted antigens of *Mtb*, then with recombinant antigens and peptides [63–65]. Upon antigen-specific stimulation, T cells produce cytokines as mediators of protection against *Mtb*. IFNγ has been classically used as the read-out system in these T-cell assays. Enzyme-linked immunosorbent assay (ELISA) measures the concentration of a single cytokine in the fluid (either serum or other body fluid or culture supernatants from *ex vivo* cell culture) produced by either a single cell or multiple cells or cell types. Multiplex assay measures amounts of numerous cytokines simultaneously in serum or culture supernatant in a single assay. These

multiple datasets can provide deeper insights into the overall picture. ELISPOT assay measures frequency of antigen-specific cells that produce a cytokine or multiple cytokines; hence, these assays enable enumeration of biologically relevant T cells. A combination of ELISA and ELISPOT, therefore, is needed to quantify the amount of cytokine produced and the frequency of antigen-specific cells, respectively. Intracellular cytokine staining by flow cytometry determines cell phenotypes based on cell surface markers, along with intracellular cytokines. This approach is best suited for determining the role of multifunctional T cells (i.e. single T cells producing relevant cytokines of protective immunity) at a given maturation stage (e.g. effector T cells or memory T cells). It has been claimed that multifunctional memory T cells are crucial for vaccine-induced protection against TB, even though further functional evidence has yet to be demonstrated [66,67]. Each of these techniques has its advantages and disadvantages and has to be appropriately selected and matched with technical feasibility in the field [68,69].

Recently, broader antigen panels of *Mtb* have been tested for immunogenicity using peripheral blood mononuclear cells (PBMC) or whole-blood assays to study antigen-specific immune responses [70]. In an ongoing effort from the GCGH Biomarkers for TB Consortium, 86 novel mycobacterial antigens have been screened in endemic populations of Africa for their ability to produce IFNγ upon stimulation in whole-blood assays [13,15,70,71]. Several studies could delineate differences between TB patients and latently infected TB individuals based on T-cell responses to antigens [72–74]. This is complemented by determinations of multiple cytokines and chemokines to elucidate their interplay in pathogenesis and protection [75,76]. Multifunctional T cells, memory T cells and different T-cell subsets have been studied to find immune markers of protection and disease progression [77,78]. An association between Th1 cell expansion and IRIS in TB has been observed, with speculation on defective immune regulation [76,79]. Combining the results from several initiatives will serve as an impetus to advance the immune marker field into effective translational uses in the field.

Vaccine trials

Immunological assays are being used for assessing immunogenicity of various vaccine candidates in clinical trials currently in progress. Conventionally, IFNγ secretion by antigen-specific CD4 T cells has been used as a biomarker of protection, but current evidence indicates that, while being an important component of the immune response, IFNγ is not a reliable biomarker of protection [80–82]. Polyfunctional T cells expressing multiple cytokines (IL-2, TNFα and IFNγ) are being increasingly studied, emphasizing the importance of multiple cytokines in conjunction. In other trials, cytokine production by specific subsets of T cells after restimulation with peptides or proteins is being determined in intracellular flow cytometric assays [83]. Protection in BCG-immunized mice after challenge with *Mtb* correlated with rapid accumulation of specific $CD8^+$ T cells in infected organs of *Mtb*-challenged mice [81]. By contrast, specific IFNγ production by specific $CD4^+$ T cells reflected the bacterial load, rather than the strength of protection [81]. A recent report in humans has shown that IFNγ does not correlate with BCG-induced protection in children, even though it is a valid marker of immunogenicity [82]. Thus, we still have no robust surrogate of protection to accelerate vaccine trials. Ideally, these markers should be available in advance, but the current scenario demands moving forward in parallel with existing tools and curetting relevant biological samples for future validation and testing of new biomarkers [68,81,82,84,85].

Concluding remarks

The FDA in the USA propagates the development of personalized medicine for patients suffering from cancer or metabolic diseases. These personalized treatment schemes will be determined by individual biomarker profiles. Initially, such an approach might seem overambitious for a disease like TB, which is most prevalent in poverty-ridden countries. Yet TB might benefit from a semi-personalized approach. Biomarkers could help explain why some people develop disease and others remain healthy. Moreover, TB is unique among bacterial infectious diseases because its treatment requires numerous drugs over several months (three to four drugs for six to nine months). Although the so-called 'DOTS' scheme has proven successful, increasing incidences of MDR- and XDR-*Mtb* strains demand a re-evaluation of this strict treatment scheme. Transcriptomic, proteomic and metabolomic profiling combined with broad-scale immunologic profiling can provide clues to burning questions in TB, which will help in the design of novel intervention strategies. All of these are currently in the explorative discovery phase but might lead to product development within the next five years. Much progress has been made in the past five years in understanding the pathogenesis in a composite way, rather than using a simple reductionist approach, but this has also raised new questions. IFNγ, which was thought to be the most reliable correlate of protection, is losing its spot and being replaced by multiple cytokines and multicytokine-producing T cells. Similarly, efforts to understand the pathogen have brought some hope of detecting it with either one or multiple moieties. The early results from the laboratory benches need to be validated in large studies in the clinics and the field before the research can be translated into applications. There is a great need to use systems biology approaches to integrate the multiple datasets from different biomics platforms along with immunological and clinical parameters to draw a more complete picture of living organisms or investigate disease as a multifactorial phenomenon [86]. A recent publication has identified early signatures predicting immune responses in humans vaccinated with a yellow fever vaccine, highlighting the importance of this approach in predicting vaccine efficacy [87].

Although biomarkers have to prove their robustness in clinical trials for vaccine and drug candidates, in the long run, they can provide tools for predicting the risk of active TB disease outbreak in latently infected individuals and for individual-based decisions on preventive and therapeutic drug treatment schemes. With increasing incidences of HIV–*Mtb* coinfected individuals and rising numbers of MDR- and XDR-TB, efficacious control will depend on such a comprehensive approach. Obviously, such personalized efforts face high hurdles that require huge concerted efforts from different stakeholders, including private industry, regulatory agencies, foundations, and governmental and nongovernmental organizations. Despite these obstacles, it would be a worthwhile endeavor to provide solutions to a disease that annually afflicts ten million people and kills two million people worldwide.

Acknowledgements

The authors thank M.L. Grossman for editorial help and D. Schad and L. Fehlig for graphic support. Studies in the authors' laboratory receive financial support from the Bill & Melinda Gates Foundation Grand Challenges in Global Health Grant No. 37772, the European Union Framework Program 6 Projects, Design and Testing of Vaccine Candidates against Tuberculosis, and Bundesministerium für Bildung und Forschung Networks PathoGenoMikPlus and Nationales Genomforschungsnetz.

References

1 Projan, S.J. (2008) Whither antibacterial drug discovery? *Drug Discov. Today* 13, 279–280
2 WHO, (2009) *Global Tuberculosis Control: Epidemiology, Strategy, Financing*. WHO/HTM/TB/2009.411 (http://www.who.int/tb/publications/global_report/2009/pdf/full_report.pdf)
3 WHO, (2009) *Global map and information on XDR-TB*. In: http://www.who.int/tb/challenges/xdr/xdr_map_sep09.pdf
4 WHO, (2008) In *Tuberculosis Control, The Burden of Tuberculosis: Economic Burden*, (Vol. 2009) (Kim, S.H., Shakow, Aaron, Castro, Arachu, Vande, Chris, Farmer, Paul, eds) In: http://www.who.int/trade/distance_learning/gpgh/gpgh3/en/index7.html
5 Kaufmann, S.H. and Parida, S.K. (2007) Changing funding patterns in tuberculosis. *Nat. Med.* 13, 299–303
6 Kaufmann, S.H. (2007) The contribution of immunology to the rational design of novel antibacterial vaccines. *Nat. Rev. Microbiol.* 5, 491–504
7 Kaufmann, S.H. and Parida, S.K. (2008) Tuberculosis in Africa: learning from pathogenesis for biomarker identification. *Cell Host Microbe* 4, 219–228
8 Stop TB, (2008) *Strategic Plan: Stop TB Partnership*. Working Group on New TB Drugs In: http://www.stoptb.org/wg/new_drugs/assets/documents/WGND%20Strategic%20Plan%20%28final%29.pdf
9 Lawn, S.D. *et al.* (2008) Immune reconstitution and ''unmasking'' of tuberculosis during antiretroviral therapy. *Am. J. Respir. Crit. Care Med.* 177, 680–685
10 Pepper, D.J. *et al.* (2007) Combined therapy for tuberculosis and HIV-1: the challenge for drug discovery. *Drug Discov. Today* 12, 980–989
11 Havlir, D.V. *et al.* (2008) Opportunities and challenges for HIV care in overlapping HIV and TB epidemics. *J. Am. Med. Assoc.* 300, 423–430
12 Lalloo, U.G. and Pillay, S. (2008) Managing tuberculosis and HIV in sub-Sahara Africa. *Curr. HIV/AIDS Rep.* 5, 132–139
13 Marais, B.J. *et al.* (2008) Use of light-emitting diode fluorescence microscopy to detect acid-fast bacilli in sputum. *Clin. Infect. Dis.* 47, 203–207
14 Voskuil, M.I. *et al.* (2003) Inhibition of respiration by nitric oxide induces a *Mycobacterium tuberculosis* dormancy program. *J. Exp. Med.* 198, 705–713
15 Black, G.F. *et al.* (2009) Immunogenicity of novel DosR regulon-encoded candidate antigens of *Mycobacterium tuberculosis* in three high-burden populations in Africa. *Clin. Vaccine Immunol.* 16, 1203–1212
16 Farhat, M. *et al.* (2006) False-positive tuberculin skin tests: what is the absolute effect of BCG and non-tuberculous mycobacteria? *Int. J. Tuberc. Lung Dis.* 10, 1192–1204
17 Stop TB, (2008) *TB Diagnostics Pipeline: New TB Diagnostics Working Group*. November 2007 (http://www.stoptb.org/retooling/assets/documents/TB%20DIAGNOSTICS%20PIPELINE%20March08.pdf)
18 FIND, (2008) *Foundation for Innovative New Diagnostics tuberculosis product deliverables 2008–2013*. In: http://www.finddiagnostics.org/programs/tb/pipeline.html
19 Huang, T.S. *et al.* (2008) Detection of *M. tuberculosis* using DNA chips combined with an image analysis system. *Int. J. Tuberc. Lung Dis.* 12, 33–38
20 Stop TB, (2008) *TB Vaccines Pipeline: Working Group on New TB Vaccines*. March 2008 (http://www.stoptb.org/retooling/assets/documents/StopTB%202008%20Vaccines%20Pipeline%20March%2008.pdf)
21 Hoft, D.F. *et al.* (2008) A new recombinant Bacille Calmette-Guerin vaccine safely induces significantly enhanced tuberculosis-specific immunity in human volunteers. *J. Infect. Dis.* 198, 1491–1501
22 Grode, L. *et al.* (2005) Increased vaccine efficacy against tuberculosis of recombinant *Mycobacterium bovis* Bacille Calmette-Guerin mutants that secrete listeriolysin. *J. Clin. Invest.* 115, 2472–2479
23 (2001) Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin. Pharmacol. Ther.* 69, 89–95
24 Braunwald, E. (2008) Biomarkers in heart failure. *N. Engl. J. Med.* 358, 2148–2159
25 Sadoff, J.C. and Wittes, J. (2007) Correlates, surrogates, and vaccines. *J. Infect. Dis.* 196, 1279–1281
26 Colburn, W.A. (2000) Optimizing the use of biomarkers, surrogate endpoints, and clinical endpoints for more efficient drug development. *J. Clin. Pharmacol.* 40, 1419–1427
27 Jacobsen, M. *et al.* (2008) Novel strategies to identify biomarkers in tuberculosis. *Biol. Chem.* 389, 487–495
28 Prentice, R.L. (2007) Epidemiologic methods developments: a look forward to the year 2032. *Ann. Epidemiol.* 17, 906–910
29 Perrin, F.M. *et al.* (2007) Biomarkers of treatment response in clinical trials of novel antituberculosis agents. *Lancet Infect. Dis.* 7, 481–490
30 Bacon, J. and Marsh, P.D. (2007) Transcriptional responses of *Mycobacterium tuberculosis* exposed to adverse conditions *in vitro*. *Curr. Mol. Med.* 7, 277–286
31 Williams, W.V. (2005) Transcriptome analysis in drug development. *Curr. Mol. Med.* 5, 1–2
32 Sorlie, T. *et al.* (2003) Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc. Natl. Acad. Sci. U. S. A.* 100, 8418–8423
33 Turner, N.C. and Jones, A.L. (2008) Management of breast cancer – part I. *BMJ* 337, 107–110
34 Turner, N.C. and Jones, A.L. (2008) Management of breast cancer – part II. *BMJ* 337, 164–169
35 Davies, D.H. *et al.* (2005) Profiling the humoral immune response to infection by using proteome microarrays: high-throughput vaccine and diagnostic antigen discovery. *Proc. Natl. Acad. Sci. U. S. A.* 102, 547–552
36 PathogenChip, (2008) *PathogenChip*. ''About the protein microarray laboratory at UC Irvine and ImmPORT: The protein microarray laboratory at UC Irvine and ImmPORT has developed an efficient method for producing all of the proteins from infectious agents like smallpox and printing them onto microarray chips. The chips can be probed with serum from vaccinated individuals to study the antibodies that are produced after vaccination or infection. The team has produced and printed microarray chips containing 16,000 different proteins from 20 infectious agents, including the organisms responsible for tuberculosis and malaria.'' In: http://archive.today.uci.edu/news/release_detail.asp?key=1716
37 Gaseitsiwe, S. *et al.* (2008) Pattern recognition in pulmonary tuberculosis defined by high content peptide microarray chip analysis representing 61 proteins from *M. tuberculosis*. *PLoS One* 3, e3840
38 Wallis, R.S. *et al.* (2003) Whole blood bactericidal activity during treatment of pulmonary tuberculosis. *J. Infect. Dis.* 187, 270–278
39 Mitchison, D.A. (1993) Assessment of new sterilizing drugs for treating pulmonary tuberculosis by culture at 2 months. *Am. Rev. Respir. Dis.* 147, 1062–1063
40 Benator, D. *et al.* (2002) Rifapentine and isoniazid once a week versus rifampicin and isoniazid twice a week for treatment of drug-susceptible pulmonary tuberculosis in HIV-negative patients: a randomised clinical trial. *Lancet* 360, 528–534
41 Repsilber, D. *et al.* (2005) Sample selection for microarray gene expression studies. *Methods Inf. Med.* 44, 461–467
42 Jacobsen, M. *et al.* (2007) Candidate biomarkers for discrimination between infection and disease caused by *Mycobacterium tuberculosis*. *J. Mol. Med.* 85, 613–621
43 Jacobsen, M. *et al.* (2006) Deconfounding microarray analysis – independent measurements of cell type proportions used in a regression model to resolve tissue heterogeneity bias. *Methods Inf. Med.* 45, 557–563
44 Mistry, R. *et al.* (2007) Gene-expression patterns in whole blood identify subjects at risk for recurrent tuberculosis. *J. Infect. Dis.* 195, 357–365
45 Wilkins, M.R. *et al.* (1996) Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. *Biotechnol. Genet. Eng. Rev.* 13, 19–50
46 Issaq, H.J. *et al.* (2007) Serum and plasma proteomics. *Chem. Rev.* 107, 3601–3620
47 White, M.Y. *et al.* (2006) When does a fingerprint constitute a diagnostic? *Lancet* 368, 971–973
48 Jungblut, P.R. *et al.* (1999) Comparative proteome analysis of *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG strains: towards functional genomics of microbial pathogens. *Mol. Microbiol.* 33, 1103–1117
49 Mattow, J. *et al.* (2003) Comparative proteome analysis of culture supernatant proteins from virulent *Mycobacterium tuberculosis* H37Rv and attenuated *M. bovis* BCG Copenhagen. *Electrophoresis* 24, 3405–3420
50 Pearson, H. (2008) Biologists initiate plan to map human proteome. *Nature* 452, 920–921

51 Agranoff, D. *et al.* (2006) Identification of diagnostic markers for tuberculosis by proteomic fingerprinting of serum. *Lancet* 368, 1012–1021

52 Rai, A.J. *et al.* (2005) HUPO Plasma Proteome Project specimen collection and handling: towards the standardization of parameters for plasma proteome samples. *Proteomics* 5, 3262–3277

53 Wang, R. and Marcotte, E.M. (2008) The proteomic response of *Mycobacterium smegmatis* to anti-tuberculosis drugs suggests targeted pathways. *J. Proteome Res.* 7, 855–865

54 Lawrie, L.C. *et al.* (2001) Application of laser capture microdissection and proteomics in colon cancer. *Mol. Pathol.* 54, 253–258

55 Mustafa, D. *et al.* (2008) Combining laser capture microdissection and proteomics techniques. *Methods Mol. Biol.* 428, 159–178

56 Ryals, J. (2004) Metabolomics – an important emerging science. In *Drug Discovery: Business Briefing. PharmaTech 2004* (Boulton, E., ed.), pp. 51–54, Business Briefings Ltd

57 Beecher, C. (2003) The human metabolome. In *Metabolic profiling: its role in biomarker discovery and gene function analysis* (Harrigan, G. and Goodacre, R., eds), pp. 311–319, Kluwer Academic Publishers

58 Glassbrook, N. and Ryals, J. (2001) A systematic approach to biochemical profiling. *Curr. Opin. Plant Biol.* 4, 186–190

59 Fiehn, O. *et al.* (2000) Metabolite profiling for plant functional genomics. *Nat. Biotechnol.* 18, 1157–1161

60 Sreekumar, A. *et al.* (2009) Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. *Nature* 457, 910–914

61 Syhre, M. and Chambers, S.T. (2008) The scent of *Mycobacterium tuberculosis*. *Tuberculosis (Edinb.)* 88, 317–323

62 Syhre, M. *et al.* (2009) The scent of *Mycobacterium tuberculosis* – part II breath. *Tuberculosis (Edinb.)* 89, 263–266

63 Launois, P. *et al.* (1994) T-cell-epitope mapping of the major secreted mycobacterial antigen Ag85A in tuberculosis and leprosy. *Infect. Immun.* 62, 3679–3687

64 Andersen, P. (1994) The T cell response to secreted antigens of *Mycobacterium tuberculosis*. *Immunobiology* 191, 537–547

65 Whalen, C.C. *et al.* (2006) Immune correlates of acute *Mycobacterium tuberculosis* infection in household contacts in Kampala, Uganda. *Am. J. Trop. Med. Hyg.* 75, 55–61

66 Harari, A. *et al.* (2008) An HIV-1 clade C DNA prime. NYVAC boost vaccine regimen induces reliable, polyfunctional, and long-lasting T cell responses. *J. Exp. Med.* 205, 63–77

67 Almeida, J.R. *et al.* (2007) Superior control of HIV-1 replication by $CD8^+$ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. *J. Exp. Med.* 204, 2473–2485

68 Hanekom, W.A. *et al.* (2008) Immunological outcomes of new tuberculosis vaccine trials: WHO panel recommendations. *PLoS Med.* 5, e145

69 Beveridge, N.E. *et al.* (2008) A comparison of IFNgamma detection methods used in tuberculosis vaccine trials. *Tuberculosis (Edinb.)* 88, 631–640

70 Lin, M.Y. and Ottenhoff, T.H. (2008) Host–pathogen interactions in latent *Mycobacterium tuberculosis* infection: identification of new targets for tuberculosis intervention. *Endocr. Metab. Immune Disord. Drug Targets* 8, 15–29

71 Pai, M. *et al.* (2008) Systematic review: T-cell-based assays for the diagnosis of latent tuberculosis infection: an update. *Ann. Intern. Med.* 149, 177–184

72 Martinez, V. *et al.* (2007) T-cell and serological responses to Erp, an exported *Mycobacterium tuberculosis* protein, in tuberculosis patients and healthy individuals. *BMC Infect. Dis.* 7, 83

73 Hougardy, J.M. *et al.* (2007) Heparin-binding-hemagglutinin-induced IFN-gamma release as a diagnostic tool for latent tuberculosis. *PLoS One* 2, e926

74 Demissie, A. *et al.* (2006) Recognition of stage-specific mycobacterial antigens differentiates between acute and latent infections with *Mycobacterium tuberculosis*. *Clin. Vaccine Immunol.* 13, 179–186

75 Djoba Siawaya, J.F. *et al.* (2007) Correlates for disease progression and prognosis during concurrent HIV/TB infection. *Int. J. Infect. Dis.* 11, 289–299

76 Nejentsev, S. *et al.* (2008) Analysis of association of the TIRAP (MAL) S180L variant and tuberculosis in three populations. *Nat. Genet.* 40, 261–262

77 Mueller, H. *et al.* (2008) *Mycobacterium tuberculosis*-specific CD4+, IFNgamma+, and TNFalpha+ multifunctional memory T cells coexpress GM-CSF. *Cytokine* 43, 143–148

78 Schuck, S.D. *et al.* (2009) Identification of T-cell antigens specific for latent *mycobacterium tuberculosis* infection. *PLoS One* 4, e5590

79 Meintjes, G. *et al.* (2008) Type 1 helper T cells and FoxP3-positive T cells in HIV-tuberculosis-associated immune reconstitution inflammatory syndrome. *Am. J. Respir. Crit. Care Med.* 178, 1083–1089

80 Langermans, J.A. *et al.* (2001) Divergent effect of Bacillus Calmette-Guerin (BCG) vaccination on *Mycobacterium tuberculosis* infection in highly related macaque species: implications for primate models in tuberculosis vaccine research. *Proc. Natl. Acad. Sci. U. S. A.* 98, 11497–11502

81 Mittrucker, H.W. *et al.* (2007) Poor correlation between BCG vaccination-induced T cell responses and protection against tuberculosis. *Proc. Natl. Acad. Sci. U. S. A.* 104, 12434–12439

82 Soares, A.P. *et al.* (2008) Bacillus Calmette-Guerin vaccination of human newborns induces T cells with complex cytokine and phenotypic profiles. *J. Immunol.* 180, 3569–3577

83 Day, C.L. *et al.* (2008) Detection of polyfunctional *Mycobacterium tuberculosis*-specific T cells and association with viral load in HIV-1-infected persons. *J. Infect. Dis.* 197, 990–999

84 Andersen, P. (2007) Vaccine strategies against latent tuberculosis infection. *Trends Microbiol.* 15, 7–13

85 Kaufmann, S.H. *et al.* (2006) Exploiting immunology and molecular genetics for rational vaccine design against tuberculosis. *Int. J. Tuberc. Lung Dis.* 10, 1068–1079

86 Young, D. *et al.* (2008) Systems biology of persistent infection: tuberculosis as a case study. *Nat. Rev. Microbiol.* 6, 520–528

87 Querec, T.D. *et al.* (2009) Systems biology approach predicts immunogenicity of the yellow fever vaccine in humans. *Nat. Immunol.* 10, 116–125