Joint TDR/EC expert consultation on biomarkers in tuberculosis

Report of the joint TDR/EC expert consultation to evaluate the potential roles of biomarkers in the management of HIV-infected and HIV-uninfected patients with tuberculosis

Geneva, Switzerland, 2–3 July 2008
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Authors:
Professor Alimuddin Zumla, Dr Robert Wallis, Professor Mark Doherty, Professor Nigel Klein, Dr Shreemanta Parida, Dr Ole Olesen, Dr Hannu Lång, Dr Mahnaz Vahedi and Dr Philip Onyebujoh
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**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BCG</td>
<td>bacille Calmette–Guérin</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
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<tr>
<td>CFP</td>
<td>culture filtrate protein</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CXCL</td>
<td>chemokine interleukin</td>
</tr>
<tr>
<td>CXCR</td>
<td>chemokine receptor</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EBA</td>
<td>early bactericidal activity</td>
</tr>
<tr>
<td>EC</td>
<td>European Commission</td>
</tr>
<tr>
<td>EDCTP</td>
<td>European and Developing Countries Clinical Trials Partnership</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ELISPOT</td>
<td>enzyme-linked immunosorbent spot</td>
</tr>
<tr>
<td>ESAT</td>
<td>early secretory antigenic target</td>
</tr>
<tr>
<td>ESR</td>
<td>erythrocyte sedimentation rate</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
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<tr>
<td>FP</td>
<td>Framework Programme</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HAART</td>
<td>highly active antiretroviral therapy</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
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<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
</tr>
<tr>
<td>ICOS</td>
<td>inducible costimulator</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>IGRA</td>
<td>interferon-gamma release assay</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>INH</td>
<td>isoniazid</td>
</tr>
<tr>
<td>IP</td>
<td>integrated project</td>
</tr>
<tr>
<td>IRIS</td>
<td>immune reconstitution inflammatory syndrome</td>
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<tr>
<td>LAG</td>
<td>lymphocyte activation gene</td>
</tr>
<tr>
<td>LAM</td>
<td>lipoarabinomannan</td>
</tr>
<tr>
<td>LTBI</td>
<td>latent TB infection</td>
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<tr>
<td>MDR</td>
<td>multidrug-resistant</td>
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<tr>
<td>MIG</td>
<td>monokine induced by interferon-gamma</td>
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<tr>
<td>MPFACS</td>
<td>multiparameter flow cytometry</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>Mtb</td>
<td>Mycobacterium tuberculosis</td>
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<tr>
<td>nHBHA</td>
<td>O-4-nitrobenzylhydroxylamine</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PMBC</td>
<td>peripheral blood mononuclear cell</td>
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<tr>
<td>PPD</td>
<td>purified protein derivative</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RT</td>
<td>reverse transcriptase</td>
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<tr>
<td>s</td>
<td>soluble</td>
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<tr>
<td>SELDI-ToF</td>
<td>surface-enhanced laser desorption/ionization time of flight</td>
</tr>
<tr>
<td>STREP</td>
<td>specific targeted research project</td>
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<tr>
<td>TB</td>
<td>tuberculosis</td>
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<tr>
<td>TBVAC</td>
<td>TB Vaccine Consortium</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TDR</td>
<td>Special Programme for Research and Training in Tropical Diseases</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>tr</td>
<td>trans-renal</td>
</tr>
<tr>
<td>TST</td>
<td>tuberculin skin test</td>
</tr>
<tr>
<td>uPAR</td>
<td>urokinase plasminogen activator receptor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>XDR</td>
<td>extensively drug-resistant</td>
</tr>
<tr>
<td>4FDC</td>
<td>four-month fixed-dose combinations</td>
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The impact of tuberculosis (TB) has been aggravated in recent years by the appearance of multidrug-resistant and extensively drug-resistant cases of TB, as well as the continued increase in number of people presenting with TB who are infected with the human immunodeficiency virus. The urgency of the present situation calls for accelerated clinical development of new vaccines, diagnostics and drugs, but a major obstacle is the lack of suitable and validated biomarkers to facilitate the early prediction of the rates of bacillary clearance and later risk of relapse and recurrences – regardless of the type or mechanism of action of the therapy under investigation – among various categories of TB patients.

The classical end-points in studies of anti-TB therapy are the proportion of subjects whose sputum fails to clear at the end of therapy (failures or reinfections) plus the proportion with recurrent disease during the subsequent two years post-therapy (relapses). While these end-points have been used successfully in the past, it would be highly desirable to identify new and more specific biomarkers that could reduce the size and duration of clinical trials of new drug candidates and define treatment efficacy, disease activity, cure and relapse. In vaccine development it is similarly important to identify reliable biomarkers for correlates of protection, which may predict the efficacy of an experimental vaccine candidate at an early stage of clinical development.

Biomarkers should be able to assess several areas of clinical management for both adults and children with TB:

- disease activity and extent
- treatment effect (response to treatment)
- treatment outcome (cure)
- disease relapse
- anticipated poor clinical outcomes (so that treatment can be modified appropriately)
- end-points of novel anti-TB drugs.

This report summarizes deliberations of an expert consultation on biomarkers in TB convened jointly by the Special Programme for Research and Training in Tropical Diseases (TDR) and the European Commission in Geneva, Switzerland, on 2–3 July 2008. It provides an overview of biomarkers under study and potential future ones for TB disease activity, treatment response and outcome. Advances in technological platforms are discussed: these will play a significant role in facilitating biomarker discovery in the future. The challenges and hurdles in biomarker research are reviewed and the importance of collaboration between researchers and funding agencies, in working together in a synchronized way to have maximal sustained outputs, is highlighted.
1. INTRODUCTION

Early evaluation of the response to anti-tuberculosis (TB) treatment may improve routine clinical management and assessment of novel anti-TB drug candidates during clinical trials. New drugs are urgently needed as existing ones are several decades old, current treatment regimens have a duration of at least six months and an alarming increase in the incidence of infections with multidrug-resistant (MDR) and extensively drug-resistant (XDR) organisms has been reported (Gandhi et al., 2006). The human immunodeficiency virus (HIV) pandemic has led to a resurgence of TB in developing countries and approximately 8.8 million people develop active TB each year, resulting in 1.6 million deaths (WHO, 2008).

Currently the ultimate success of chemotherapy is measured by the rate of relapse within the first two years after completion of treatment. The long duration of clinical trials that rely on this outcome renders clinical TB research, despite its importance, unattractive to the pharmaceutical industry. This has to be seen in the context of only 1 in every 12 drugs that enter clinical trials eventually reaching the markets, in addition to the questionable profitability of the anti-TB drug market, which will mainly be focused on resource-constrained settings.

For clinical management of TB, the length of anti-TB therapy (six months of directly observed therapy) has negative implications for patient adherence, which in turn puts pressure on health-care systems in developing countries. There is evidence that individuals responding early to treatment might only require a shortened course of antibiotic therapy (Balasubramanian et al., 1990; Hong Kong Chest Service/British Medical Research Council, 1991). The ability to classify TB patients at diagnosis or early on during chemotherapy into risk groups requiring different durations of treatment might improve

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**Box 1. Biomarkers studies in clinical stages of M. tuberculosis (Mtb) in the human host: infection, active disease, latency, reactivation and relapse**

- Markers and correlates of protection from:
  - Mtb infection
  - developing latent Mtb infection
  - developing early TB disease
  - developing late TB disease (reactivation)

- Developing severe TB disease
- TB disease activity
- TB cure
- TB relapse

*Source: Rieder HL et al. (1989)*

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*Courtesy of T. Ottenhoff*
adherence and thus treatment outcome. Health-care providers in resource-limited settings may then be able to focus more attention on patients who have a high risk of poor treatment outcomes. Therefore the need arises to find surrogate biomarkers (biological markers) that provide an indication of treatment efficacy early on during chemotherapy or markers that stratify patients into risk groups even prior to the start of therapy. Such biomarkers would not only improve therapeutic strategies and possibly reduce drug resistance due to non-adherence, but would also be crucial in validation of novel anti-TB drug candidates, thereby accelerating new drug development through shortening of clinical trials.

Clinical trials have historically used clinically important parameters as end-points (Box 1). These variables reflect how a patient feels, functions or survives. In anti-TB treatment trials, end-points historically have been the sum of failures plus relapses. Because relapses occur in only a small proportion of adequately treated patients, and because they can occur up to two years after completion of therapy, such trials have required large sample sizes and long total durations to ensure adequate enrolment and statistical power. This problem is compounded in the case of TB vaccine trials, where the rate of incident cases, even in highly TB-endemic populations, is typically less than 1% and development of disease after exposure can take years to become obvious. The urgency of the global TB and HIV pandemics, including the emergence of MDR- and XDR-TB, requires the acceleration of current research. Biomarkers may contribute significantly to this effort.

2. IDEAL CHARACTERISTICS OF MARKERS AND SURROGATE END-POINTS

Biomarkers are measurable characteristics that indicate normal biological or pathogenic processes, or pharmacological responses to a therapeutic intervention (Biomarkers working group, 2001) (Box 2). In clinical trials they may form the basis of a surrogate end-point that can substitute for a clinical end-point, based on epidemiological, therapeutic, pathophysiological or other scientific evidence. To be valuable as a surrogate end-point, a biomarker should measure an event that is directly involved in pathogenesis or protection and that changes early during treatment. Experience with measurement of plasma HIV RNA indicates the potential of surrogate end-points to accelerate research (Holodniy, 2006). However, other research has indicated the ease with which apparently appropriate biomarkers may be dissociated from clinically meaningful events. For example, although ventricular premature contractions occur frequently in people at risk of sudden death due to tachyarrhythmia, suppression of these contractions with flecainide, encainide, or moracizine was unexpectedly found to increase mortality after myocardial infarction (CAST II investigators, 1992; Echt et al., 1991). This experience indicates that early markers of disease activity may not necessarily be satisfactory predictors of ultimate therapeutic success. The validation of surrogate end-points is therefore critically important for the field to advance.

Biomarkers and surrogate end-points may be classified in several ways (Box 3). Static assays measure levels of an analyte in a clinical sample, whereas dynamic or functional assays measure a process, such as a response to a stimulus, either in vivo or in vitro. Some markers are disease specific: such markers will not be confounded by concomitant illnesses or therapies, and may also serve as diagnostic tests for study entry. End-points may be single measurements, or may be highly multiplexed biosignatures, such as those from gene expression microarrays. Finally, analytes may be of either host or pathogen origin.
BOX 2. Definitions

- **Biomarker (biological marker)**
  A measurable characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathological processes, or physiological or pharmacological responses to a therapeutic intervention.

- **Biosignature**
  A group of biomarkers used together that form highly multiplexed biosignatures.

- **Surrogate end-point**
  - A biomarker that is intended to substitute for a clinical end-point based on epidemiological, therapeutic, pathophysiological or other scientific evidence.
  - Predicts clinical outcome in terms of benefit, or harm or lack of benefit.

- **Clinical end-point**
  A characteristic or variable that reflects the final outcome of disease in terms of function, effect, progress, recovery, survival or death.

- **Surrogates of protection**
  Validated markers of correlates of protection.

- **Correlates of protection**
  Measurable sign(s) in a host in response to an infectious agent indicating whether the individual is being protected against becoming infected and/or developing disease.

*Courtesy of R. Wallis and S. Parida*

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BOX 3. Biomarker classification

- **Static vs dynamic (functional)**
- **Disease specific vs nonspecific**
- **Single vs multiplex**
- **Pathogen vs host analytes**
- **Patient management vs clinical drug or vaccine development**
- **Early-stage vs later-stage clinical trials**

*Courtesy of R. Wallis*
3. THE TB AND HIV PANDEMICS: COINFECTION ISSUES

The global burdens of HIV and TB are currently estimated at 33 million and 14.4 million cases, respectively (WHO, 2008). The two pandemics are closely intertwined, with HIV being the most common predisposing factor for TB, and TB the most common presenting illness for acquired immunodeficiency syndrome (AIDS). Anti-TB therapy presently must be continued long past the time required for resolution of symptoms. Ensuring adherence throughout therapy places a large burden on TB control programmes that hinders TB elimination.

Current knowledge for combined treatment of HIV and TB is inadequate. Immune reconstitution, a general goal of antiretroviral therapy, can have deleterious immediate consequences in HIV-infected TB patients (designated immune reconstitution inflammatory syndrome or IRIS). Physicians presently lack tools to accurately assess and manage risks and optimize long-term outcomes in dually infected people. Reports in some regions have associated HIV coinfection with anti-TB drug resistance (both MDR and XDR) (Gandhi et al., 2006). Although some of this risk represents enhanced susceptibility to transmitted resistant TB strains, HIV/AIDS appears to particularly predispose to the emergence of acquired anti-TB drug resistance during intermittent therapy. The interplay of the biological and pharmacological factors responsible for resistance in this circumstance is inadequately understood (Wallis, Weyer & Fourie, 2008). The potential role for new vaccines and immunotherapeutics in the prevention and management of drug-resistant and HIV-associated TB remains largely untapped. The time required for traditional efficacy end-points is a major challenge to development of new drugs, vaccines and adjunctive immunotherapies.

4. CLINICAL NEEDS FOR MARKERS

Different types of markers can be distinguished in TB biomarker discovery, including markers of extent of disease, treatment effect and treatment outcome (Box 4).

4.1. Biomarkers of extent of disease

A biomarker for extent of disease would be measured at diagnosis before initiation of treatment and would allow patients to be stratified into different groups according to disease severity. If patients are stratified into different groups, drug regimens can be adjusted accordingly, enhancing adherence and treatment outcome by ensuring that only patients with increased risk for slow response or relapse are placed on lengthy treatment regimens. Furthermore, homogenous patient groups are desirable for clinical trials, to reduce the required sample size.
Box 4. Biomarkers needs in the context of TB

There is a need for biomarkers:
- of bacterial clearance (clinical end-point) – needed for assessing potential drug candidates
- of immune protection – needed for assessing potential vaccine candidates
- of infection
- of disease activity
- of cure
- of treatment outcome
- for detecting prognostically risk of relapse

4.2. Biomarkers of treatment effect

Markers for treatment effect will correlate with bactericidal and sterilizing activities of drugs and should change during therapy to reach control levels as the disease is brought under control. Such markers would be extremely useful in clinical trials and could allow shortening of trial duration.

4.3. Biomarkers of treatment outcome

Markers of eventual treatment outcome and especially of relapse after initial cure would be of particular importance, as high relapse rates necessitate the long duration of therapy that is currently employed worldwide.

The clinical needs for host and pathogen biomarkers in HIV-infected and HIV-uninfected adults and children with TB fall into several areas. From a clinical viewpoint, biomarkers needed are those that will be important in monitoring TB disease activity, therapeutic success (cure), and relapse or reinfection. From a research perspective, tools are needed to accelerate development of new drugs, immunotherapies and vaccines. Such tools might be used to identify or predict a rapid, non-relapsing cure in patients with active TB, assess the risk of reactivation or the effectiveness of latent TB infection (LTBI) treatment or identify protective responses to vaccination. Some biomarkers may serve dual roles (i.e. predicting success for both drug therapies and preventive vaccines) or may be useful to show the interactions of combined therapies.

Validated biomarkers may ultimately also be used in management of individual patients, for example, to identify those TB patients at low risk of relapse, for whom an ultrashort regimen might be appropriate. Substantially greater predictive accuracy will be required for biomarkers used in this fashion. It is a particular challenge facing developers of TB biomarkers that Mycobacterium tuberculosis (Mtbo) infection results in multiple bacillary subpopulations with distinct anatomical localizations and metabolic and biosynthetic profiles. Latent infection is thought to be due to a distinct non-replicating population contained in granulomas. Multiple populations can coexist within individual patients. Replicating bacilli are thought to outnumber non-replicating bacilli by several orders of magnitude in patients with cavitary TB. However, the small non-replicating population is thought to give rise to relapses. The ability of biomarkers to distinguish differential drug effects on these two populations will be critical for their success in predicting long-term clinical outcomes.
Clinical markers, such as extent of cavitation on chest radiography, cannot distinguish between active and inactive TB and even though associated with bacterial load are not suitable as a marker for treatment response, since improvement on chest radiographs generally lags behind overall treatment response (Perrin et al., 2007). Additionally chest X-ray facilities and the skills to perform radiological grading of the extent of disease are frequently not available in resource-limited settings. Nevertheless, severity of cavitary lung disease and the extent of lung tissue involved have been shown to be associated with and predictive of relapse in several independent studies (Benator et al., 2002; Mallory et al., 2000; Nettles et al., 2004; Sonnenberg et al., 2001).

4.4. Approaches to studying biomarkers

There are several approaches to studying markers:

- targeting specific known pathogen/microbiological biomarkers
- targeting specific known host clinical and immune markers
- exploiting advanced platforms (e.g. transcriptomics, proteomics, metabolomics, glycomics and lipidomics) to study common markers or signatures of response.

A range of host biomarkers have been studied (Table 1 and Box 5) using a variety of clinical specimens:

- sputum and saliva
- urine
- whole blood, serum/plasma
- cerebrospinal fluid, pleural and pericardial effusions
- breath.

Taken together, most currently used microbiological and clinical markers for anti-TB treatment response have not been validated nor do they have sufficient predictive value on an individual level. These markers and their limitations have recently been reviewed extensively (Perrin et al., 2007; Wallis, 2007; Walzl et al., 2008). Therefore the focus of this review is on a range of possible alternative host biomarkers (immune parameters) found in readily available body fluids, mainly serum, and it assesses the strength of the evidence of the individual studies that have investigated these putative markers. Additionally, emerging novel technologies involving genomic, proteomic and metabolomic approaches assisting in “fishing” are discussed, as well as targeted searches for biomarkers. Finally, insight is provided into the challenges and difficulties TB biomarker discovery is faced with.
<table>
<thead>
<tr>
<th>Candidate biomarker</th>
<th>Association</th>
<th>Study size and positive treatment outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chest radiography</strong></td>
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<tr>
<td>Baseline chest X-ray</td>
<td>Recurrence</td>
<td>46/938</td>
<td>Mallory et al., 2000</td>
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<tr>
<td>Baseline chest X-ray</td>
<td>Relapse</td>
<td>74/830</td>
<td>Benator et al., 2002</td>
</tr>
<tr>
<td>Baseline chest X-ray</td>
<td>Relapse</td>
<td>4/227</td>
<td>Nettes et al., 2004</td>
</tr>
<tr>
<td>Baseline chest X-ray</td>
<td>Recurrence</td>
<td>24/175</td>
<td>Sonnenberg et al., 2001</td>
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<tr>
<td><strong>Serial sputum microbiology</strong></td>
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<td></td>
<td></td>
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<tr>
<td>M2 SCC</td>
<td>Recurrence</td>
<td>Many patients</td>
<td>Mitchison, 1993</td>
</tr>
<tr>
<td>Serial sputum CFU counts</td>
<td>Superior sterilizing activity</td>
<td>Few patients</td>
<td>Davies et al., 2006; Rustome et al., 2008a</td>
</tr>
<tr>
<td>Serial MGIT™ or BACTEC™ time to positivity</td>
<td>Anti-TB treatment response, failure and relapse</td>
<td>Many patients</td>
<td>Epstein et al., 1998; Wallis et al., 1998</td>
</tr>
<tr>
<td><strong>Early bactericidal activity</strong></td>
<td>None</td>
<td>Many patients</td>
<td></td>
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<td><strong>TB-specific biomarkers</strong></td>
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<td>Sputum antigen 85B RNA</td>
<td>Anti-TB treatment response</td>
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<td>Desjardin et al., 1999</td>
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<td>Treatment response</td>
<td>2/40</td>
<td>Wallis et al., 1998; Wallis et al., 2000; Wallis et al., 2001b</td>
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<td>Sputum antigen 85</td>
<td>Drug evaluation</td>
<td>40</td>
<td>Wallis et al., 2001b</td>
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<td>Urine Mtb DNA</td>
<td>Anti-TB treatment response</td>
<td>20</td>
<td>Cannas et al., 2008</td>
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<tr>
<td>Urine lipoprotein</td>
<td>Infection, active disease</td>
<td>Many patients</td>
<td>Wallis et al., 2001b</td>
</tr>
<tr>
<td>Anti-ESAT-6, 36 kDa protein, alanine dehydrogenase, malate synthetase</td>
<td>Extent of disease, treatment response</td>
<td>168</td>
<td>Azurri et al., 2006</td>
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<td>IGRA</td>
<td>Anti-TB treatment response</td>
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<td>Carrara et al., 2004</td>
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<td><strong>Breath biomarkers</strong></td>
<td>Culture plates data</td>
<td>19/23</td>
<td>Syhre &amp; Chambers, 2008; Phillips et al., 2007</td>
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<td><strong>Nonspecific biomarkers of immune activation</strong></td>
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<td></td>
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<td>NKT cells at diagnosis</td>
<td>M2 SCC</td>
<td>8/21</td>
<td>Veemstra et al., 2006</td>
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<tr>
<td>Sputum IFN-γ</td>
<td>Anti-TB treatment response</td>
<td>15</td>
<td>Ribeiro-Rodrigues et al., 2002</td>
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<td>sIL-2R</td>
<td>Anti-TB treatment response</td>
<td>44</td>
<td>Chan et al., 1999</td>
</tr>
<tr>
<td>sTNF-R, granzyme B at diagnosis</td>
<td>M2 SCC</td>
<td>18/36</td>
<td>Brahmi et al., 2006</td>
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<td>Neopterin</td>
<td>Anti-TB treatment response, relapse</td>
<td>11/39</td>
<td>Immanuel et al., 2001</td>
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<td>C-reactive protein</td>
<td>Anti-TB treatment response, death</td>
<td>100</td>
<td>Bajaj, Rattan &amp; Ahmad, 1999</td>
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<td>sICAM-1</td>
<td>Treatment response</td>
<td>30</td>
<td>Demir et al., 2002</td>
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<tr>
<td>suPAR</td>
<td>Death</td>
<td>101</td>
<td>Eugen-Olsen et al., 2002</td>
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<td><strong>Functional studies of TB protection</strong></td>
<td>Vaccine effect</td>
<td>Many patients</td>
<td>7/14</td>
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<td>ELISPOT and QuantiFERON®</td>
<td>Immune eradication of Mtb infection</td>
<td>Many patients</td>
<td>5/8</td>
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<td>QFN</td>
<td>Prediction of disease in untreated contacts (using upper bound cut-off)</td>
<td>6/6</td>
<td>Diel et al., 2008</td>
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<td>Whole blood killing</td>
<td>TST effect</td>
<td>12</td>
<td>Cheon et al., 2002; Kampmann et al., 2000</td>
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<td>Whole blood killing</td>
<td>BCG effect</td>
<td>10</td>
<td>Cheon et al., 2002; Hoff et al., 2002; Kampmann et al., 2000</td>
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<td>Whole blood killing</td>
<td>AIDS effect</td>
<td>22</td>
<td>Tena et al., 2003</td>
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<td>Whole blood killing</td>
<td>Combined antiretroviral therapy effect</td>
<td>15</td>
<td>Kampmann et al., 2006</td>
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<td>Whole blood killing</td>
<td>TNF monoclonal antibody effect</td>
<td>20</td>
<td>Salu et al., 2006</td>
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<tr>
<td>Whole blood killing</td>
<td>Vitamin D effect</td>
<td>192</td>
<td>Martineau et al., 2007</td>
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<tr>
<td><strong>Functional studies of anti-TB treatment</strong></td>
<td>LTBI treatment response</td>
<td>38</td>
<td>Ewer et al., 2006</td>
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<td>IGRA</td>
<td>Anti-TB treatment response</td>
<td>5/18</td>
<td>Carrara et al., 2004</td>
</tr>
<tr>
<td>Whole blood killing</td>
<td>Anti-TB treatment response</td>
<td>Many patients</td>
<td>Wallis et al., 2001a</td>
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<td>Whole blood killing</td>
<td>Correlation between serial CFU slope and M2 SCC</td>
<td>36</td>
<td>Wallis et al., 2003</td>
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<td>Anti-TB treatment response</td>
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<td>Janulionis et al., 2004</td>
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<td><strong>Highly multiplexed assays</strong></td>
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<td>Transcriptomics</td>
<td>TB disease and infection</td>
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<td>Mistry et al., 2007</td>
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<td>Proteomics</td>
<td>TB disease</td>
<td>–60</td>
<td>Agronoff et al., 2006</td>
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<tr>
<td>Metabolomics</td>
<td>TB disease</td>
<td>NA</td>
<td>S. Parida, in preparation</td>
</tr>
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</table>

BCG, bacille Calmette–Guerin; CFU, colony forming unit; ESAT, early secretory antigenic target; IFN, interferon; IGRA, interferon-gamma release assay; IL, interleukin; LTBI, latent TB infection; MGIT™, Mycobacterium Growth Indicator Tube; M2 SCC, month-two sputum culture conversion; Mtb, Mycobacterium tuberculosis; NA, not available; NKT, natural killer T cells; sICAM-1, soluble intercellular adhesion molecule type 1; suPAR, soluble urokinase plasminogen activator receptor; TNF, tumour necrosis factor; TST, tuberculin skin test.
BOX 5. *M. tuberculosis* – pathogen and host biomarkers

A. Pathogen biomarkers

1. **Mycobacterial load**
   a. Mycobacterial count in sputum
   b. Early bactericidal activity decrease in colony forming units (CFU) in sputum during early phase of treatment
   c. Sputum culture: conversion after two months’ therapy (time to detection)
   d. Sputum culture: conversion after two years’ therapy

2. **Mycobacterial cell wall products**
   a. Lipoarabinamannan
   b. Phenolic glycolipid-1
   c. Dectin

3. **Mycobacterial antigens**
   a. Antigen 85
   b. Antigen 85B
   c. Antigen 85B RNA

4. **Mycobacterial nucleic acids**
   a. Mycobacterial DNA and its fragments (cannot distinguish live from dead bacilli)
   b. Mycobacterial mRNA and its fragments

5. **Mycobacterial volatile metabolites in breath**

B. Host biomarkers

1. **Clinical markers**
   a. Symptoms (nausea, anorexia, weight loss, cough, chest pain, sputum, haemoptysis, wasting, night sweats)
   b. Skin tests
   c. Chest X-ray (extent of disease, severity of disease, cavitary disease)

2. **Inflammatory markers**
   a. C-reactive protein
   b. Erythrocyte sedimentation rate

3. **Biochemical markers and liver function tests**
   a. Adenosine deaminase
   b. Liver enzymes (serum glutamic pyruvic transaminase, serum glutamic oxaloacetic transaminase, alkaline phosphatase)
   c. Volatile metabolites (breath markers)

4. **Immune markers**
   a. B-cell markers – antibody/*M. tuberculosis* antigen studies
   b. T-cell markers
      i. associated with Th1 responses: LAG-3V3, splice variant of LAG
      ii. associated with Th2 responses: sCD30, total IgE, CCL22
   c. Immune cytokines
      i. Type 1 (Th1) cytokines (IL-2, IL-7, IL-12, IFN-γ)
      ii. Type 2 (Th2) cytokines (IL-4, IL-4/IL-4 ratios, TNF-α, IL-10)
      iii. Inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-10)

5. **T-cell stimulation responses to mycobacterial antigens**

6. **Interferon-γ production studies (ELISPOT, QuantiFERON®)**
   a. Macrophage markers and chemokines
      IL-8, MIP-1α, RANTES, IFN-gene STAT7, ribosomal proteins CXCR4, CCR5, neopterin
   b. Apoptosis mediators
      Fas, Fas ligand, TNF-α, TNF-R1, TNF-R2, TRAF2, caspase 8, FLIP, bcl-2 and bax

7. **Serological markers**
   a. Host proteins
      i. Markers that correlate with Th1 responses (LAG3V3)
      ii. Markers that correlate with Th2 responses (sCD30, total IgE, CCL22)
   b. Antigen/antibody tests – ELISA for IgG, IgM, IgA to *M. tuberculosis* antigens

CXCR, chemokine receptor; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; Ig, immunoglobulin; IL, interleukin; Th, T helper; TNF, tumour necrosis factor.
5. CANDIDATE MICROBIOLOGICAL MARKERS

The global emergency of TB can only be controlled if new tools such as diagnostics, vaccines and drugs become available. This brings with it a challenge of discovering and implementing a biomarker or biosignature that will allow the new drugs to be tested in clinical trials expeditiously. A summary of research to date on TB biomarkers is given in Table 1 and Box 5.

The only unequivocally accepted end-points for TB clinical trials are bacteriological cure at the completion of therapy and the absence of relapse during the following 6–24 months (Fox, Ellard & Mitchison, 1999). Anti-TB drug therapy is characterized by multiple phases (Mitchison, 1985). At its onset, the total bacillary burden is large, consisting mainly of metabolically active extracellular bacilli that are replicating in lung cavities. The first phase of treatment must reduce this population by several orders of magnitude, while preventing the emergence of resistance. Bactericidal drugs such as isoniazid (INH) – capable of killing replicating bacteria – have an important role in this phase. Ethambutol and other bacteriostatic drugs have an important but secondary role in this phase, preventing the emergence of resistance. Bactericidal activity can be measured in short-term trials by examining the rate of decline of sputum colony forming unit (CFU) counts during the first two days of therapy (termed early bactericidal activity or EBA) or during the first five days (termed extended EBA) (Gillespie & Charalambous, 2003; Gosling, Heifets & Gillespie, 2003).

The second phase of treatment proceeds at a greatly reduced rate compared with the first. This appears to reflect a semi-dormant bacillary population with reduced susceptibility to killing. Such bacteria may arise spontaneously and be selected by chemotherapy, or they may represent an adaptation to specific microenvironments in different anatomical compartments (Mitchison, 1985). In vitro, such adaptation can be demonstrated by oxygen and/or nutrient deprivation, in broth culture or in artificial granulomas (Karakousis et al., 2004; Wayne, 1994). Some drugs, such as INH, lack activity against hypoxia-adapted Mtb; others, such as metronidazole, are active despite inactivity against aerobically grown bacilli (Wayne & Sramek, 1994b). The eradication of this bacillary population in clinical trials has been termed sterilization. It is traditionally measured by the relapse rate. The long duration of trials to study relapse as an end-point has focused biomarker discovery on a search for early indicators of sterilization.

5.1. The basis and plausibility of bacteriological biomarkers

Work by the Medical Research Council Tuberculosis and Chest Diseases Unit, United Kingdom, has indicated an association between delayed sputum conversion and cavitation. It has been shown that cavitation is associated with a higher bacterial load. The extent of cavitation as measured by computed tomography scanning is correlated with the bacterial load. This provides, for the first time, an explanation for the relationship between cavitation and the longer time taken to render sputum culture negative that is observed in clinical practice and reported in clinical trials.

It also provides an explanation for the validity of the currently accepted surrogate of two-month culture negativity as the dynamics of anti-TB treatment indicate that this measure is directly related to the initial bacterial load and the bactericidal efficacy of the treatment regimen (Davies et al., 2006; Gillespie & Charalambous, 2003). Thus, although bacterial load measurements are both the most logical and currently the only accepted surrogate measure of treatment response, there are significant challenges to their implementation in clinical practice. Several studies have shown significant variation in measures of bacterial load both within and between subjects and between experimental sites (Sirgel et al., 2000). It is believed that measurements of bacterial load coupled with mathematical modelling of data...
over the first two months of therapy can provide a good indicator of subsequent failure and relapse rates. Other indicators of viability, such as quantitative messenger RNA (mRNA) detection, may add to these assessments.

5.2. Mycobacterial load

Currently the marker with which there is greatest experience as a predictor of non-relapsing cure is sputum culture status after two months of anti-TB therapy (Mitchison, 1993; Wallis & Johnson, 2006) (Table 2). These data may be examined at three levels. Across studies, a significant relationship exists between two-month culture conversion rate and relapse rate \((R = -0.753, \ P<0.01)\). However, this depends heavily on a single study arm (6SH, i.e. six-months’ treatment with streptomycin and isoniazid) with an atypically high relapse rate (29%), without which no statistical relationship is identified. In practice, however, two-month culture conversion and relapse are compared within individual clinical trials. Figure 1 therefore shows the incremental effect of an additional drug on two-month culture conversion and relapse rates in this context. Symbols indicate distinct trials. All data points but one lie in quadrants 2 or 4, indicating an inverse relationship \((R = -0.764, \ P = 0.038\) by Spearman rank correlation). Lastly, limited data indicate this marker may also be of value in individual patients. In Study 22 of the Tuberculosis Trials Consortium Study, for example, two-month culture positivity was an independent predictor of relapse, with a hazard ratio of 2.8 (Benator et al. 2002.). However, the marker was relatively insensitive (identifying only half of all relapses) and lacked adequate positive predictive value (18%) for use as a guide to treatment of individual patients.

Figure 1. Correlation of change in two-month sputum conversion rate with change in relapse rate

It is important to note that the number of drugs used in treatment does not correlate with either rate. In the last study cited in Table 2, for example, the addition of ethambutol improved neither two-month conversion nor relapse rate. This observation supports the hypothesis that specific drugs, rather than their total number, are the main determinant of sterilization.
### TABLE 2. Relationship between month-two sputum culture conversion and relapse

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Month-two sputum culture conversion</th>
<th>Relapse</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No of subjects</td>
<td>Rate (%)</td>
<td>No of subjects</td>
</tr>
<tr>
<td><strong>Rifampicin added</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>6SH</td>
<td>154</td>
<td>49</td>
<td>112</td>
</tr>
<tr>
<td>6SHR</td>
<td>148</td>
<td>69</td>
<td>112</td>
</tr>
<tr>
<td>2SHZ/5S%H₂Z₂</td>
<td>129</td>
<td>72</td>
<td>129</td>
</tr>
<tr>
<td>2SHRZ/3 or 5S%H₂Z₂</td>
<td>261</td>
<td>92</td>
<td>269</td>
</tr>
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<td><strong>Pyrazinamide added</strong></td>
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<td></td>
</tr>
<tr>
<td>6SH</td>
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<td>49</td>
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</tr>
<tr>
<td>6SHZ</td>
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<tr>
<td>6SHR</td>
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<td>2SHRZ/4TH or 4S%H₂Z₂</td>
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<td>75</td>
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<td>2SHRZ/4 or 6TH</td>
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<td>6SHR</td>
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<td>88</td>
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<td>2SHRZ/4 or 6S%H₂Z₂</td>
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<td><strong>Ethambutol added</strong></td>
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<tr>
<td>6SHR</td>
<td>148</td>
<td>88</td>
<td>143</td>
</tr>
<tr>
<td>2SHRE/4 or 6S%H₂E₂</td>
<td>171</td>
<td>81</td>
<td>168</td>
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<tr>
<td><strong>Standard short course therapy</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>2HREZ/4HR</td>
<td>415</td>
<td>18.8</td>
<td>415</td>
</tr>
</tbody>
</table>

E, ethambutol; H, isoniazid; R, rifampicin; S, streptomycin; T, thiacetazone; Z, pyrazinamide.


* Numerals indicate months of treatment; subscript numerals indicate number of weekly doses if treatment is not administered daily.
Limited data indicate that two-month culture status may also be of value in individual patients. In Tuberculosis Trials Consortium Study 22, for example, two-month culture positivity was an independent predictor of relapse, with a hazard ratio of 2.8 (Benator et al., 2002). However, it was relatively insensitive (identifying only half of all relapses) and lacked adequate positive predictive value (18%) for use as a guide to treatment of individual patients.

Other factors may also limit the utility of this parameter. In recent fluoroquinolone studies, regional differences have emerged with regard to the proportion of subjects positive and the magnitude of the drug effect. Further research is required to determine whether these reflect differences in clinical populations, mycobacterial strains or specific laboratory methods (for example, the use of commercial Lowenstein-Jensen slants versus locally prepared medium). Additional studies using time to positivity in automated liquid culture systems may help clarify the basis of these divergent observations.

5.2.1. Quantitative sputum microbiological studies
Two quantitative approaches have been suggested to improve the prognostic and statistical power of sputum microbiology. In the first, the frequency of sampling is increased from once to twice monthly or weekly, with time to sputum culture conversion by Kaplan-Meier analysis as the outcome measure. In the second, sputum CFU counts are measured at weekly intervals during the first month of therapy beginning on day 2, with the rate of decline through to day 28 as the outcome measure (Davies et al., 2006; Rustomjee et al., 2008a). Omission of the first two days of treatment removes effects on replicating, rapidly killed bacteria (EBA) that are unrelated to treatment outcome. Experience with this approach is limited but promising, as is experience using time to positivity in automated liquid culture systems (Epstein et al., 1998; Wallis et al., 1998).

5.3. Mycobacterial antigens: antigen 85 and antigen 85B
Several studies have examined other microbial markers in sputum. Two studies have examined levels of Mtb antigen 85 by enzyme-linked immunosorbent assay (ELISA). In the first, the magnitude and duration of increases in this protein during the first week of therapy predicted subsequent relapse in 4 of 42 subjects (Wallis et al., 1998). Induction of antigen 85 occurs due to INH: it does not occur in INH-resistant strains, requires new protein synthesis, and is not due to release of existing protein by dying cells (Garbe, Hibler & Deretic, 1996). In a second trial, induction of antigen 85 by INH in sputum was prevented by concomitant administration of rifampicin and by the higher of two doses of rifalazil (Wallis et al., 2001b). However, induction would not be anticipated in patients not treated with INH or with INH-resistant infections, potentially limiting the application of this marker.

One study has examined antigen 85B RNA (Desjardin et al., 1999), finding that it was cleared more rapidly from sputum compared with viable colony counts. One patient in that study who subsequently relapsed could not be distinguished from others based on his early RNA response. Additional research is needed to determine whether other RNA species, such as those associated with dormancy, might have greater predictive value.

5.4. Mycobacterial markers in urine
The collecting and processing of sputum has historically been a limiting factor for TB diagnosis and monitoring. Some patients, such as children, are unable to produce adequate specimens. In others, there is substantial inhomogeneity within and among repeated specimens. Urine has been viewed as a potential alternative source of diagnostic material that might be more readily collected, and that might more uniformly reflect total body bacillary burden. Several studies have examined mycobacterial DNA fragments, lipoarabinomannan (LAM) and other protein antigens in urine.
5.4.1. Trans-renal mycobacterial DNA

Several studies have described the presence in urine of small mycobacterial DNA fragments due to apoptosis of mammalian cells (Botezatu et al., 2000; Su et al., 2004b; Su et al., 2004a). These fragments, which appear in the soluble fraction and appear to be of renal origin, have been termed trans-renal (tr)DNA to distinguish them from full-length DNA species arising from cells shed into the lower urinary tract (Umansky & Tomei, 2006). One study has reported the presence of small fragments of \textit{Mtb} IS6110 DNA in urine of 79\% (34/43) of patients with pulmonary TB but not in urine of healthy controls (Cannas et al., 2008). None of 20 patients positive at diagnosis remained positive after two months of standard therapy. Responses have not yet been evaluated at earlier time points or in relation to sputum culture conversion. The method presently requires nested polymerase chain reaction (PCR) amplification; assay sensitivity may not be sufficient for \textit{Mtb} strains with low IS6110 copy numbers (Lok et al., 2002). However, the approach may be particularly useful in situations where sputum cannot be readily obtained, such as in children, and could potentially be adapted to detect drug-resistance mutations, thus serving multiple roles in diagnosis and monitoring. Collaborative European Commission (EC) 7th Framework Programme-funded studies on (tr)DNA are ongoing between Germany (Ludwig-Maximilians-Universität), the United Kingdom (University College London), United Republic of Tanzania (Mbeya Medical Research Programme) and Zambia (University of Zambia) (see Annex 4, section A4.3).

5.4.2. Urine mycobacterial LAM

Detection in urine of mycobacterial LAM and other antigens has been reported in some TB patients and in animals with experimental \textit{Mtb} infection (Boehme et al., 2005; Choudhry & Saxena, 2002; Kashino et al., 2008; Napolitano et al., 2008; Singh et al., 2003; Tessema et al., 2002). No studies have yet examined the clearance of these antigens during treatment or established a relationship to clinical outcome or to another surrogate end-point.

5.5. Volatile mycobacterial markers in breath

It has been suggested that exhaled breath contents (volatile chemicals, hydrogen peroxide, etc.) could be used to predict the prognosis of non-responsive asthma in a non-invasive manner. Volatile metabolites from \textit{Mtb} organisms may be exploited to diagnose TB rapidly and could be used as biomarkers of cure and relapse (Phillips et al., 2007). The advent of solid phase microextraction and gas chromatography/mass spectrometry now makes it possible to investigate whether these metabolites in patient breath indicate an infection with the organisms.

Four specific compounds have been identified by Syhre & Chambers (2008): methyl phenylacetate, methyl p-anisate, methyl nicotinate and o-phenylalanisole. In culture these are detectable before visual appearance of colonies. This may lead to a useful non-invasive diagnostic test for TB and may serve as biomarkers. Phillips et al. (2007) studied volatile organic compounds above cultures of \textit{Mtb} and in breath of confirmed 23 TB patients, 19 suspects in whom TB had been ruled out, and 59 healthy controls. Among the most commonly detected volatile organic compounds were derivatives of heptane, naphthalene, hexane and benzene. Profiles were identified by fuzzy logic or principal components analysis that differentiated TB cases from other ill patients with high sensitivity and moderate specificity. This is an area of much interest and may lead to a practical, non-invasive way of diagnosing TB at site of care. However, there is no evidence yet that these markers can predict relapse, i.e. distinguish tissue sterilization from mere inhibition of bacterial growth and metabolism.
6. CANDIDATE HOST MARKERS

When looking at host markers, the potential field of approaches is much broader, but the practical utility of the various biomarkers so far analysed is much more poorly defined, and validation of the most promising approaches (Table 1) is urgently needed. The approaches most vigorously developed at present fall into two categories:

- assessment of host biomarkers that can be used to monitor response to treatment, similarly to the assessment of bacterial products described in section 5;
- biomarkers that can potentially be used to identify the risk of developing disease in Mtb-exposed individuals.

Some tests can potentially be used in both settings.

6.1. Interferon-gamma release assays (IGRAs)

TB is also an immunological disease, both in its pathogenesis and the importance of the immune response in bringing about a long-term cure. The key component of the adaptive immune response against Mtb is the cellular immune system, particularly human leukocyte antigen (HLA) class II- and class I-restricted antigen-specific Th 1-type IFN-γ-secreting CD4+ T cells and CD8+ T cells. As in infections with other intracellular pathogens, a key determinant of the frequency of pathogen-specific effector T cells measured ex vivo is the antigen load in vivo (Pathan et al., 2001). Two industrial companies have developed cellular immune response assays that detect IFN-γ production when peripheral blood mononuclear cells (PBMCs) are incubated with Mtb antigens (QuantiFERON®-TB Gold from Cellestis and T-SPOT®.TB from Oxford Immunotec), which can differentiate between Mtb-uninfected and Mtb-infected individuals. The best characterized of these tests are the IGRAs that use a combination of two (T.SPOT) or three (T.SPOT®+, Quantiferon®-TB Gold, in-tube) antigens that are present in members of the TB complex but not the bacille Calmette–Guérin (BCG) vaccine strain (designated RD antigens). Multiple studies have shown that these responses are more specific for Mtb infection than the current tuberculin skin test (TST) and correlate better with exposure to an index case (where exposure can be quantified) (Arend et al., 2007; Lalvani et al., 2001). These tests have the advantage that conversion to positivity appears to occur relatively rapidly, allowing early identification of infected cases.

There is also evidence that these responses disappear rapidly on treatment, suggesting that they may be able to serve for monitoring response to therapy. In one study, enzyme-linked immunosorbent spot (ELISPOT) responses to RD antigens by PBMCs from British schoolchildren following point source TB exposure declined by 68% following LTBI treatment, although only 6 of the 38 children converted to negative. Responses declined in 7 of 14 children with borderline TST and positive ELISPOT who did not receive INH (Ewer et al., 2006). In one study of patients with active TB, ELISPOT responses to RD1 antigens declined from baseline to three months in all 13 patients with an adequate clinical response, but remained elevated in five treatment failures (Carrara et al., 2004). However, given that responses in some treated patients using ELISA may apparently be maintained for decades after the conclusion of therapy (Wu-Hsieh et al., 2001), these results should be treated with caution.

6.2. Antibodies to mycobacterial enzymes and 38 kDa antigen

Severity of TB disease has been shown to be correlated with levels of antibody to mycobacterial enzymes; the amount of antibodies to two mycobacterial enzymes (alanine dehydrogenase and malate synthetase) before treatment directly correlated with treatment failure (Azzurri et al., 2006). In

1 IFN, interferon; Th, T helper.
a different study, high levels of antibodies against the 38 kDa antigen were associated with advanced disease (Sartain et al., 2006). The levels of antibodies against some mycobacterial proteins have been reported to increase in serum well before diagnosis of TB can be made on bacteriological or clinical grounds (Gennaro et al., 2007). These antibodies should therefore be further evaluated as predictive biomarkers in future studies. Recently, antigen-specific markers have also been identified using proteomic techniques (see section 7.2) (Bahk et al., 2004).

### 6.3. Host nonspecific immune parameters as biomarkers for treatment response

The role of different immune parameters during active TB and anti-TB therapy has been studied by several laboratories. Even though many of these markers are not specific for TB and often occur during immune responses with other pathogens, they have been shown to be associated with extent of disease and/or anti-TB treatment response. A list of selected immune parameters can be found in Table 1. IFN-γ measured in sputum has been associated with response to therapy and bacterial clearance (Ribeiro-Rodrigues et al., 2002); however, the study was too small to establish a relationship to sputum culture conversion or relapse. Serum soluble tumour necrosis factor (sTNF)-R1 and 2 as well as granzyme B have been associated with sputum conversion (Brahmbhatt et al., 2006). Similarly soluble interleukin (sIL)-2R relates to treatment response (Chan et al., 1995). Although these markers have been shown to be sensitive to treatment, there is no evidence that they are useful in predicting treatment outcome.

### 6.4. Soluble intercellular adhesion molecule type 1 (sICAM-1)

ICAM-1 is mainly expressed by endothelial cells, is a ligand for leucocyte integrins and therefore involved in cell adhesion and leukocyte rolling. A soluble form of this molecule, sICAM, is released into the bloodstream. Its levels are elevated in TB patients at diagnosis, in proportion to disease extent, and decrease in response to anti-TB treatment (Demir et al., 2002; Lai et al., 1993; Mukae et al., 2003; Walzl et al., 2008). No studies to date have established a relationship to clinical or other end-points.

### 6.5. Other nonspecific immune parameters as predictors of treatment outcome

Selected immune parameters that have not only been shown to be markers for extent of disease and/or treatment response, but also qualify as candidate biomarkers for treatment outcome, have been summarized by others (Perrin et al., 2007; Walzl et al., 2008). Some of these are discussed below.

#### 6.5.1. Neopterin

Neopterin, a catabolic product of guanosine triphosphate (GTP), is produced by and released from macrophages upon stimulation with IFN-γ and is a biochemical marker of cellular immune responses. Neopterin levels are increased at TB diagnosis and decline during treatment (Immanuel et al., 2001; Turgut et al., 2006; Wallis et al., 1996). Levels are highest in patients with concomitant HIV infection, in whom they predict mortality; however, these deaths appeared to be related to HIV, and not active TB per se (Wallis et al., 1996). Like β-2 microglobulin, neopterin is recognized as a prognostic indicator in HIV/AIDS (Fahey et al., 1990). Its prognostic value for TB-specific outcomes in HIV-coinfected patients may therefore be limited. In HIV-uninfected TB patients, the decrease in neopterin levels during therapy was greater in patients with limited radiological lesions compared with patients with extensive lesions. In patients matched for pulmonary extent of disease, elevated levels after completion of treatment were associated with relapse. The association between elevated neopterin levels at the end of treatment and relapse was also shown previously by Hosp et al. (1997). Although the sample sizes of these three studies investigating neopterin levels during anti-TB treatment are relatively small, with
a maximum of 39 active TB patients per study, the consistency of the findings strengthens the case for neopterin levels as predictor for relapse. Therefore serum neopterin may represent a promising biomarker that requires further evaluation.

6.5.2 *Erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP)*

ESR is one of the oldest markers of diseases activity and inflammation known to date. Its routine use has decreased since the discovery of CRP, although ESR is still commonly used in health settings where facilities for measuring CRP are not available.

CRP is an acute-phase protein produced by the liver and known to opsonize pathogens, bind to CRP receptors on macrophages and thereby facilitate phagocytosis. Serum CRP is increased in TB; highest levels occur in patients with far-advanced disease (Bajaj, Rattan & Ahmad, 1989; Lee & Chang, 2003; Plit et al., 1998). CRP levels fall with therapy and are reported to correlate with clinical response (Bajaj, Rattan & Ahmad, 1989; Baynes et al., 1986). One study in paediatric TB indicated that levels fall after one month of treatment. Very high baseline levels of CRP (>20 mg/litre) have been shown to be associated with adverse treatment outcome (Scott, Murphy & Gemidjioğlu, 1990). These factors may indicate that stratification by CRP at baseline may promote equality among treatment groups. Its role as an early indicator of treatment response is uncertain.

6.5.3 *Soluble urokinase plasminogen activator receptor (suPAR)*

The urokinase plasminogen activator receptor is mainly expressed by macrophages and monocytes and is involved in cell adhesion and motility. The soluble form of this receptor was elevated in patients with active TB and correlated directly with the number of mycobacteria in sputum (Eugen-Olsen et al., 2002). During follow-up of 101 patients with active TB, a decrease in suPAR levels to those of controls was observed after eight months of anti-TB treatment. Additionally, the level of suPAR before treatment was positively associated with poor prognosis and mortality during therapy. Like CRP, suPAR could therefore be a candidate marker for the prognosis of anti-TB treatment outcome, although more studies are needed to validate its usefulness. The value of suPAR for the diagnosis of TB in sputum-negative patients is currently being tested by ViroGates (Denmark) in a clinical trial in Guinea-Bissau (study number: LSSP-CT-2005-012173).

6.6. *Host proteins*

A marker that correlates with Th1 responses is LAG-3V3 (a soluble splice variant of lymphocyte activation gene or LAG); markers related to Th2 responses are sCD30, total immunoglobulin (Ig) E and the chemokine CCL22. Their usefulness for monitoring effects of drug therapy or disease relapse needs to be addressed. Assays for these are now available. Measurement of specific IgE binding to *Mtb* may turn out to be a more precise measure of Th2 activity directed at *Mtb* itself.

6.7. **IL-4/IL-4α2 ratio**

The presence of IL-4α2 in TB was discovered by the European Union (EU)-funded University College London -VACSEL/VACSIS group in longitudinal cohort studies set up in 2000. IL-4 itself, IL-13 and IL-4 α2 were all found to be raised in freshly isolated PMBCs from TB patients, and their levels correlated with severity of disease (Seah, Scott & Rook, 2000). Subsequent work from these projects has confirmed that this increase is seen not only in blood cells, but also in freshly isolated bronchoalveolar lavage cells from TB patients (Dheda et al., 2005a; Dheda et al., 2005b). Interest in IL-4α2 in TB increased when studies in Ethiopia and the Gambia revealed that in individuals with stable long-established LTBI, there was a disproportionate increase in mRNA encoding IL-4α2, compared with that encoding IL-4 (Demissie et al., 2004). This was in sharp contrast to the increased IL-4 seen in recently exposed
individuals with strong IFN-γ responses to early secretory antigenic target (ESAT)-6, who are known to be at risk of disease (Demissie, 2006b). Moreover, in the United Kingdom, in TB patients after six months of treatment, the expression of IL-4 mRNA tended to rise relative to levels of IL-4 mRNA. A study performed in South Africa confirmed the inverse relationship between the IL-4/IL-4 mRNA ratio and severity of disease (Walzl et al., 2008).

Thus IL-4 mRNA appears to have potential as a biomarker for TB activity and cure. In a preliminary study, the levels of expression of IL-4, IL-4 mRNA and IFN-γ were followed over time in Ethiopian TB patients and their contacts (Wassie et al., 2008). The following results were obtained, leading to the overall conclusion that the ratio of IL-4/IL-4 mRNA was a correlate of immunity:

- the ratio was higher in healthy contacts than in patients
- the ratio rose after treatment
- the ratio fell in contacts developing symptoms
- the ratio tended to rise in those developing stable LTBI.

With several new anti-TB drug clinical trials commencing, there is a valuable opportunity to comprehensively investigate and characterize this phenomenon. This would, if successful, facilitate the translation of measurement of this biomarker into a simple test that will assist physicians in their management of patients with TB.

### 6.8. Whole blood killing assays

Several studies have examined the capacity of blood or blood cells to kill intracellular mycobacteria in ex vivo cultures. As originally described, inhibition of intracellular replication of *M. microti* in mononuclear cell cultures served as an indicator of BCG vaccine effect (Cheng et al., 1988). Recent studies have substituted whole blood for mononuclear cells, and have used alternative readouts (light production by *lux*-transformed indicator strains, or time to positivity in BACTEC™) (Cheon et al., 2002; Kampmann et al., 2000). Immune control of growth has been shown to be inferior in TST-negative people, enhanced by BCG vaccination or vitamin D administration, impaired by T-cell depletion or HIV infection, and restored by antiretroviral therapy (Cheon et al., 2002; Hoft et al., 2002; Kampmann et al., 2000; Kampmann et al., 2004; Kampmann et al., 2006; Martineau et al., 2007; Saliu et al., 2006; Tena et al., 2003). In using whole blood rather than mononuclear cell culture the requirement for washing of infected cells to remove extracellular bacteria is eliminated. As a result, concentrations of administered drugs in the cultures mimic those in vivo at the time of phlebotomy. The approach therefore can readily be used to examine pharmacokinetic/pharmacodynamic relationship as well as the combined effects of immunotherapy and chemotherapy on intracellular mycobacteria. One study found that whole blood bactericidal activity during anti-TB therapy correlated with the decline in sputum CFU counts and was superior in two-month sputum culture converters (Wallis et al., 2003). Two studies reported that regimens for drug-sensitive TB were superior to those for MDR-TB, consistent with required treatment durations and outcomes (Janulionis et al., 2004; Wallis et al., 2001a). Antagonism has been demonstrated between some drug and immune effects (Wallis et al., 2004). The whole blood models may be particularly suited to exploring the dose–response relationship of second-line anti-TB drugs, as well as the combined effects of drug and immunotherapies for MDR/XDR TB in short, early phase II trials, together with assessment of EBA.

### 6.9. Use of sets of markers to increase the predictive value

Some concerns emerge regarding the use of single host markers such as cytokines as biomarkers for the assessment of therapy. This makes host biomarker sensitivity and specificity challenging. It therefore is generally accepted that the model validating the end-point should be designed on a
set of markers rather than a single marker. So far only a few studies have integrated combinations of markers to predict treatment outcome. A report by Wallis et al. (2000) used multiple regression analysis to identify combinations of early microbiological markers that predicted two relapses occurring in a study of 43 patients. The concentration of antigen 85 in sputum on day 14 of therapy and days to positivity in BACTEC™ on day 30 were the strongest independent predictors. Brahmbhatt et al. (2006) and Veenstra et al. (2006) showed that this type of modelling with a combination of immune parameters could be used to distinguish fast from slow responders to treatment early after the start of anti-TB therapy. They found that a particular lymphocyte subtype, namely CD3dim/CD56+ natural killer (NK) T cells, was more prominent in TB patients compared with controls, but on their own CD3dim/CD56+ NK T cells did not correlate sufficiently with treatment response. However, when a combination of only two variables, CD3dim/CD56+ NK T cells and total NK cells, was used in a support vector machine discriminant analysis, classification of patients into slow and fast responders could correctly be made in a high percentage of patients. This study had small sample numbers in the responder groups, with a total of only 21 patients; therefore the role of this particular cell type in predicting final treatment outcome and recurrence needs to be assessed further. Similarly using discriminant analysis, Brahmbhatt et al. (2006) showed that the percentages of correct predictions of fast and slow responders were 88% and 67% respectively at diagnosis and 78% and 83% respectively at week 4 of treatment when combining white blood cell count, serum granzyme B and serum sTNF-R as predictive markers for week 8 sputum smear status.

6.10. Research questions arising

Can stable cure be predicted by measuring relapse? Culture negativity at two months is the only validated surrogate of cure in TB currently available (Aber & Nunn, 1978), but this is dependent on the drugs used in the continuation phase (Jindani, Nunn & Enarson, 2004). Evidence from previous trials has indicated that the potency of the regimen can alter the point of association to be either earlier or later (Jindani, Nunn & Enarson, 2004; Kennedy et al., 1996). A number of potential biomarkers have been proposed for TB but none to date have been evaluated in the context of a large-scale clinical trial. Only by validating markers in the context of new anti-TB drug clinical trials will a sufficient body of evidence be generated to permit successful markers to be used in further trials to reduce patient numbers or to be introduced into clinical practice. It is not clear if the differences in regimen potency and point of association reflect the difference between populations, between the assay techniques used, or genuinely differentiate between cure and remission of symptoms. In addition, there are few data on the stability of positive and negative responses using IGRAs over time. Longitudinal studies to assess all of these issues are urgently required in HIV-infected and HIV-uninfected adults and children. These could be incorporated into planned and ongoing drug trials with relatively little extra expense.

Similar uncertainties exist for the use of these tests to diagnose infection status prior to initiation of therapy, the most important being the degree to which these tests can differentiate recent or progressive infection from and distant or latent infection. Inability to distinguish these two states will greatly reduce the potential for these tests to add value to clinical decision-making in TB-endemic regions, where a majority of adults would be expected to have been exposed to *Mtb*. There are many studies that suggest that the magnitude of the immune response reflects the magnitude of the bacterial load and two longitudinal studies in untreated contacts suggest that the highest responders are at greatly elevated risk of TB (Diel et al., 2008; Doherty et al., 2002), but these studies are relatively small and lack detailed sequential testing routines. Thus the kinetics and duration of the differences are unknown; however, the fact that the two studies produced similar results in very different populations and environments (Ethiopia and Germany) is encouraging. Studies to address the questions of remote versus recent infection, predictive value and optimization of the assays, and their usefulness in HIV-infected patients should prove very valuable.
6.11. Further research on host immune markers

Further research in two areas may help advance this work: (i) adding additional antigens as stimulation, and (ii) using additional cytokines as the readout. There are some data to suggest that combining the RD antigens (expressed highly by actively growing bacteria) with antigens expressed by the bacteria under stress may improve differentiation between distant and recent infection (Demissie et al., 2006a; Hougardy et al., 2007). Alternatively, other cytokines or chemokines such as chemokine interleukin (CXCL)-10 may prove to be more sensitive than IFN-γ alone (Ruhwald et al., 2007) and assessment of levels of expression of multiple cytokines may prove more effective than reliance on a single marker (Wassie et al., 2008). Indeed, the latter manuscript suggested that assaying expression of multiple host markers (in this case, the cytokines IFN-γ, IL-4 and IL-4δ2) directly ex vivo without in vitro antigen stimulation can provide useful insight into the progress of disease, and may in fact be more representative of the ongoing immune response, since it reflects in vivo antigen stimulation, and therefore presumably the existing effector response. However, there is the potential downside that these responses are not necessarily TB-specific, a weakness that may be addressed by the identification of biosignatures – multiple signals driven by specific host–pathogen interaction (see Annex 4, section A4.7) (Jacobsen et al., 2008; Walzl et al., 2008).

7. EXPLOITING ADVANCED TECHNOLOGICAL PLATFORMS TO STUDY COMMON MARKERS OR SIGNATURES OF HOST RESPONSE

The recent development of advanced technological platforms such as proteomics, transcriptomics and metabolomics has led to novel approaches where “fishing” rather than targeted search can lead to identifying component networks involved in disease, pathogenesis and treatment response (Box 6). They are reviewed in depth by several authors (Jacobsen et al., 2008; Walzl et al., 2008). These biomarkers studies are ongoing (see Annex 4, section A4.7) and promise new data. The data sets obtained from these analyses are complex, requiring highly skilled interpretation of data by bioinformatics experts. The combined data from these new methodologies could be assembled into biosignatures, which could be put into biological context and may provide insight into host-pathogen interactions, TB disease state and treatment response.

The use of surface enhanced laser desorption/ionization time of flight (SELDI-ToF) mass spectrometry is a novel approach to biomarker discovery. This technique is able to provide a rapid protein expression profile from a variety of clinical and biological samples. The discovery, identification and validation of proteins associated with a particular disease state is a difficult and laborious task and often requires numerous samples.

The integration of transcriptomic, proteomic and metabolomic data will allow us to gain an overall understanding of the component networks involved in disease and treatment response.

Other advanced technological platforms such as glycomics and lipidomics may also provide valuable information. Glycomics is the comprehensive study of glycomes (the entire complement of glycan structure; carbohydrates, in free as well as in more complex molecules, of an organism), including genetic, physiological, pathological and other aspects; lipidomics: is the systematic study of lipids in a cell or organism.
**Box 6. Advanced technological platforms for biomarker discovery**

<table>
<thead>
<tr>
<th>Platform</th>
<th>Characteristics</th>
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<tbody>
<tr>
<td><strong>Transcriptomics</strong></td>
<td>Differentially expressed genes that distinguish latent from active TB</td>
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<tr>
<td></td>
<td>RNA</td>
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<td></td>
<td>(metabolites &lt;1500 Da)</td>
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<tr>
<td></td>
<td>100 000 transcripts</td>
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<tr>
<td><strong>Proteomics</strong></td>
<td>Differentially expressed proteins that distinguish latent from active TB</td>
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<td></td>
<td>Proteins</td>
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<td></td>
<td>1 000 000 proteins</td>
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<tr>
<td><strong>Metabolomics</strong></td>
<td>Metabolomics exploits the identification of small metabolites</td>
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<tr>
<td></td>
<td>Biochemical analytes</td>
</tr>
<tr>
<td></td>
<td>2400 compounds</td>
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<tr>
<td><strong>Combinations</strong></td>
<td>of above three platforms</td>
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</tbody>
</table>

Courtesy of S. Parida

### 7.1. Transcriptomics

Elucidation of the human genome and identification of approximately 30 000 genes has focused attention on gene polymorphisms and differential expression of genes and their implications for human disease. The concept of differential gene expression in TB patients versus healthy individuals is relatively new. In a recent study gene expression profiles of PBMCs from 40 TB patients were compared with those of 37 latently infected individuals by a combination of microarray analysis and quantitative reverse transcriptase (RT) PCR (Jacobsen et al., 2007; Figure 2). Using discriminant analysis, it was shown that a group of only three genes predominantly involved in microbial defence and intracellular trafficking (CD64, lactoferrin and Rab33A) could discriminate successfully between active TB cases and latently infected individuals. In a similar study, Mistry et al. (2007) identified differentially expressed genes in RNA extracted from whole blood of patients with active, recurrent, cured or latent TB using microarray analysis. Although the sample size of this study was very small, with only 10 individuals per study arm, discriminant analysis required only a combination of nine differentially expressed genes to stratify the patients into their respective groups. The identified genes are Ras and Rab interactor 3 (RIN3), lymphocyte antigen 6 complex/locus G6D (LY6G6D), testis-expressed gene 264 (TEX264), chromosome 14 open reading frame 2 (C14orf2), suppressor of cytokine signalling 3 (SOCS3), hypothetical protein KIAA2013, adenosine triphosphate (ATP)-binding arsenite transporter (ASNA1), mitochondrial ATP synthase (ATP5G1) and nucleolar protein family A/member 3 (NOLA3). These data suggest that whole-blood gene-expression profiling might also be used for prediction of high risk for relapse of TB.

The expression of RIN3 was found to be increased in CD8+ T cells in a gene expression study in which blood was stimulated with live *Mtbc* (Cliff et al., 2004). More studies of transcriptomics are therefore needed, in which such markers can be tested and validated in larger groups and different populations.
7.2. Proteomics

As genomics and transcriptomics have to date been inadequate with regard to comprehensive insight into disease processes, the focus has turned to the proteins that are encoded by the genes and their respective mRNAs. The expression and structure of the proteins can give valuable insight into their function and biological effects. Recently proteomic techniques have also been used in TB research to identify mycobacterial as well as host proteins that could potentially be used as biomarkers. One study showed that two mycobacterial antigens (rRV3369 and rRV3874) identified by proteomic techniques from culture filtrate could be used to distinguish 200 serum samples from TB patients and healthy controls with a sensitivity of 60–74% and a specificity of 96–97% (Bahk et al., 2004). Using a similar approach, Sartain et al. (2006) identified four *Mtb* antigens – 38 kDa PstS1 protein, heat shock protein X (HspX), MPT64 and TrxC – that were able to differentiate sera from cavitary and non-cavitary TB patients in serological tests. Antibodies against the 38 kDa protein are particularly found in patients with advanced TB disease.

Proteomic fingerprinting of patient serum recently lead to identification of host diagnostic markers for TB (Agranoff et al., 2006). In this study 349 serum samples from 179 TB patients and 170 controls with other infective and non-infective inflammatory conditions were profiled on protein chip arrays by SELDI-ToF mass spectrometry. Support vector machine modelling using three biomarkers, transthyretin, CRP and neopterin, discriminated correctly between TB patients and controls with high accuracy (82% sensitivity, 86% specificity). Further studies should be conducted with the aim of identifying...
proteomic signatures for treatment response and outcome, which then could lead to development of cost-effective immunoassays.

Challenges remain for proteomic techniques. Proteins undergo post-translational modifications such as phosphorylation and glycosylation, which increase the functional complexity of the proteins. Often signals of low-abundance proteins such as cytokines in serum can be obscured by high-abundance proteins such as albumin and protein fractionation techniques need to be applied before sample analysis.

7.3. Metabolomics

Metabolomics exploits the identification of small metabolites (small molecules <1500 Da) in body fluids such as serum or urine by means of mass spectrometry in order to identify biomarkers of the biochemical consequences of disease and drug treatment. Although transcriptomic and proteomic techniques are very powerful in biomarker research, comparative sample analysis from humans and animal models can be hampered because across-species genes and proteins of similar function possess different nucleotide and amino acid sequences (van der Greef et al., 2007). In contrast metabolites often have identical structures across species. Additionally the metabolome is downstream of the proteome and small changes in the proteome are thereby amplified, which makes the analysis of metabolites more discriminating than other techniques (Kell, 2006). Thus metabolomics may aid biomarker research profoundly and complement transcriptomic and proteomic techniques. However, the metabolite pattern found in individuals will vary greatly depending on time of the day when the blood or urine sample is taken, and food, fluid or drug intake before sample collection, etc. For clinical trials, standardization of patients will become crucial. Although no published results exist for TB biomarker discovery using advanced technological platforms, metabolomics has been used to analyse several other conditions (reviewed in Kell, 2006) and there is good reason to believe that similar approaches might be of benefit in TB biomarker discovery. Host as well as mycobacterial metabolites might be able to provide insight into host–pathogen specific interactions as well as disease state and treatment response and might be able to predict final outcome.

**BOX 7. Multiple host response profiling in biomarker research**

**Transcriptomic host response profiling approaches**
- Multiplex ligation probe amplification and related dedicated platforms
- Genome or (innate + adaptive) immunome-wide arrays

**Multiplex protein host response profiling approaches**
- Cytokines and chemokines
- Effector molecules
- M. tuberculosis killing/growth inhibition

**Need for sophisticated data collection, management and analysis**
- Bioinformatics
- Multivariate analysis
Box 8. Biomarkers: ideal performance characteristics

- Reflect immune correlates of protection
- Assist in diagnosis and management
- Have high levels of sensitivity and specificity
- Assays are practical and easy to perform
- Assays provide quick results
- Can be translated for use at site of care
- Are cost effective

7.4. Multiplex assessments (biosignatures)

Multiplex assessments have proven to have significant prognostic/diagnostic value in cancer (Bullinger et al., 2004; Burczynski et al., 2005), HIV infection (Ockenhouse et al., 2005) and malaria (Griffiths et al., 2005). Several recent studies suggest the same is true in TB, implying that highly multiplexed assays may be superior to single immune biomarkers. Agranoff et al. (2006) found that TB could be differentiated from other infectious and inflammatory conditions based on proteomic fingerprinting study of serum by SELDI-ToF mass spectrometry. Serum amyloid A and transthyretin were among the candidate biomarkers. The study was limited, however, by the relative insensitivity of the techniques used. Similarly, a recent report using DNA microarrays identified signatures involving expression profiles of nine genes in blood cells that could distinguish active TB, LTBI, cure and TB recurrence (Mistry et al., 2007). TB patients were evaluated in this study at the time of diagnosis. Taking a complementary approach, an ongoing study by Willem Hanekom and colleagues in BCG-vaccinated infants in South Africa’s Western Cape has used multiplex ELISA and DNA microarray to identify profiles associated with resistance to \textit{Mtb} infection after BCG vaccination (W. Hanekom, personal communication, 2008).

Further research is needed to determine whether these findings can be reproduced in other clinical populations, and to verify the suggestions by these reports that recurrent TB (whether due to relapse or to reinfection) in treated patients or protection induced by vaccination can be predicted by gene expression profiles.

8. OBSTACLES IN BIOMARKER DISCOVERY

A major challenge in biomarker research will be the analysis and integration of data from transcriptomic, proteomic and metabolomic investigations, which is required to understand the “bigger picture” of anti-TB treatment response and outcome (Jacobsen et al., 2008; Walzl et al., 2008). Highly skilled bioinformaticists will be required to interpret the enormously complex data sets before the results are put into a biological context. Despite the identification of a number of biomarkers that show promise for monitoring response to treatment and of treatment outcome, much remains to be done. Currently these markers have not been sufficiently validated to persuade funding bodies to support lengthy and costly clinical trials to evaluate these markers.
Prospective studies for validation of such markers would require substantial funding, advanced logistics and special field site characteristics. Ideally, biomarker studies for relapse would collect suitable patient samples from pretreatment throughout the six-month therapy period and from the subsequent 18–24 months, and would require sufficient clinical and microbiological characterization at uniform time points. Treatment adherence, drug susceptibility, concurrent illness and HIV status represent just some of the important parameters for which data need to be collected. To achieve a significant number of patients with recurrent disease (e.g. 20–40) after initial cure, a study would have to include between 500 and 1000 patients, assuming a recurrence rate of 5% and a true relapse rate that is even lower. It should be noted that frequent reinfections with different bacterial strains have been reported in high-incidence settings (Wang et al., 2007; Warren et al., 2002), where such studies would presumably be conducted.

DNA fingerprinting of strains is necessary for both disease episodes to enable the correct characterization as true relapse rather than reinfection. This also implies that patients would have to be culture positive at enrolment. The patients should ideally all fall under the same trial protocol to ensure standardization. Such a trial, whether at one site or multisite in nature, would require substantial funding and site preparedness. The choice of field sites will, in the first instance, be dictated by the requirement of sufficient numbers of HIV-uninfected first-time, culture-positive pulmonary TB cases. Follow-up for more than two years must be feasible and cure rates should be acceptable (demonstrating the establishment of a functional TB control programme that is required for successful conduction of such a trial). Laboratory facilities for culture, drug sensitivity testing and DNA fingerprinting must be available and storage of samples will require reliable electrical supply and liquid nitrogen supply for cryopreservation of cells. As the ideal sample type for reliable biomarkers is currently unknown, the collection of serum, plasma, RNA, sputum, urine and PBMCs may be required. The sample volumes required for this purpose constitute an additional challenge on participant recruitment and retention in such studies. Advanced data and sample management systems must also be in place.

The high prevalence of HIV coinfection in TB patients complicates matters further, as immune deficiency may seriously affect host biomarker expression. A further complicating factor could include the presence of previous TB in a high proportion of patients, which would slow down recruitment rates significantly. New patients with a first episode of TB would be needed for such studies, to exclude confounding factors such as partially treated patients and drug resistance.

9. ETHICAL IMPLICATIONS OF BIOMARKER RESEARCH

Storage of samples for future analysis must be covered by suitable participant consent and institutional review board permissions. Where samples are collected for a variety of biomarker discovery laboratory techniques, it is important that consent forms are worded to allow testing and validation of as yet unidentified biomarkers. Regardless of ethical permissions for sample acquisition, social and cultural issues may also impact on such studies as spiritual values may be attached to certain samples types (such as blood). Blood volumes that can be obtained differ significantly from site to site and from country to country.
10. NEED FOR FURTHER STUDIES

Research is urgently needed to further evaluate and validate candidate biomarkers and surrogate end-points in HIV-infected and HIV-uninfected adults and children with TB and their contacts using conventional and new advanced technological platforms. The development plan for these biomarkers should provide for small initial studies that should increase progressively in size and duration, as occurs in clinical testing of new drugs. The studies should be conducted to the clinical and laboratory standards that are necessary for licensing of new drugs and diagnostic tests.

11. CONCLUSIONS

The search for surrogate markers that can provide primary measurements of treatment effectiveness and clinical prognosis has to be intensified. Only once suitable markers are found and validated accordingly will development of new therapeutic strategies (the right treatment for the right patient at the right time) be possible. Additionally drug tolerance and resistance due to suboptimal treatment would be minimized and clinical trials of new anti-TB drugs accelerated and shortened. The interest in finding such biomarkers is growing, judged by the emphasis placed on biomarker research by the World Health Organization (WHO), the European and Developing Countries Clinical Trials Partnership, the United Kingdom Medical Research Council and the Bill & Melinda Gates Foundation. Although the TB biomarker field is challenging, the pursuit of this research area must urgently be prioritized to aid the quest of improved therapy and the control of the TB pandemic.

12. RECOMMENDATIONS TO TDR AND THE EC

12.1. Culture-based biomarker development

It is recommended that culture-based biomarker development emphasize the use of automated liquid culture systems, since there is increasing recognition of the growing clinical role of these systems worldwide. Surrogate end-points developed using these methods would therefore be most readily incorporated into new standards of care.

12.2. Drug-resistance testing

Similarly, resistance testing, using line probe or conventional assays, will be required to determine drug susceptibility on entry and throughout study participation. Such tests will be important in differentiating primary from secondary (acquired) resistance, and in uncovering mixed infections.
12.3. Mycobacterial speciation

Mycobacterial speciation will also be essential, as, with the increasing use of liquid culture, mixed cultures with non-tuberculous mycobacteria are increasingly recognized as a potential diagnostic confounder.

12.4. Exploratory studies

For some markers of treatment efficacy, initial studies may be required that are short (e.g. one-week drug exposure) and small (15 subjects per arm). Such studies may assess, for example, the dose–response relationship to second-line anti-TB drugs, using quantitative sputum microbiology (EBA), whole blood bactericidal activity, and pharmacokinetic sampling. These exploratory studies could be conducted in patients with drug-sensitive TB, at sites familiar with methods for quantitative sputum microbiology. It should be assumed that this brief duration of treatment will not be sufficient to detect host immune biomarker responses, unless specific data suggest otherwise.

12.5. Pharmacokinetics and whole blood bactericidal studies

Parallel studies of the pharmacokinetic and whole blood bactericidal activity of selected interventions in healthy volunteers will permit the analysis of immune/drug interactions, and may identify new candidate interventions for subsequent study in TB patients.

12.6. Breath biomarkers studies

Only preliminary studies have been performed on mycobacterial colonies. Further work to identify the metabolites in the breath of TB patients needs to be performed in appropriate controlled conditions. Once the assays are standardized these could be developed as biomarkers and tested in the relevant population groups.

12.7. Markers relevant to treatment monitoring

The equivalent of phase II trials for markers relevant to treatment monitoring may be best conducted in patients with MDR-TB or XDR-TB. The efficiency of clinical research is enhanced in such patients, due to the higher proportion of subjects reaching clinical end-points of treatment failure or relapse. The wide potential range of resistance patterns represents both a challenge and an opportunity. Some larger phase II trials will require stratification based on the number of drugs to which *Mtb* is resistant and possibly on the presence of resistance to key drugs, such as the fluoroquinolones or other injectable anti-TB drugs. Smaller phase II trials will be possible with regimens consisting of single drugs or of limited combinations of drugs and immunotherapies. Such trials will probably be considered ethical if other treatment options do not exist and if the study is likely to increase medical knowledge of importance to other patients. Site selection for these trials will be dependent on the availability of appropriate patient populations, laboratory facilities (including appropriate biosafety containment) and the necessary clinical and laboratory expertise to conduct trials at good clinical practice and good laboratory practice levels. These trials must be of sufficient duration to collect data regarding treatment success and relapse (i.e. a minimum of one-year follow-up after 12–18 months treatment). Pathogen-based and host immune markers should both be evaluated. Biomarkers based on quantitative sputum microbiology should be performed using both quantitative CFU and time to detection in automated liquid culture systems. The study sizes will be sufficiently small that both static and functional biomarkers should be included.
12.8. Markers of protective immune response

To identify biomarkers of protective immune responses, different approaches will be needed. For vaccine studies, we are unlikely to obtain conclusive data until the completion of the first successful phase III trial, due to the difficulty of defining protection in the general population. Some data may be obtained in phase II studies, though they will be at best indicative. Phase II studies, however, will be small enough that in-depth analysis is possible, and to this end, where possible a variety of approaches incorporating both static and functional biomarkers, as noted above, should be tried. Even with a small number of end-points expected, the information gathered may be able to inform other studies – particularly if the data can be compared with responses predictive of successful cure, latency and relapse. A less expensive and faster approach than phase III vaccine trials is to conduct retrospective analyses in large numbers of exposed individuals – with the necessary caveat that success in such studies is not guaranteed. To be most useful, however, such studies should also be conducted to the same high standards as registration trials, and include both static and functional biomarkers: this requirement is most easily met by nested case–control analyses. However, not all assays can be performed retrospectively – inclusion of simple whole blood assays throughout the course of the studies should therefore be considered. Since little is known about the kinetics and stability of the different markers assessed, collection of multiple sequential samples should be strongly encouraged, which means addressing issues of sample storage. In all cases, neglected issues, but ones that need to be addressed, are validation of potential markers identified in different populations and geographical regions. In particular, assessment/validation of potential biomarkers in at-risk populations such as HIV-positive subjects, infants, children and elderly patients has been poorly served, despite the obvious need.

12.9. Paediatric TB and biomarkers

Paediatric TB remains understudied and there are very few markers that have been studied in that population. A brief summary of the status is given in Annex 5. Basically most of the studies recommended for adults also apply to paediatric TB.

12.10. Clinical trials and markers studies

12.10.1 Phase III treatment trials

Phase III treatment trials may be best conducted in patients with drug-sensitive TB. Such studies must be of sufficient size to collect adequate numbers of TB recurrence. Initial and recurrent strains must be tested by restriction fragment length polymorphism (RFLP) or other methods to distinguish reinfection from true relapse. Because only a small number of patients will reach the end-point of relapse, biomarkers that can be evaluated retrospectively in a case–control sub-study using banked specimens will be favoured.

12.10.2 Markers studies during usual patient care

Alternatively, markers that will be routinely evaluated in the course of usual patient care (or with minor modifications) may also be appropriate. At least two studies, conducted in Africa and either Asia or South America, will be required. Studies in all three regions may be particularly helpful to resolve the questions that have arisen in the course of the moxifloxacin studies described in Annex 4, section A4.4).
12.10.3 Opportunities for collaborative work

Opportunities may arise for the coordination of these studies with ongoing or planned drug trials intended to test strategies for shortening anti-TB treatment. Currently the Global Alliance for TB Drug Development/European and Developing Countries Clinical Trials Partnership-funded clinical trial on the use of moxifloxacin substitution of INH (REMox Trial) for shortening the duration of chemotherapy has a clinical biomarkers study integrated with one of the trial sites (Durban, Medical Research Council). Such coordination would decrease costs and enhance study value.

12.10.4 Biomarker work within national TB programme conditions

Alternatively, it may be possible to conduct phase III biomarker trials under national TB programme conditions. In this case it will be essential that additional support be provided to ensure adequate follow-up after the usual programme end-point of completion of therapy. A minimum of one additional year of follow-up will be required, making the probable total study duration three years. Collection of serum samples at regular intervals for studies of transcriptomics, proteomics, and metabolomics will be essential. Finally, the equivalent of phase IV trials may be considered in which a biomarker is used to modify patient treatment under programme conditions. The design and implementation of such studies will be highly dependent on the outcomes of earlier phase studies.

There is an urgent need for clinical trials of newer anti-TB drug regimens to be conducted in parallel with biomarkers studies in adults and children. These can be used for studying all the markers described in this report.

13. DESIGNING FURTHER BIOMARKER STUDIES AND FUNDING ISSUES

Currently, markers and surrogate end-point studies are dependent on a number of variables (funding availability, scientific interest, appropriate study sites with good clinical practice and good laboratory practice facilities, clinical trials limitations on amount of blood taken and frequency of bleeding, cohort studies, laboratory expertise, and the priority agenda of funding agencies). Studies in children, HIV-infected people, and those in confined institutions have ethical considerations that might be restrictive. These factors influence the direction of future studies.

Markers studies involve the state-of-the-art technologies that require expensive equipment and reagents. With a competitive scientific atmosphere for the restricted availability of funding, only a limited number of applications get funded for a three- or five-year duration. Preliminary data obtained on promising biomarkers cannot be taken forward because of lack of funding thereafter to sustain and continue the work. The expert consultation noted that the current competitive research funding market leads duplicity and cryptic research, providing no guaranteed follow-up funding. Suggestions were made that call for an innovative change to funding important issues. These suggestions include the following.

- New funding for biomarkers should include adequate budgets for the planned studies and should be provided for a longer duration (around 10 years).
- To avoid competition and allow for collaboration, markers studies should involve all expert groups who are willing to work together.
• The studies should, wherever possible, be carried out at the field sites so that local capacity development and training can take place.

These studies should be commissioned after an expert consultative review of what is required in terms of priorities of experienced, multi-centre partnerships covering wide geographical areas. Annual meeting of investigators with funding agencies to stimulate further studies and dissemination of knowledge must be part of the contracts. It was thought that these issues must be discussed at political and scientific levels by existing research and development funding bodies and a precedence could be set by the EC and TDR. They could eventually partner with American, Japanese and European funders to create a multiplier effect for coordinated funding.

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Demissie A et al. (2006b). The 6-kilodalton early secreted antigenic target-responsive, asymptomatic contacts of tuberculosis patients express elevated levels of interleukin-4 and reduced levels of gamma interferon. *Infection and Immunity*, 74(5):2817–2822.


Hong Kong Chest Service/British Medical Research Council (1991). Controlled trial of 2, 4, and 6 months of pyrazinamide in 6-month, three-times-weekly regimens for smear-positive pulmonary tuberculosis, including an assessment of a combined preparation of isoniazid, rifampin, and pyrazinamide. Results at 30 months. The American Review of Respiratory Disease, 143(4 Pt 1):700–706.


## Day 1

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<thead>
<tr>
<th>Time</th>
<th>Item</th>
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<tbody>
<tr>
<td>09:00–09:20</td>
<td>Welcome and introductions by attendees</td>
<td>Alimuddin Zumla</td>
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<tr>
<td></td>
<td>TDR introductory remarks</td>
<td>Robert Ridley</td>
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<tr>
<td>09:20–09:40</td>
<td>Importance and background to the meeting</td>
<td>Philip Onyebujo</td>
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<tr>
<td>09:40–10:00</td>
<td>EU perspective on TB research and biomarkers</td>
<td>Ole Olesen/Hannu Lång</td>
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<tr>
<td>10:20–10:50</td>
<td>Discussion</td>
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<td>10:50–11:10</td>
<td>Coffee break</td>
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<tr>
<td>11:10–11:40</td>
<td>Review of currently available biomarkers in TB and TB/HIV. Definition, need and approaches for identifying markers for new drugs, vaccines and immunotherapy</td>
<td>Robert Wallis</td>
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<tr>
<td>11.40–12:10</td>
<td>Immune markers in tuberculosis – current and possibilities for the future</td>
<td>Mark Doherty</td>
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<tr>
<td>12.10–12.30</td>
<td>TB biomarkers studies in Europe</td>
<td>Tom Ottenhoff</td>
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<td>12:30–13.00</td>
<td>Discussion</td>
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<td>13.00–14.00</td>
<td>Lunch break</td>
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<tr>
<td>14:00–14:30</td>
<td>New insight into the promising biomarkers of TB: prospects for improved interventions</td>
<td>Shreemanta Parida</td>
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<td>14:30–15:00</td>
<td>Designing studies for TB and TB/HIV biomarkers: challenges and opportunities</td>
<td>Andrew Nunn</td>
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<td>15:00–15:45</td>
<td>Discussion</td>
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<td>15.45–16:15</td>
<td>Coffee break</td>
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<tr>
<td>16:15–17:30</td>
<td>Working groups I and II: allocation to groups and preliminary discussions for Thursday’s deliberations.</td>
<td>Working group I (Mark Doherty, Chair)</td>
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<td>WGI (host biomarkers): Review existing and potential biomarkers and candidates and priorities for research.</td>
<td>Working group II (Robert Wallis, Chair)</td>
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<td></td>
<td>WGII: (pathogen biomarkers): Existing biomarkers and priorities for their validation/evaluation. Potential adoption in different field settings.</td>
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Closure 1st day
## Day 2

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<tr>
<td>09:00–10:30</td>
<td>Working groups I and II (discussions, priority settings and preliminary draft report)</td>
<td>Robert Wallis (WGI)</td>
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<tr>
<td></td>
<td></td>
<td>Mark Doherty (WGII)</td>
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<tr>
<td>10:30–10:45</td>
<td>Coffee break</td>
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<tr>
<td>10:45–12:45</td>
<td>Presentation of WG draft reports and discussion</td>
<td>Robert Wallis and Mark Doherty</td>
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<td>12:45–14:00</td>
<td>Lunch break</td>
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<tr>
<td>14:00–15:45</td>
<td>Presentation of WG reports and final inputs</td>
<td>Robert Wallis and Mark Doherty</td>
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<td></td>
<td>Final comments and discussions</td>
<td>Everyone</td>
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<tr>
<td>15:45–16:00</td>
<td>Coffee break</td>
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<tr>
<td>16:00–16:30</td>
<td>Conclusions and consensus summary</td>
<td>Alimuddin Zumla</td>
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<tr>
<td>16:30–16:45</td>
<td>TDR and EC viewpoints</td>
<td>Philip Onyebujoh, Ole Olesen/Hannu Lång</td>
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<tr>
<td>16:45–17:00</td>
<td>Vote of thanks</td>
<td>Robert Ridley</td>
</tr>
<tr>
<td>17:00–18:00</td>
<td>Writing assignments</td>
<td>Alimuddin Zumla, Robert Wallis and Mark Doherty</td>
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Closure of the meeting
ANNEX 2. LIST OF PARTICIPANTS

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ANNEX 3. BACKGROUND TO THE MEETING

Despite the availability of inexpensive, effective chemotherapy, TB persists as a major infectious disease, contributing to significant global morbidity and mortality, killing more than 1.6 million people annually worldwide (WHO, 2008). Many people with TB go undiagnosed each year and globally accurate diagnosis still relies on the 120-year-old method of sputum examination for acid-fast mycobacteria and culture. On average only 60% of people with active TB are diagnosed by routine microscopy – nearly 40% of active TB cases are missed. Several groups of patients – HIV-positive adults and children, those with extrapulmonary TB, and those with IRIS – have few tubercle bacilli in their sputum and are often missed by sputum microscopy. Clinical methods of monitoring the management of patients with active TB depend on subjective assessment of clinical signs or the patient’s perception of well-being, which are notoriously unreliable. Latent infections are difficult to monitor and are only dealt with when they progress and manifest with clinical disease. Mechanisms of reactivation are unknown and it is currently not possible to predict which group of patients with latent infections will reactivate.

The impact of tuberculosis has been aggravated in recent years by the appearance of MDR and XDR cases of TB, as well as the continued increase in number of people presenting with TB who are infected with HIV. The urgency of the present situation calls for accelerated clinical development of new vaccines, diagnostics and drugs, but a major obstacle is the lack of suitable and validated biomarkers to facilitate the early prediction of the rates of bacillary clearance and later risk of relapse and recurrences – regardless of the type or mechanism of action of the therapy under investigation – among various categories of TB patients.

The classical end-points in studies of anti-TB therapy are the proportion of subjects whose sputum fails to clear at the end of therapy (failures or reinfections) plus the proportion with recurrent disease during the subsequent two years post-therapy (relapses). While these end-points have been used successfully in the past, it would be highly desirable to identify new and more specific biomarkers that could reduce the size and duration of clinical trials of new drug candidates and define treatment efficacy, disease activity, cure and relapse. In vaccine development it is similarly important to identify reliable biomarkers for correlates of protection, which may predict the efficacy of an experimental vaccine candidate at an early stage of clinical development.

Biomarkers should be able to assess several areas of clinical management for both adults and children with TB:

- disease activity and extent
- treatment effect (response to treatment)
- treatment outcome (cure)
- disease relapse
- anticipated poor clinical outcomes (so that treatment can be modified appropriately)
- end-points of novel anti-TB drugs.

In the post-licensing clinical setting, biomarkers may also assist in the early identification of patients with poor anticipated clinical outcomes, so that their treatment may be appropriately modified.

It is in the context of these challenges that TDR and the Poverty-related Diseases unit, EC, jointly agreed to hold an informal consultation to identify the research gaps in the area of biomarkers for TB/HIV and to develop a research priority list that will inform the research and development efforts of academics, research institutions, policy-makers and funding agencies.
A number of aspects made the convening of this meeting particularly urgent, and suggested that such a meeting would yield important information on existing biomarkers, and priorities for biomarkers research and development.

A3.1 Link between TB and HIV pandemics

The HIV and TB pandemics are closely intertwined, with HIV being the most common predisposing factor for TB, and TB the most common presenting illness for AIDS. Immune reconstitution, a general goal of antiretroviral therapy, can have deleterious immediate consequences (IRIS) in HIV-infected TB patients. In addition, TB appears to accelerate the course of HIV, via activation of the nuclear transcription factor NF-κB: rapid diagnosis of TB in the early stages of infection may allow targeting of preventive therapy. Physicians presently lack tools to accurately assess and manage risks and optimize long-term outcomes in HIV-infected TB patients.

A3.2 Link between HIV infection and anti-TB drug resistance

Reports in some regions have associated HIV infection among anti-TB patients with TB drug resistance (both MDR- and XDR-TB). Although some of this risk presumably represents enhanced susceptibility to transmitted resistant TB strains, HIV/AIDS appears to particularly predispose to the emergence of acquired anti-TB drug resistance during intermittent therapy. The interplay of the biological and pharmacological factors responsible for resistance in this circumstance is inadequately understood. Robust and reliable biomarkers that could be used in TB and HIV-infected TB patients would greatly accelerate research and facilitate patient management in this regard. Immunological biomarkers of treatment success would be useful in drug regimen, vaccine and immunotherapy trials, reducing costs and decreasing the long development timeline.

A3.3 Untapped potential role of TB therapeutic agents

The potential role of new anti-TB drugs, vaccines and immunotherapeutic agents in the prevention and management of drug-resistant and HIV-associated TB remains largely untapped. A major challenge to drug, vaccine and adjunctive immunotherapeutic candidate development is the length of time required for assessment of efficacy due to dependence on long-term clinical outcomes.

A3.4 Need to identify relapse and to stratify patients

The ultimate success of anti-TB therapy is measured by the rate of relapse within the first two years after treatment. The long duration of clinical trials that rely on this outcome constitute a disincentive for the pharmaceutical industry to develop new anti-TB drugs. Biomarkers for relapse that can be measured early during treatment would advance the field significantly.

Additionally, baseline markers that allow stratification of TB patients at diagnosis or early during treatment into groups with different risks for adverse treatment outcome would facilitate validation of novel anti-TB drug candidates by ensuring equality of groups in clinical trials. Such markers could also allow stratification of patients into groups with different treatment requirements (different regimens and treatment duration) for clinical use. Currently the earliest validated marker of treatment effect is month-two sputum culture conversion. Markers that are measurable earlier during therapy may also accelerate new drug development through shortening of clinical trials.
A3.5 Meeting objectives and expected outcomes

Objectives
1. To review the current candidates for biomarkers for TB in adults and children with and without HIV infection.
2. To evaluate their utility for measuring disease activity and progression, relapse, reinfection and disease protection (correlates of protection).
3. To review ongoing research on biomarkers and identify and prioritize the research needed in this area of work.
4. To discuss the requirements for validating the utility of candidates biomarkers.

Expected outcomes
1. Identification of current pathogen (Mtb) and host biomarkers described in the literature.
2. Discussion and list of potential biomarkers for further evaluation in the context of new drugs, vaccines and immunotherapy.
3. Review of new technological platforms (transcriptomics, proteomics and metabolomics) for biomarker studies.
4. Recommendations on priority research for the development of biomarker candidates and their potential adoption in different settings.
5. Requirements for validating the role, adoption and evaluation of biomarkers in HIV-positive and HIV-negative adults and children with TB.

A3.6 Meeting outline
The agenda and list of participants are listed in Annex 1 and Annex 2, respectively. Presentations were given by experts on specific biomarker issues and challenges for the development and use of biomarkers to contribute to the existing control activities for TB (Annex 4):

- global TB control in 2008 – achievements, challenges and the need for better tools;
- pathogen biomarkers;
- host immune biomarkers
- TB biomarkers studies in Europe;
- review of current biomarkers for use in adults and children with and without HIV infection (definition, need and approaches for identifying markers);
- development and potential use of biomarkers for clinical trials (new anti-TB drugs), new vaccines and adjunctive immunotherapy;
- new insights into promising biomarkers for TB and prospects for improved interventions;
- designing studies for TB and TB/HIV exploring the role of biomarkers in relapse and treatment failure cases.

Two working groups looked at and discussed the following topics:
- pathogen biomarkers (current and future biomarkers for detection of disease activity, cure, relapse and treatment failure, as well as new technological platforms);
- host biomarkers (biomarkers for monitoring disease activity, cure and relapse and treatment failure for application to clinical trials of new anti-TB drugs, vaccines, adjunctive immunomodulation and IRIS).
ANNEX 4. PRESENTATIONS AT THE MEETING

Summary texts and slides are provided.

A4.1. Background and role of TDR

Dr Robert Ridley, TDR, Geneva, Switzerland

As a result of changes in the research environment since TDR was created in 1975, TDR has revised its vision and strategy and is changing to meet new challenges.

TDR’s mandate has been extended: its new vision is to foster an effective global research effort on infectious diseases of poverty in which disease endemic countries play a pivotal role. There is now an emphasis on greater social contextualization of research, bringing it closer to control needs, and while TDR still has a technical focus, it also leverages scale, policy and impact.

In order to fulfil its vision, TDR has adopted a three-pronged strategy:

1. stewardship – to harmonize global research efforts
2. empowerment – to develop disease endemic country leadership in research
3. research on neglected priority needs – to enhance access to superior interventions.

This new vision and strategy was endorsed by the TDR Joint Coordinating Board in 2007. The Board includes TDR’s four cosponsors – the United Nations Children’s Fund, the United Nations Development Programme, the World Bank and WHO – as well as representatives of governments of developing and developed countries.

Stewardship constitutes a major new role for TDR, as facilitator and knowledge manager, to support comprehensive research needs assessments, priority setting, progress analysis and advocacy, and to provide a neutral platform for partners to discuss and harmonize their activities. A major output will be a biennial report on the status of tropical disease research.

Empowerment moves beyond traditional research training to build leadership in disease endemic countries at individual, institutional and national levels. As for its stewardship functions, there is functional decentralization, with TDR research teams based in developing country institutions.

Research on neglected priority needs for disease control that are not adequately addressed by other partners focuses on three functions, aiming to foster:

- innovation for product discovery and development
- research on development and evaluation of interventions in real-life settings
- research for access to interventions.

To achieve these objectives, TDR has restructured its operations, creating nine research lines. The research line Evidence for treatment policy for HIV-infected TB patients aims to optimize treatment and case management and delivery of care for all patients’ populations with TB and TB/HIV coinfection, including patients with additional co-morbid diseases. A specific objective in developing evidence for the management of HIV-infected TB patients involves determining the role of bio/surrogate markers...
and immunomodulation for optimal care of patients presenting with IRIS and HIV-infected patients with TB.

Biomarker research also brings together important aspects of two other research lines:
- the TDR targets database (part of Lead discovery for drugs for infectious diseases);
- a range of specimen banks and evaluation networks (part of Accessible quality-assured diagnostics).

This expert consultation, bringing together TDR, the EC and researchers from both developed and developing countries to explore an innovative area of research, represents a microcosm of TDR’s new strategy – fostering an effective global research effort … in which disease endemic countries play a pivotal role.
New TDR Vision

Covers ‘infectious diseases of needy populations’

Social contextualisation of research

Technical, but leverages scale, policy and impact

What we want to achieve

1. Stewardship
   Harmonised global Research efforts

2. Empowerment
   DEC leadership in Research

3. Research on Neglected Priorities
   Enhanced Access to Superior Interventions
Stewardship / Knowledge Management

- Comprehensive research and priority assessments
- Biennial Report 'status of tropical disease research'

Stewardship decentralisation

- Reference groups for research and priority assessments

Empowerment / Capacity Building

- Focus on leadership
  - National
  - Institution
  - Individual
**Interventions: Quality-assured Diagnostics**

**Objective**
To promote and facilitate the development, evaluation and application of diagnostic tests appropriate for use in developing countries

**Achievement Highlights (for TB)**
- Market analysis for TB diagnostics (with FIND)
- Validated PCR-based tests to assess TB resistance
- Demonstrated non-validity of 19 serological TB tests currently on the market
- Systematic reviews led to policy change in TB
- 2008 Specimen bank / Evaluation networks

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**Interventions: Evidence for TB/HIV Treatment**

**Overall Objective**
To optimize treatment and case management/delivery of care for all patients' populations with tuberculosis and tuberculosis/HIV co-infection, including patients with additional co-morbid diseases

**Specific Objectives**
- Develop the evidence for shortening and simplification of TB treatment in TB and HIV-infected TB patient populations
- Develop the evidence for management of HIV-infected TB patients:  
  - concomitant use of anti-TB and antiretroviral drugs
  - optimal timing of highly active antiretroviral therapy (HAART)
  - more effective anti-TB chemotherapy regimen for treatment with HAART
- Role of biomarkers and immunomodulation for optimal care of patients presenting with AIDS and HIV-infected TB.
- Develop strategies for operational implementation of TB and HIV/AIDS case management and treatment strategies in resource-limited settings of high burden countries in Africa.

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**TDR - inter-agency and inter-governmental**

[Diagram showing inter-agency and inter-governmental cooperation]

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TDR Vision

To foster:

An effective global research effort on infectious diseases of poverty in which disease endemic countries play a pivotal role.
A4.2. Overview of the global TB problem and background to the meeting

Dr Philip Onyebujoh, TDR, Geneva, Switzerland

Context: needs, opportunity and epidemiology

Needs
The convergence of TB and HIV has created a new disease, requiring:

- new diagnostics and new approaches to treatment;
- evidence for strategies and policies for optimized case management of TB/HIV;
- improved case detection, drug susceptibility testing at the point of care, and optimized case-holding;
- improved algorithms for detecting sputum-negative TB cases;
- clinical studies to evaluate safety and efficacy of new drug and diagnostic entities;
- shortened clinical trials.

Clinical trials currently cost about US$ 1.5 million–2 million per year over about five years. Any shortening of trials would therefore result in significant cost savings. Surrogate markers are urgently needed to help achieve this.

Such markers may also be able to complement current case-detection tools and strategies.

Opportunities

- Scaling up access to antiretroviral drugs brings with it the need to develop new strategies and tools for TB control (e.g. treatment and diagnostics).
- There is a renewed interest in research for evidence-driven policy.
- The Stop TB Partnership’s "retooling" strategy will help to prepare for the adoption, introduction and implementation of new diagnostics, drugs and vaccines in the control of TB.
- The emergence of XDR-TB in KwaZulu-Natal, South Africa, has confirmed that other treatment strategies (e.g. adjunctive immunotherapy) should be used to enhance current treatment strategies.
- There is an unprecedented availability of funds for "essential health research".

Epidemiology

In 2006, there were 9.2 million new TB cases globally; the number of prevalent cases was 14.4 million. Of these, there were 4.1 million new smear-positive cases and 0.7 million HIV-positive cases. The leading countries by TB burden are India, China, Indonesia, South Africa and Nigeria. There were 1.5 million deaths from TB in HIV-negative people and 0.2 million among HIV-positive people (WHO, 2008).

There were 0.5 million cases of MDR-TB, representing 4.8% of all new TB cases, with China, India and the Russian Federation having the highest burden. A total of 45 countries have reported at least one case of XDR-TB. There is a high proportion of XDR-TB among MDR-TB cases in the former Soviet Union, Japan and the Republic of Korea, whereas in Rwanda and the United Republic of Tanzania, there is no XDR-TB and levels of MDR-TB are low (WHO, 2008).
TDR's TB/HIV portfolio

In TDR’s revised strategy, a research line – Evidence for treatment policy for HIV-infected TB patients – has been set up to coordinate TDR’s TB/HIV activities. It focuses on the optimization of care for HIV-infected TB patients within the context of available health systems capacity, through the development and deployment of evidence-for-policy in real time. Its overall objective is to optimize treatment, case management and delivery of care for all patient populations with TB and HIV-infected TB, including patients with additional co-morbid diseases.

The research activities will be embedded in national TB control programmes, further building their capacity. The specific objectives are to develop:

- evidence for shortening and simplifying anti-TB treatment in TB and HIV-infected TB patient populations (gatifloxacin-containing four-month fixed-dose combinations (4FDC) studies);
- evidence for management of HIV-infected TB patients:
  - concomitant use of anti-TB and antiretroviral drugs;
  - optimal timing of highly active antiretroviral therapy (HAART);
  - more effective anti-TB chemotherapy regimen (rifabutin) for use with HAART;
  - role of bio/surrogate markers and immunomodulation for optimal care of patients presenting with IRIS and HIV-infected TB;
- strategies for operational implementation of TB and HIV/AIDS case management and treatment strategies in resource-limited settings of high-burden countries in Africa.

The deliverables are:

- simpler, shorter anti-TB treatments, including:
  - gatifloxacin-containing 4FDC (by 2010);
  - safety and efficacy of 4FDC for HIV-positive and HIV-negative TB cases (by 2011);
- evidence for the optimal timing and concomitant use of anti-TB treatment and HAART (by 2013);
- use of rifabutin-containing TB regimen (rifampicin-free regimen) for the management of HIV-infected TB patients failing first-line antiretroviral drugs (by 2013);
- evidence for the utility of bio/surrogate markers and immunomodulation for IRIS and HIV-infected TB (by 2013);
- evidence for a new policy framework for care of HIV-infected TB patients (by 2010).

Issues and challenges for conducting clinical studies for TB/HIV care

- Is the current clinical trial approach relevant to the current needs for appropriate and urgent control of TB and HIV-infected TB cases? Should new guidelines be developed based on safety and efficacy studies or best clinical practice?
- Should study types that are mid-way between classical regulatory trials and academic trials be considered? What levels of monitoring are needed and are the current trial endpoints still relevant?
- Can surrogate markers be sufficiently robust to predict non-relapsing cure in TB trials and pass regulatory criteria?
- Can surrogate markers be used to predict which populations will best respond to specific interventions (e.g. adjunctive immunomodulation) or to complement current case detection approaches?
- Is there a role for immunological markers of host response in predicting IRIS?
WHO/TDR and EC Expert Consultation on Biomarkers
2-3 July, 2008

P Onyejobu, PhD; FRCPP
TB/HIV research coordinator, TDR

Context: Needs and Opportunities

Needs
- Convergence of TB + HIV creates new disease & new Dx & Rx approaches
- Evidence for strategies/programs for optimized care management of TB/HIV
- Improved case detection and DST at “point of care” and optimised case finding
- Improved algorithms for detecting syphilis–TB cases
- Clinical studies to evaluate safety & efficacy of new drug and diagnostic entities
- Shortened clinical trials – surrogate markers (improvements)
- Potential for markers to complement current case detection tools

Opportunities
- ART scale-up, new strategies/tools for TB (Rx, Dx)
- Renewed interest in research for evidence-driven policy
- Step TB Partnership “ dormant ” strategy
- Emergence of WHO-TB (ICN)
- Unprecedented availability of funds for “essential health research”
Epidemiological Context

- 9.2m New TB cases (2006); 14.4M prevalent cases (2006)
- 4.1M New SM+ cases; 0.7M HIV+ TB Cases
- 409,139 MDR TB, representing 4.8% of all new TB cases
  (China, India and Russia have the highest burden)
- Leading Countries by TB burden: India, China, Indonesia,
  South Africa & Nigeria
- 1.5m deaths from TB in HIV- & 0.2 m among HIV+ (2006)
- 45 countries have reported at least one XDR-TB case
- High proportion of XDR-TB among MDR-TB cases (Former
  Soviet Union, Japan and republic of Korea)
- Rwanda and Tanzania: No XDR-TB low levels of MDR-TB

Estimated numbers of new cases, 2006

Estimated HIV prevalence in new TB cases, 2006
The TB/HIV Portfolio

This portfolio focuses on the optimisation of care for HIV-infected TB patients within the context of available health systems capacity, through the development and deployment of evidence-for-policy "in real-time".

The TB/HIV Portfolio (BL 8): Overall Objective

- To optimize treatment, case management and delivery of care for all patients’ populations with tuberculosis and HIV-infected tuberculosis, including patients with additional co-morbid diseases.
Joint TDR/EC expert consultation on biomarkers in tuberculosis • Not to be reproduced or quoted

**TB/HIV Background & Outlook**

- Disease interactions:
  - Increased disease burden
  - Faster progression of both
  - Higher mortality
  - Resistance
  - IRIS

- Drug interactions:
  - More toxic
  - Less effective
  - Resistance
  - IRIS

Research embedded in control programmes
Research capacity

**TDR’s TB/HIV Research Portfolio**

- **Specific Objectives**
  - Develop the evidence for shortening and simplification of TB treatment in TB and HIV-infected TB patient populations (Gatifloxacin & 4FDC studies)
  - Develop the evidence for management of HIV-infected TB patients:
    - optimal timing & concomitant use of anti-TB and antiretroviral drugs (TB-HAART)
    - more effective anti-TB chemotherapy regimen for treatment with HAART (Rifabutin)
    - biomarker markers and ImRx for IRIS and HIV-infected TB
    - strategies for operational implementation of TB and HIV/AIDS case management and Rx in DEDs (OR studies)

**Deliverables**

- **Simpler/shorter TB treatments, including:**
  - Gatifloxacin-containing 4FDC for 4 months Rx (2010)
  - Safety & efficacy of 4FDC for HIV+ve TB (2011)
- **Evidence for the optimal timing and concomitant use of TB treatment and HAART (TB-HAART), (2012)**
- **Use of rifabutin-containing TB regimens (rifampicin-free regimen) for management of HIV-infected TB patients failing 1st line ARVs (2013)**
- **Evidence for the utility of bio/surrogate markers and immunomodulation for IRIS and HIV-infected TB. (2013)**
- **Evidence for new policy framework for care of HIV-infected TB patients (2010)**
Issues and Challenges for the conduct of clinical studies for TB/HIV care

- Are the current clinical trial approach relevant to the current needs for appropriate and urgent control of TB and HIV infected TB cases? (safety and efficacy studies vs best clinical practice for new GLs?)
- Should we be considering study types that are midway between classical regulatory trials and academic trials (what levels of monitoring are needed and are the current trial endpoints still relevant?)
- Can surrogate markers be sufficiently robust to predict non-relapsing cure in TB trials and pass regulatory criteria?
- Can surrogate markers be utilized in predicting populations that will best respond to specific interventions (adjunctive immuneRx) or complement current case detection approaches?
- Is there a role for immunological markers of host response in predicting IRIS?
A4.3. EU perspective on TB research and biomarkers

Dr Ole Olesen and Dr Hannu Lång, Infectious Diseases, Health Directorate, European Commission, Brussels, Belgium

This presentation gives a short overview of the EU Framework Programmes (FPs) as a mechanism for research funding. TB is one of the priority areas in the EC’s health research agenda, and TB biomarkers is a putative future topic in FP7 in the infectious diseases area. From the EU perspective, it is important to understand the current status of different TB biomarkers and to get suggestions for priorities in this field from the scientific community. In addition, this presentation gives some guidance on how to participate in FP7.

The FP is the most important mechanism for research funding in the EU. It is administered by the EC as an multi-year research programme, but with an annually updated work programme that presents a number of open calls for proposals in specific research topics. FP7 runs from 2007 to 2013, and has a total budget for health research of approximately €6.1 billion. While this is a significant amount of funding, it should nevertheless be kept in mind that the FP7 only constitutes about 6% of total public spending on research and development in the EU. Similar to previous FPs, the major part of FP7 is based on support to collaborative research between several research groups from different nations. Research groups from any country in the world can participate in the projects, although it is normally a requirement that at least three partners from three different European countries are included in the consortium behind a project. Research projects are normally funded for a period of 3–5 years, meaning that many projects of FP6 are still running.

FP6

During FP6, the EC invested a total of €458 million into research of the three major poverty-related diseases, HIV/AIDS, malaria and TB. By taking advantage of different funding instruments, the EC was able to support both discovery, translational as well as clinical research projects in these areas, and thereby establish a mixed portfolio of projects across the entire product development pipeline. Approximately €87 million was channelled to discovery-oriented research. These projects are mostly small, high-risk projects with a significant degree of innovation. Individual projects have a typical duration of three years, and receive an EC contribution of €1 million–3 million. These projects are mostly exploring new concepts for drug or vaccine development, or generating information about basic biological mechanisms.

Translational research has been supported by a total contribution of €134 million to large multidisciplinary research consortia that are organized as integrated projects (IPs). Each of the IPs comprises a critical mass of high-level and complementary research groups that can jointly move promising drug and vaccine candidates from discovery phases to early human testing. The average IP comprises 10–20 research groups, and receives an EC contribution of 10 million–15 million during a five-year period.

Clinical research and clinical capacity-building has foremost been supported by the EC through a contribution of €200 million to the European and Developing Countries Clinical Trials Partnership (EDCTP). The EDCTP is an independent organization, based in The Hague in the Netherlands, with the objective of supporting late-stage clinical trials and capacity-building in sub-Saharan Africa.

Out of the total EC contribution to poverty-related diseases, approximately 24% was allocated to research in TB or TB-related activities (while 25% and 51% was allocated to malaria and HIV/AIDS, respectively). This made it possible to support 3 IPs and 16 specific targeted research projects (STREPs), as well as a number of TB activities in the EDCTP. Whereas the STREPs are expected
to increase our knowledge of many specific aspects of TB, the IPs – NM4TB (New Medicines for Tuberculosis), TB-VAC (an integrated project for new vaccines against TB) and MUVAPRED (Mucosal vaccines for poverty-related diseases) – constitute the core of the programme, and are expected to deliver results that are likely to lead to real applications in the near future. These projects typically include research on biomarkers, either embedded in the overall plan of the project, or as a separate work package.

**FP7**

In 2007, FP6 was replaced by FP7. As in FP6, health research is a major theme for collaborative research, with a total budget of more than €6 billion over its seven years’ duration. The objectives of health research under FP7 are to:

- improve the health of European citizens;
- increase competitiveness of European health-related industries and businesses;
- address global health issues, such as antimicrobial resistance, HIV/AIDS, malaria, TB and emerging pandemics.

During the first two years of FP7, approximately €37 million have been committed by the EC to support 10 collaborative projects on TB-related research. These projects have been selected for funding following calls for proposals in research topics such as:

- development of rapid tests for the diagnosis of multidrug-resistant strains of HIV, malaria parasite and Mtb, and of LTBI;
- highly innovative approaches for research into host–pathogen interaction in TB;
- a European network for the study and clinical management of anti-TB drug resistance;
- highly innovative research in HIV/AIDS, malaria and TB between Indian and European partners.

The deadline for the next call for proposals (the third call of FP7) is early December 2008. The topics of this call are focused around a common theme of vaccine research, and are expected to result in the establishment of projects in the following areas:

- identification and preclinical testing of new vaccine candidates for TB
- mucosal and topical vaccines for HIV/AIDS, malaria and/or TB
- translational vaccine research for HIV/AIDS, malaria and/or TB.

Biomarkers for TB could be a topic in subsequent calls for proposals in the Health programme, which is still at an early planning stage. This expert consultation on biomarkers could contribute to supporting and justifying the inclusion of a biomarker topic in the work programme for Health research under FP7. Therefore, the objectives of the meeting from the EC point of view are: to set priorities for the future and to identify the most important research areas. An extensive report of the meeting and recommendations for future actions are essential parts of our future planning activities. The most concrete result from our perspective would be specific call topic examples and suggestions.
EU Perspective on TB Research & Biomarkers

Hanna Lõng, Ole Olsen
Infectious Diseases, Health Directorate
DG Research - European Commission

Geneva, 2-3 July 2006

What's the Framework Programmes?

- The Framework Programmes (FP) are the European Union's multi-annual Research funding mechanism
- FPs are based on collaborative research between several research groups from different nations (3 partners from 3 countries).

Reminder:
The European Commission currently manages about 6% of total public spending on R&D in the European Union.

Funding Instruments of the FPs

- Discovery: Small consortia (1-3 M€, 3-10 partners, 2-3 years)
  - FP6: Approximately 70 projects for PRD
- Translational: Medium/Large consortia, Preclinical research up to early clinical testing (3-12 M€, 10-30 partners, 4-6 years)
  - FP6: 10 projects for PRD
- Clinical Trials: Programme funding to phase II and III clinical trials in Sub-Saharan Africa (200 M€, 5-7 years)
  - FP6: Funding to the EDCTP programme

<table>
<thead>
<tr>
<th></th>
<th>Discovery</th>
<th>Translational</th>
<th>Clinical (incl EDCTP)</th>
<th>Total (%)</th>
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<tbody>
<tr>
<td></td>
<td>87 M€</td>
<td>134 M€</td>
<td>237 M€</td>
<td>458 M€</td>
</tr>
<tr>
<td></td>
<td>(19%)</td>
<td>(29%)</td>
<td>(52%)</td>
<td>(100%)</td>
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Collaborative Research in the Funding Pipeline

Translational Clinical capacity


Distribution of EC funding in Infectious Diseases FP6 (2002-2006)

TB funding: 61 M€
Total EC support to PRD: 258 M€

FP6 TB Projects:

Integrated Projects:
- NMATS: New Medicines for Tuberculosis
- TB-VAC: An Integrated Project for New Vaccines against Tuberculosis
- MUVAPRED: Mucosal Vaccines for Poverty Related Diseases

STREPS:
- IMM-TB: Transcriptionomics
- ImmunoVacTB: Immune systems
- Vaccines4TB: Immune systems
- NewTB-Drugs: Drug Development
- TB-DRUG: Drug Development
- scRN-SILICO: M to macrophage interaction
- MYCOMANCY: Cell cycle and dormancy
- TBMACS: TB-OLIGOCOLOR: Drug Resistance

CSI LB TADAPT: bacterial adaptability
MILD-TB: Latency
FASTEST-TB: Diagnostics
SERO-TB: Diagnostics
TB-4/DNA: Diagnostics

~ € 61 MIL
FP7 (2007-2013)

Indicative breakdown (million €)

Total = € 50.4 billion for 7 years
(40% increase compared to FP6)

Health Research in FP7
(2007-13)

Main policy drivers:
• Improving health of European citizens
• Increasing competitiveness of European health-related industries and businesses
• Addressing global health issues, including emerging epidemics

Budget:
➢ €6.1 billion over 7 years (2007-2013)
➢ ~900 M€/year

Health research in FP7

Pillar 1: Biotechnology, generic tools and medical technologies for human health
Pillar 2: Translating research for human health
Pillar 3: Optimising the delivery of health care to European citizens

Small and Medium-sized Enterprises
Child Health
Health of the Ageing population
International Cooperation
Infectious Diseases in FP7

Translational research in major infectious diseases: to confront major threats to global public health from

- Poverty-related diseases:
  - HIV/AIDS, Malaria, TB
- Anti-microbial resistance
- Emerging infectious diseases:
  - Influenza
  - Emerging diseases
- Neglected Infectious diseases

Topics in TB research from 1st & 2nd calls of FP7

1st call (2007-a):
- Development of fast tests for the diagnosis of Multi-Drug-Resistant strains of HIV, malaria and tuberculosis and of latent tuberculosis infection (LTBI) (Small)
- Highly innovative approaches for research into host-pathogen interaction in tuberculosis (Small)

2nd call (2007-b):
- European network for study and clinical management of TB drug resistance (large-scale project)
- Highly innovative research in HIV/AIDS, malaria, and tuberculosis between Indian and European partners (Small)
- Coordination of European research activities with global initiatives, including Public-Private Partnerships (Coordination or Support action)
- Next generation of researchers for HIV/AIDS, malaria, tuberculosis and neglected infectious diseases (Coordination or Support action)

Total EC commitment to TB so far in FP7: ~€ 37 million (10 projects)

Topics for 3rd and 4th Calls in FP7 Tuberculosis

3rd
- Identification and pre-clinical testing of new vaccine candidates for tuberculosis (Large scale integrating project)
- Mucosal and topical vaccines for poverty related diseases (HIV/AIDS, malaria and/or tuberculosis) (Large scale integrating project)
- Translational vaccine research for poverty related diseases (HIV/AIDS, malaria and/or tuberculosis) (FRP)

4th
- Lead compounds for Tuberculosis Drug Development?
- Biomarkers for TB?
Objectives of the Biomarkers Workshop
- Commission point of view

Priorities
- Identification of most important research areas

Recommendations
- Draft report of the meeting and recommendations for future actions

Publication
- Review article of TB Biomarkers

Suggestions for a Call Topic in FP7
- Examples of possible call topics

Suggestions for a Call Topic
- Example: HEALTH-2008-2.3.2-4: Mucosal and topical vaccines for PRD. Projects should take advantage of the newest available genetic and immunological information to design and develop vaccine candidates against PRD for local application, such as mucosal and/or transcutaneous. Projects should include elements to identify new immunogens and adjuvants with a potential to elicit a prophylactic or therapeutic immune response. Wherever appropriate the inclusion of research groups from industrial partners and disease-endemic areas is highly encouraged to strengthen the research.

Funding scheme: Collaborative Project (Large-scale focused research project with maximum EC contribution between EUR 6 000 000 and EUR 12 000 000).

Information & useful addresses

National Contact Points: www.cordis.europa.eu/health
www.fp7-bio.eu (Biotechnology)

ERC webpage: http://erc.europa.eu

TB research: Hannu Läng: Hannu.Laang@ec.europa.eu

Infectious Diseases unit http://ec.europa.eu/research/health/poverty-diseases/index_en.html

Framework Programme 7: http://cordis.europa.eu/fp7

EDCTP: http://www.edctp.org
A4.4. Review of currently available biomarkers and surrogate end-points in TB and TB/HIV: definition, need and approaches for identifying specific biomarkers

Dr Robert Wallis, PPD, Washington DC, USA

Introduction

The global burdens of HIV and TB are currently estimated at 33 and 14.4 million cases, respectively (slide 2). Cases are concentrated in resource-constrained settings: TB, primarily in Asia (slide 3) and HIV/AIDS primarily in Africa (slide 4). Neither epidemic has been adequately controlled by present strategies. The two pandemics are closely intertwined, with HIV being the most common predisposing factor for TB, and TB the most common presenting illness for AIDS.

Anti-TB therapy presently must be continued long past the time required for resolution of symptoms. Ensuring adherence throughout therapy places a large burden on TB control programmes that hinders TB elimination. Current knowledge for combined treatment of HIV and TB is inadequate. Immune reconstitution, a general goal of antiretroviral therapy, can have deleterious immediate consequences in HIV/TB coinfected people (designated IRIS). Physicians presently lack tools to accurately assess and manage risks and optimize long-term outcomes in dually infected people. Reports in some regions have closely associated HIV coinfection with anti-TB drug resistance (both MDR and XDR). Although some of this risk represents enhanced susceptibility to transmitted resistant TB strains, HIV/AIDS appears to particularly predispose to the emergence of acquired anti-TB drug resistance during intermittent therapy. The interplay of the biological and pharmacological factors responsible for resistance in this circumstance is inadequately understood (Wallis, Weyer & Fourie, 2008). The time required for traditional efficacy end-points is a major challenge to development of new drugs, vaccines and adjunctive immunotherapies. The potential role for new vaccines and immunotherapeutics in the prevention and management of drug-resistant and HIV-associated TB remains largely unexplored.

Biomarkers (biological markers) are measurable characteristics that indicate normal biological or pathogenic processes, or pharmacological responses to a therapeutic intervention (slide 7). In clinical trials they may form the basis of a surrogate end-point that can substitute for a clinical end-point, based on epidemiological, therapeutic, pathophysiological or other scientific evidence. To be valuable as a surrogate end-point, a biomarker should measure an event that is directly involved in pathogenesis or protection and that changes early during treatment (slide 8). Experience with measurement of plasma HIV RNA indicates the potential of surrogate end-points to accelerate research (Holodniy, 2006). However, other research has indicated the ease with which apparently appropriate biomarkers may be dissociated from clinically meaningful events. For example, although ventricular premature contractions occur frequently in people at risk of sudden death due to tachyarrhythmias, suppression of these contractions with flecainide, encaainide, or moracizine was unexpectedly found to increase mortality after myocardial infarction (CAST II investigators, 1992; Echt et al., 1991). This experience indicates that early markers of disease activity may not necessarily be satisfactory predictors of ultimate therapeutic success. The validation of surrogate end-points is therefore critically important for the field to advance. Biomarkers and their corresponding surrogate end-points may be classified in several ways (slide 9). Static assays measure levels of an analyte in a clinical sample, whereas dynamic or functional assays measure a process, such as a response to a stimulus, either in vivo or in vitro.

Some markers are disease specific. Such markers will not be confounded by concomitant illnesses or therapies, and may also serve as diagnostic tests for study entry. End-points may be single measurements, or may be highly multiplexed signatures, such as those from gene expression microarrays. Analytes may be of either host or pathogen origin.
Potential role for TB/HIV biomarkers

Clinical trials have historically used clinically important parameters as endpoints. These variables reflect how a patient feels, functions or survives. In anti-TB treatment trials, endpoints historically have been the sum of failures plus relapses. Because relapses occur in only a small proportion of adequately treated patients, and because they can occur up to two years after completion of therapy, such trials have required large sample sizes and long total durations to ensure adequate enrollment and statistical power. This problem is compounded in the case of TB vaccine trials, where the rate of incident cases even in highly TB-endemic populations is typically less than 1% and development of disease after exposure can take years to become obvious. The urgency of the global TB and HIV epidemics, including the emergence of MDR- and XDR-TB, requires the acceleration of current research. Biomarkers may contribute significantly to this effort.

Clinical needs for biomarkers

The clinical needs for TB biomarkers fall into several areas. From a research perspective, tools are needed to accelerate development of new drugs, immunotherapies and vaccines (slide 6). Such tools might identify or predict a rapid, non-relapsing cure in patients with active TB, assess the risk of reactivation or the effectiveness of treatment in people with latent infection, or identify protective responses to vaccination. Some biomarkers may serve dual roles (i.e. predicting success for both drug therapies and preventive vaccines) or may be useful to show the interactions of combined therapies. Validated biomarkers may ultimately also be used in management of individual patients, for example, to identify those TB patients at low risk of relapse, for whom an ultrashort regimen might be appropriate. Substantially greater predictive accuracy will be required for biomarkers to be used in this fashion. It is a particular challenge facing developers of TB biomarkers that $Mtb$ infection results in multiple bacillary subpopulations with distinct anatomical localizations and metabolic and biosynthetic profiles. Latent infection is thought to be due to a distinct non-replicating population contained in granulomas. Multiple populations can coexist within individual patients. Replicating bacilli are thought to outnumber non-replicating bacilli by several orders of magnitude in patients with cavitary TB. However, the small non-replicating population is thought to give rise to relapses. The ability of biomarkers to distinguish differential drug effects on these two populations will be critical for their success in predicting long-term clinical outcomes.

Table 1 summarizes the available literature on biomarkers that have been studied in TB. Many clinical parameters weakly predict relapse risk and are present at baseline. These include chest radiography (slide 10), baseline sputum CFU count and non-specific inflammatory markers erythrocyte sedimentation rate, CRP and gamma globulin. Currently the marker with which there is greatest experience as a predictor of non-relapsing cure is sputum culture status after two months of anti-TB therapy (Mitchison, 1993; Wallis & Johnson, 2006) (slide 11, Table 2). These data may be examined at three levels. Across studies, a significant relationship exists between two-month culture conversion rate and relapse rate ($R = -0.753, P<0.01$). However, this depends heavily on a single study arm (6SH, i.e. six-months’ treatment with streptomycin and isoniazid) with an atypically high relapse rate (29%), without which no statistical relationship is identified. In practice, however, two-month culture conversion and relapse are compared within individual clinical trials. Figure 1 therefore shows the incremental effect of an additional drug on two-month culture conversion and relapse rates in this context. Symbols indicate distinct trials. All data points but one lie in quadrants 2 or 4, indicating an inverse relationship ($R = -0.764, P = 0.038$ by Spearman rank correlation). Lastly, limited data indicate this marker may also be of value in individual patients. In Study 22 of the Tuberculosis Trials Consortium Study, for example, two-month culture positivity was an independent predictor of relapse, with a hazard ratio of 2.8 (Benator et al. 2002,). However, the marker was relatively insensitive (identifying only half of all relapses) and lacked adequate positive predictive value (18%) for use as a guide to treatment of individual patients. Experience using automated liquid culture systems in this regard is limited, as most studies have focused on solid culture media. Regional differences have also emerged in recent studies of moxifloxacin with regard to the proportion of subjects positive, the extent of difference between cultures on solid and liquid medium, and the effect of the study drug. Further research is required to
determine whether these reflect differences in clinical populations, mycobacterial strains or specific laboratory methods (for example, the use of commercial versus locally prepared solid media).

**Improving sputum microbiology**

Two quantitative approaches have been suggested to improve the prognostic and statistical power of sputum microbiology (slide 11). In the first, the frequency of sampling is increased from once to twice monthly or weekly, with time to sputum culture conversion by Kaplan-Meier analysis as the outcome measure. In the second, sputum CFU counts are measured at weekly intervals during the first month of therapy beginning on day 2, with the rate of decline through day 28 as the outcome measure (Davies et al., 2006; Rustomjee et al., 2008a). The omission of the first two days of treatment removes effects on replicating, rapidly killed bacteria (EBA) that are unrelated to treatment outcome. Experience with this approach is limited but promising, as is experience using time to positivity in automated liquid culture systems (Epstein et al., 1998; Wallis et al., 1998).

**Other microbial markers in sputum**

Several studies have examined other microbial markers in sputum (slide 12). Two studies have examined levels of *Mtb* antigen 85 by ELISA. In the first, the magnitude and duration of increased levels of this protein in sputum during the first week of therapy predicted subsequent relapse in 4 of 42 subjects (Wallis et al., 1998). Induction of antigen 85 occurs due to INH: it does not occur in INH-resistant strains, requires new protein synthesis, and is not due to release of existing protein by dying cells (Garbe, Hibler & Deretic, 1996). In a second trial, induction of antigen 85 by INH in sputum was prevented by concomitant administration of rifampicin and by the higher of two doses of rifalazil (Wallis et al., 2001b). However, induction would not be anticipated in patients not treated with INH or with INH-resistant infections, potentially limiting the application of this marker.

One study has examined antigen 85B RNA (Desjardin et al., 1999), finding that 85B RNA was cleared more rapidly from sputum than viable bacilli. One patient in that study who subsequently relapsed could not be distinguished from others based on his early RNA response. Additional research is needed to determine whether other RNA species, such as those associated with dormancy, might have greater predictive value.

**Urine biomarkers**

There are several pathogen markers that can be measured in urine and interest in this is growing, particularly because of easy non-invasive access of the specimen for studies on children.

**Trans-renal mycobacterial DNA and RNA**

One study has reported the presence of small fragments of *Mtb* IS6110 DNA in urine of 79% (34/43) of patients with pulmonary TB but not in urine of healthy controls (Cannas et al., 2008). The (tr)DNA fragments are thought to arise due to apoptosis of host cells. None of 20 patients positive at diagnosis remained positive after two months of standard therapy. Responses have not yet been evaluated at earlier time points or in relation to sputum culture conversion. The method presently requires nested PCR amplification; assay sensitivity may not be sufficient for *Mtb* strains with low IS6110 copy numbers (Lok et al., 2002). However, the approach may be particularly useful in situations where sputum cannot be readily obtained, such as in children, and could potentially be adapted to detect drug-resistance mutations, thus serving multiple roles in diagnosis and monitoring. The EU FP7 has funded a consortium of African and European countries to investigate the usefulness of (tr)DNA detection for detection of mycobacterial DNA (see Annex 4, section A4.3).

**Mycobacterial LAM in urine**

Detection in urine of mycobacterial LAM and other antigens has been reported in some TB patients and in animals with experimental *Mtb* infection (Boehme et al., 2005; Choudhry & Saxena, 2002; Kashino et al., 2008; Napolitano et al., 2008; Singh et al., 2003; Tessema et al., 2002). No studies have yet examined the clearance of these antigens during treatment or established a relationship to clinical outcomes.
outcome or to another surrogate end-point. However, one study has indicated a correlation of urinary antigen with sputum bacillary burden at diagnosis; this may indicate a potential role as a biomarker.

**Whole blood killing assays**

Several studies have examined the capacity of blood or blood cells to kill intracellular mycobacteria in ex vivo cultures. As originally described, inhibition of intracellular replication of M. microti in mononuclear cell cultures served as an indicator of BCG vaccine effect (Cheng et al., 1988). Recent studies have substituted whole blood for mononuclear cells, and have used alternative readouts (light production by lux-transformed indicator strains, or time to positivity in BACTEC™) (Cheon et al., 2002; Kampmann et al., 2000). Immune control of growth has been shown to be inferior in TST-negative people, enhanced by BCG vaccination or vitamin D administration, impaired by T-cell depletion or HIV infection, and restored by antiretroviral therapy (Cheon et al., 2002; Hoft et al., 2002; Kampmann et al., 2000; Kampmann et al., 2004; Kampmann et al., 2006; Martineau et al., 2007; Saliu et al., 2006; Tena et al., 2003) (slide 19). By using whole blood rather than mononuclear cell culture, the requirement for washing of infected cells to remove extracellular bacteria is eliminated. As a result, concentrations of administered drugs in the cultures mimic those in vivo at the time of phlebotomy. The approach therefore can readily be used to examine pharmacokinetic/pharmacodynamic relationship as well as the combined effects of immunotherapy and chemotherapy on intracellular mycobacteria. One study found that whole blood bactericidal activity during anti-TB therapy correlated with the decline in sputum CFU counts and was superior in two-month sputum culture converters (Wallis et al., 2003) (slide 20). Two studies reported that regimens for drug-sensitive TB were superior to those for MDR-TB, consistent with required treatment durations and outcomes (Janulionis et al., 2004; Wallis et al., 2001a). Antagonism has been demonstrated between some drug and immune effects (Wallis et al., 2004). The whole blood models may be particularly suited to explore the dose–response relationship of second-line anti-TB drugs, as well as the combined effects of drug and immunotherapies for MDR- and XDR-TB in short, early phase II trials, together with assessment of EBA.

**Candidate host immune markers**

Several studies have examined nonspecific markers of immune activation as prognosticators of TB outcome (slides 13–15) (Table 1). These appear most valuable in *Mtb*/HIV coinfected subjects, in whom they predict mortality. Much of this predictive value appears to be related to mortality due to untreated HIV infection. In contrast, TB outcomes seem to be poorly predicted by these markers.

Several studies have examined TB-specific induced responses. The best characterized of these tests are the IGRAs (slide 18). One study of ELISPOT responses to RD antigens by PBMC from British schoolchildren following point source TB exposure, response declined by 68% following LTBI treatment, although only 6 of the 38 children converted to negative. Responses declined in 7 of 14 children with borderline TST and positive ELISPOT who did not receive INH (Ewer et al., 2006). In one study of patients with active TB, ELISPOT responses to RD1 antigens declined from baseline to three months in all of 13 patients with an adequate clinical response, but remained elevated in five treatment failures (Carrara et al., 2004). However, given that responses in some treated patients using ELISA may apparently be maintained for decades after the conclusion of therapy (Wu-Hsieh et al., 2001), these results must be confirmed in prospective trials. Similarly, two longitudinal studies in untreated contacts suggest that the highest responders are at greatly elevated risk of TB (Diel et al., 2008; Doherty et al., 2002). Studies to address the questions of remote versus recent infection, predictive value and optimization of the assays should prove very valuable.

**New technical platform for biomarker studies**

Finally, a small number of studies suggest that specificity and higher predictive value could be achieved for otherwise non-specific tests by measuring multiple parameters (slide 16). The advanced technological platforms using transcriptomics, proteomics and metabolomics now bring a new dimension to biomarkers research. Agranoff et al. (2006) found that TB could be differentiated from other infectious and inflammatory conditions based on proteomic fingerprinting study of serum by SELDI-ToF mass
spectrometry. Serum amyloid A and transthyretin were among the candidate biomarkers. The study was limited, however, by the relative insensitivity of the techniques used. Similarly, a recent report using DNA microarrays identified signatures involving expression profiles of nine genes in blood cells that could distinguish active TB, LTBI, cure, and TB recurrence (Mistry, 2007) (slide 17). TB patients were evaluated in this study at the time of diagnosis. Taking a complementary approach, an ongoing study by Willem Hanekom and colleagues in BCG-vaccinated infants in South Africa’s Western Cape has used multiplex ELISA and DNA microarray to identify profiles associated with resistance to Mtb infection after BCG vaccination (W. Hanekom, personal communication, 2008). Further research is needed to determine whether these findings can be reproduced in other clinical populations, and to verify the suggestions by these reports that recurrent TB (whether due to relapse or to reinfection) in treated patients or protection induced by vaccination can be predicted by gene expression profiles.

**Recommendations**

1. Research is urgently needed to further evaluate and validate candidate TB and TB/HIV biomarkers in adults and children with and without HIV infection. These should now include the use of the new technological platforms of transcriptomics, proteomics and metabolomics. The development plan for these biomarkers should provide for small initial studies, which should increase progressively in size and duration as occurs in clinical testing of new drugs. The studies should be conducted to the clinical and laboratory standards that are necessary for licensing of new drugs and diagnostic tests. It is recommended that culture-based biomarker development emphasize the use of automated liquid culture systems, since there is increasing recognition of the growing clinical role of these systems worldwide. Surrogate end-points developed using these methods would therefore be most readily incorporated into new standards of care. Similarly, resistance testing, using line probe or conventional assays, will be required to determine drug susceptibility on entry and throughout study participation. Such tests will be important in differentiating primary from secondary (acquired) resistance, and in uncovering mixed infections. Mycobacterial speciation will also be essential, as, with the increasing use of liquid culture, mixed cultures with non-tuberculous mycobacteria are increasingly recognized as a potential diagnostic confounder.

2. For some markers of treatment efficacy, initial studies may be required that are short (e.g. one-week drug exposure) and small (15 subjects per arm). Such studies may assess, for example, the dose–response relationship to second-line anti-TB drugs, using quantitative sputum microbiology (EBA), whole blood bactericidal activity, and pharmacokinetic sampling. These exploratory studies could be conducted in patients with drug-sensitive TB, at sites familiar with methods for quantitative sputum microbiology. It should be assumed that this brief duration of treatment will not be sufficient to detect host immune biomarker responses, unless specific data suggest otherwise. Parallel studies of the pharmacokinetic and whole blood bactericidal activity of selected interventions in healthy volunteers will permit the analysis of immune/drug interactions, and may identify new candidate interventions for subsequent study in TB patients.

3. The equivalent of phase II trials for markers relevant to treatment monitoring may be best conducted in patients with MDR- or XDR-TB. The efficiency of clinical research is enhanced in such patients, due to the higher proportion of subjects reaching clinical end-points of treatment failure or relapse. The wide potential range of resistance patterns represents both a challenge and an opportunity. Some larger phase II trials will require stratification based on the number drugs to which Mtb is resistant and possibly on the presence of resistance to key drugs, such as the fluoroquinolones or other injectable anti-TB drugs. Smaller phase II trials will be possible with regimens consisting of single drugs or of limited combinations of drugs and adjunctive immunotherapies. Such trials will probably be considered ethical if other treatment options do not exist and if the study is likely to increase medical knowledge of importance to other patients. Site selection for these trials will be dependent on the availability of appropriate patient populations, laboratory facilities (including appropriate biosafety containment) and the
necessary clinical and laboratory expertise. These trials must be of sufficient duration to collect data regarding treatment success and relapse (i.e. a minimum of one-year follow-up after 12–18 months’ treatment). Pathogen-based and host immune markers should both be evaluated. Biomarkers based on quantitative sputum microbiology should be performed using both quantitative CFU and time to detection in automated liquid culture systems. The study sizes will be sufficiently small that both static and functional biomarkers should be included.

4. Phase III treatment trials may be best conducted in patients with drug-sensitive TB. Such studies must be of sufficient size to collect adequate numbers of TB recurrence. Initial and recurrent strains must be tested by RFLP or other methods to distinguish reinfection from true relapse. Because only a small number of patients will reach the end-point of relapse, biomarkers that can be evaluated retrospectively in a case–control substudy using banked specimens will be favoured. Alternatively, markers that will be routinely evaluated in the course of usual patient care (or with minor modifications) may also be appropriate. At least two studies, conducted in Africa and either Asia or South America will be required. Studies in all three regions may be particularly helpful to resolve the questions that have arisen in the course of the moxifloxacin studies described earlier. Opportunities may arise for the coordination of these studies with ongoing or planned drug trials intended to test strategies for shortening anti-TB treatment. Such coordination would decrease costs and enhance study value. Alternatively, it may be possible to conduct phase III biomarker trials under programme conditions. In this case it will be essential that additional support be provided to ensure adequate follow-up after the usual programme end-point of completion of therapy. A minimum of one additional year of follow-up will be required, making the likely total study duration three years. Collection of serum samples at regular intervals for the new platform studies of transcriptomics, proteomics, and metabolomics will be essential.

5. The equivalent of phase IV trials may be considered in which a biomarker is used to modify patient treatment under programme conditions. The design and implementation of such studies will be highly dependent on the outcomes of earlier phase studies.
TB Biomarkers
Needs and approaches

RS Wallis, MD, FIDSA
Medical Director, PPD

Global disease burdens

Estimated number of new TB cases, 2006
Clinical needs

- Detection
- Management
- Prevention

Clinical needs - biomarkers

- Detection
- Management
  - Early identification of suboptimal Rx response
  - Accelerate development of new drugs and immunotherapies
- Prevention
  - Assessment of TB risk
  - Assessment of TB protection
  - Accelerate development of new vaccines
### NIH working group definitions

- **Clinical endpoint**
  - A characteristic or variable that reflects how a patient feels, functions, or survives.
- **Biomarker (biological marker)**
  - A measurable characteristic that is an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention.
- **Surrogate endpoint**
  - A biomarker that is intended to substitute for a clinical endpoint based on epidemiological, therapeutic, pathophysiologic, or other scientific evidence.

### Desirable characteristics

- Involved in pathogenesis or protection
- Surrogacy should be independent of type of treatment (i.e., drug class)
- Validation is key - lesson of antiarrhythmics
  - PVCs are associated with ventricular tachycardia and sudden death
  - Flecainide, encaïnide, moricizine prevent PVCs after MI yet increase the risk of death

### Biomarker classifications

- Static vs. dynamic (functional)
- Specific vs. non-disease specific
- Single vs. multiplex
- Pathogen vs. host analyte(s)
- Patient management vs. clinical drug or vaccine development
  - Early vs. later stage clinical trials
### Baseline chest radiography

<table>
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<th>Association</th>
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<td>Recurrence</td>
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<td>Mallory, 2000</td>
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<td>Relapse</td>
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<td>Relapse</td>
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<td>Nettles, 2004</td>
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<tr>
<td>Recurrence</td>
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### Serial sputum microbiology

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<td>M2 SCC</td>
<td>Recurrence, 3X risk</td>
<td>many</td>
<td>Mitchison, 1993</td>
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<tr>
<td>Serial sputum CFU slope</td>
<td>Superior sterilizing activity</td>
<td>few</td>
<td>Rustomjee, 2008, Davies, 2006</td>
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<td>EBA</td>
<td>none</td>
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### TB-specific biomarkers

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<td>Sputum 85B RNA</td>
<td>Treatment response</td>
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<td>Desjardin, 1999</td>
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<td>Sputum Ag 85</td>
<td>Treatment response</td>
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<td>Wallis, 1996,99</td>
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<td>Drug evaluation</td>
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<td>Wallis, 2001</td>
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<td>Urine TB trDNA</td>
<td>Treatment response</td>
<td>20</td>
<td>Cannas, 2008</td>
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<td>Anti-ESAT-6, 38kD, alanine dehydrogenase, malate synthetase</td>
<td>Extent of disease, treatment response</td>
<td>168</td>
<td>Azzuri, 2006</td>
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<td>RD1 ELISPOT</td>
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### Nonspecific biomarkers immune activation

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<td>NKT cells at dx</td>
<td>M2 SCC</td>
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<td>Veenstra, 2006</td>
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<td>Sputum IFNγ</td>
<td>Treatment response</td>
<td>15</td>
<td>Ribeiro-Rodriguez, 2002</td>
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<td>sIL-2R</td>
<td>Treatment response</td>
<td>44</td>
<td>Chan, 1995</td>
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<td>Granzyme B, sTNFR, at dx</td>
<td>M2 SCC</td>
<td>18/36</td>
<td>Brahmbhatt, 2006</td>
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### Nonspecific biomarkers immune activation (cont’d)

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<td>CRP</td>
<td>Treatment response, death</td>
<td>105 100 18</td>
<td>Lawn, 2001 Bajaj, 1989 Scott, 1990</td>
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<td>sICAM-1</td>
<td>Treatment response</td>
<td>30</td>
<td>Demir, 2002</td>
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<td>suPAR</td>
<td>Death</td>
<td>101</td>
<td>Eugen-Olsen, 2002</td>
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### Representative studies

![Graphs of CRP and sTNFR2](image-url)
### Highly multiplexed assays

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<tr>
<td>Transcriptomics</td>
<td>Recurrence Active vs. latent</td>
<td>10/40 ?</td>
<td>Mistry, 2007 O’Garra, unpublished</td>
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<td>Proteomics</td>
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<td>Metabolomics</td>
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### Gene expression profiles

![Gene expression profiles]

### LTBI TB risk

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<tr>
<td>QFN Gold</td>
<td>TB risk</td>
<td>39/111 TB contacts with abnormal CT</td>
<td>Higuchi, 2008</td>
</tr>
<tr>
<td>T-spot TB</td>
<td>LTBI treatment response</td>
<td>85/226</td>
<td>Chee, 2007</td>
</tr>
<tr>
<td>E6/C10 skin test</td>
<td>TB risk</td>
<td>BCG vs naïve guinea pigs or mice</td>
<td>Weldingh, 2008</td>
</tr>
<tr>
<td>TST</td>
<td>TB risk</td>
<td>Large vs small reaction, 10X TB risk</td>
<td>Edwards, 1973</td>
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### TB protection functional assays

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<th>Candidate</th>
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<tr>
<td>ELISpot</td>
<td>Vaccine effect</td>
<td>many</td>
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<td>Whole blood killing</td>
<td>TST effect</td>
<td>12</td>
<td>Kampmann, Cheon, 2002</td>
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<td></td>
<td>BCG effect</td>
<td>10 (10) 50</td>
<td>Cheon, 2002</td>
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<td></td>
<td>AIDS effect</td>
<td>22</td>
<td>Tena, 2003</td>
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<td></td>
<td>cART effect</td>
<td>15</td>
<td>Kampmann, 2006</td>
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<td>TNF mAb effect</td>
<td>20</td>
<td>Saliu, 2006</td>
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<td></td>
<td>Vit D effect</td>
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### TB treatment functional assays

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<tr>
<td>Whole blood killing</td>
<td>Treatment effect</td>
<td>8 drug regimens</td>
<td>Wallis, 2001</td>
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<td></td>
<td>Correlation with serial CFU slope and 2M SCC</td>
<td>36</td>
<td>Wallis, 2003</td>
</tr>
<tr>
<td></td>
<td>Treatment effect</td>
<td>10</td>
<td>Janulionis, 2004</td>
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### GSK Action TB

- Large cohort
- Standard therapy
- Close follow-up
- Microbiologically defined endpoints
A4.5. Immune markers in TB: results from the EU FP6-funded VACSIS studies

Professor Mark Doherty, Statens Serum Institute, Copenhagen, Denmark

Introduction

TB was one of the first diseases for which the causative agent was identified, and a vaccine and antibiotics are widely available. Yet it remains one of the great killers, causing 2–3 million deaths and an estimated 8–10 million new cases a year (Doherty & Andersen, 2005). Although the disease is currently endemic only in developing countries, epidemics in industrialized countries are only prevented by ceaseless monitoring and treatment. Thus, by virtue of its very high mortality and morbidity, Mtb inflicts substantial economic losses across the globe (WHO, 2008). The reasons for this are manifold, but the most serious is the fact that reliable correlates of protection against Mtb have not yet been identified, greatly hindering vaccine development. It has been demonstrated in animal models that IFN-γ and TNF-α are essential for protection and this is supported by the susceptibility of humans with genetic defects in these pathways and by the accidental reactivation of LTBI in humans who received anti-TNF-α treatment as therapy for rheumatoid arthritis (Winthrop, 2006). However, vaccine studies in animals also strongly suggest that although IFN-γ is essential, production of this cytokine alone is not a good predictor of vaccine efficacy (Agger & Andersen, 2001; Mittrucker et al., 2007; Wedlock et al., 2007). The use of a proxy marker is thus a very active field of research.

Current consensus

There is now general recognition that measuring single cytokines – even when they clearly play a significant role – gives a very limited picture of the ongoing immune response. As a result, the technology that has so far primarily been used for monitoring vaccine studies (IFN-γ ELISA and ELISPOT) while sufficient for monitoring immunogenicity, tells us little or nothing about efficacy (Agger & Andersen, 2001; Mittrucker et al., 2007; Wedlock et al., 2007). It has been hypothesized that one reason for this is that the nature and specificity of the cytokine-producing cells is at least as important as the total number of cytokine-producing cells or the magnitude of the response. Several recent studies support this. For example, as shown in Figure A4.1, we can deliberately induce large numbers of IFN-γ-producing T cells that offer no detectable protection – or alternatively, we can induce a similar number of IFN-γ-producing T cells – and get more than 90% reduction in Mtb numbers.

These data suggest that we need to look at the source(s) of the immune response(s) being measured. This is not a particularly radical idea: it has been suggested before. The data in Figure A4.1 – using ELISPOT and ELISA (Bennekov et al., 2006) – suggest that the presence of a subset of the responding cells seems to be best associated with vaccine efficacy. Recent studies using multiparameter flow cytometry (MPFACS) in another animal vaccination model support this hypothesis and suggest that the high-yield cells are, in fact, multifunctional T cells producing IFN-γ, TNF-α and IL-2 (Darrah et al., 2007). It appears that certain subsets of IFN-γ-producing CD8+ cells may also be important for a protective immune response (Mittrucker et al., 2007). Both of these studies relied on the use of MPFACS for analysis. Studies carried out by the VACSIS consortium using quantitative RT-PCR show that patients with acute TB alter their cytokine production over the course of infection to one characterized not so much by increased IFN-γ but by an improved ratio of IFN-γ to IL-4, while TB contacts who develop signs of early TB show the opposite pattern (Figure A4.2, 2C and 2D).
These results again emphasize the importance of examining individual cytokine markers with caution and the benefit of measuring them in context. It also highlights the limitations of the current technology – while RT-PCR can look at multiple cytokines simultaneously, it is not well suited to examination of antigen-specific responses, nor do these experiments tell us anything about the phenotypes of the responding cells. The phenotype also includes the cells’ antigen specificity. Just as the balance between different cytokines alters over time (Figure A4.2, 2A and 2B) we (and others) have shown that the response to specific antigens from \textit{Mtb} typically differs between infected individuals who control the infection (and develop LTBI) and those who develop acute disease (Demissie et al., 2006a; Hougardy et al., 2007).


Although similar numbers of antigen reactive interferon (IFN)-γ-producing cells are induced (spot-forming units or SFU), those on the left are primarily CD8+ and produce only small amounts of IFN-γ per cell. Those shown on the right have a greater percentage of CD4+ cells and a clearly visible subpopulation (of unknown phenotype) that produce very high levels of IFN-γ. It is the presence of these cells that correlates best with protection.
FIGURE A4.2. Cytokine mRNA expression in TB patients and in contacts developing TB-like symptoms


Quantitative PCR of messenger RNA (mRNA) at entry to the study and nine months later, from unstimulated peripheral blood mononuclear cells of TB patients (A, B) and their household contacts, who were healthy at entry, but subsequently developed TB or TB-like symptoms. A and C show raw cytokine mRNA expression, B and D show the ratio of message for interferon (IFN)-γ to interleukin (IL)-4 or IL-4Δ2 to IL-4.

Currently, almost nothing is known about this process, neither the kinetics nor the cell types involved, for here we are coming up against the limits of our current technology. MPFACS analysis should enable us to begin to identify the phenotype of antigen-specific T-cell subsets and their contribution to the overall immune response by stimulating cells from patient samples with different antigens and measuring multiple cytokines and identifying the cells that produce them simultaneously. The utility of this approach to vaccination studies – especially with antigens such as antigen 85A or antigen 85B, where an immune response may exist prior to vaccination (McShane et al., 2004) – is obvious.
In total, despite the limitations of the technology, the data suggest that it is possible to identify cytokine profiles that correlate with protection better than IFN-γ alone, and that these responses can be best understood in the context of the cell types involved, their antigen specificity and the relation of their cytokine production to other cytokines. There are multiple technologies available for resolving these questions, each with specific advantages/limitations:

- quantitative PCR, preferably multiplex or mini-array assays, which have the advantage that they generate a great deal of information from a very limited number of cells – always an issue in clinical studies where sample volume is limited;
- MPFACS, which has the advantage that it identifies the cell types and antigen specificity that produce the markers of interest;
- multiplex ELISA assays, which have the advantage that they can assay antigen reactivity more readily and less expensively than MPFACS.

These technologies are only as good as the samples they are used to analyse and the quality of the analysis, however, so the basic requirement on which these technologies must rest is the careful clinical definition of study cohorts and robust multivariate statistical analyses. Combined, these should allow us to more effectively assess the response to vaccination in humans and offer in addition the possibility of better identifying correlates of immunity.
Joint TDR/EC expert consultation on biomarkers in tuberculosis

**VACSIS/VACSEL Study Sites**

- AHRI, Addis Ababa
- Regional hospital, Hosanna
- Regional hospital, Butajira

**Recruitment in Ethiopia**

- **Community Control (CC, n=40)**
  - smear/culture negative
  - X-ray normal
  - Asymptomatic
  - No known TB contact

- **Index cases (TB, n=76)**
  - smear/culture positive
  - X-ray confirmed
  - Symptomatic

- **Household Contact (HC, n=234)**
  - no diagnosis of TB

**Methods**

Flowchart showing the process of blood and plasma handling:
- PBMC
- Frozen in liquid N₂
- Thawed
- Culture with specific antigens
- Culture supernatant
- IFN-γ ELISA
- IFN-γ ELISPOT
- Multicolor FACS
Joint TDR/EC expert consultation on biomarkers in tuberculosis

Differential cytokine expression in clinical cohorts

Healthy but infected individuals give us our best definition of a protective immune response.

But identifying them has been a stumbling block.

IFN-γ to ESAT-6 (but not PPD) differentiates infected from uninfected healthy individuals both from DK and ET.

Identifying latent infection in CC

Recruitment in Ethiopia

Community Control (CC, n=40)
- smear/culture negative
- X-ray normal
- Asymptomatic
- No known TB contact

True Control (TC, n=24)
- smear/culture negative
- X-ray normal
- Asymptomatic
- No known TB contact, ESAT-6 negative

Index cases (TB, n=76)
- smear/culture positive
- X-ray confirmed
- Symptomatic

Latently infected (LTBI, n=16)
- smear/culture negative
- X-ray normal
- Asymptomatic
- No known TB contact, ESAT-6 positive

Household Contact (HC, n=234)
- no diagnosis of TB
Joint TDR/EC expert consultation on biomarkers in tuberculosis • Not to be reproduced or quoted

Differential cytokine expression in CC

Conclusions

Careful definition of clinical cohorts is essential to interpreting your results.

So, what happens to TB patients who are treated?
Or to people who self-cure?
Do they end up looking like LTBI?

Changing cytokine expression in TB over time
Recruitment in Ethiopia

- **Community Control (CC, n=40)**
  - smear/culture negative
  - X-ray normal
  - Asymptomatic
  - No known TB contact

- **True Control (TC, n=24)**
  - smear/culture negative
  - X-ray normal
  - Asymptomatic
  - No known TB contact, ESAT-6 negative

- **Latently infected (LTBI, n=16)**
  - smear/culture negative
  - X-ray normal
  - Asymptomatic

- **Healthy Household Contact (HHC, n=150)**
  - smear/culture negative
  - X-ray normal
  - Asymptomatic

- **Symptomatic Household Contact (XHC, n=84)**
  - smear/culture negative
  - X-ray often, but not always, suspicious
  - Symptomatic

---

**Cytokines in HC changing from symptomatic to asymptomatic**

- IFN-γ and IL-4 all increased in those who became asymptomatic while IL-4 levels (high to start with) did not change.

- There was an increase in the IL-4/IL-10 ratio associated with "self-cure" or spontaneous remission of symptoms.

**Cytokines in HC changing from asymptomatic to symptomatic**

- IFN-γ, IL-4, and IL-10 all increased in those who became symptomatic though only the increase in IL-4 was significant.

- There was a decrease in the IL-4/IL-10 and IFN-γ/IL-4 ratios, associated with development of TB-like symptoms.
Conclusions II

Rather than a classical Th1 to Th2 switch, infection with MTB appears to stimulate IL-4 and IFN-γ.

In all cases, protection is correlated with an increase in IFN-γ and/or IL-4 relative to IL-4 and disease with a decrease in these ratios.

All of this says that IL-4 is important. What then, is its role?

TNF-α and the apoptotic pathway

IL-4 directly counteracts the induction of the TNF-α pathway by mycobacteria
Apoptotic gene expression in CD14+ PBMC

Apoptotic gene expression in CD14- PBMC

TNF receptor is shed at high levels in TB patients

The low expression of TNF receptors on monocytc cells may inhibit clearance of infected cells by apoptosis and may be exacerbated by the shedding of soluble TNF receptor.
Conclusions III

What we are seeing in TB is not a Th1 to Th2 shift but something more insidious: immune deviation driven by IL-4 (and probably other agents), promoting necrosis and bacterial release.

These data expand earlier mouse studies indicating that the combination of TNF-α and IL-4 promotes pathology and provide a mechanism for animal and human studies suggesting that a high ratio of IL-4 to IL-4Rα is associated with pathology, rather than infection.

Proteins involved in this process may prove to be useful biomarkers which have barely been looked at.

Acknowledgements

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A4.6. Human correlates and biomarkers of TB: the TB Vaccine Consortium (TBVAC) experience and outlook

Professor Tom Ottenhoff, Leiden University Medical Centre, the Netherlands

The TBVAC Biomarker Group has identified and prioritized a set of promising markers, which need further validation. The Group has proposed to move the most promising findings and assays from these efforts forward into assay development and validation, complemented by discovery of new biomarkers to fill the pipeline.

We think it is important to focus on assays that measure the functional capacity of human cells in the innate and adaptive immune response to Mtb, including Mtb killing assays, polyfunctional and memory T-cell subsets, innate immune responses (macrophage/dendritic cell responses, granuloma models and other relevant models). Activities need to be integrated with other international activities, including those of WHO, Bill & Melinda Gates Foundation, the Stop TB Partnership, Aeras Global TB Vaccine Foundation, etc. Future biomarker discovery and validation will need to include studies in larger cohorts at relevant TB-endemic sites, in both Africa and Asia.

Organization

Activities thus far have been organized by the Group leadership into two parallel tracks.

Track 1
- Translation of already identified candidate biomarkers into assays.
- Optimization, harmonization and validation of assays.
- Development of new promising assays.

Track 2
- Discovery of new TB biomarkers and signatures of protection.

Track 2 candidates could move to track 1 once validated in small cohorts of at least 20 TB patients, 20 BCG vaccinees and 20 healthy controls. Structuring TB biomarker activities along these two tracks has been useful in prioritizing and selecting or discontinuing potential markers for further evaluation.

Overview of some TBVAC1 Work Package 4 biomarkers and assays, and future plans

Track 1

Markers of protection
- Whole blood killing assay for purified protein derivative (PPD)-induced IFN-γ following BCG vaccination.
- O-4-nitrobenzylhydroxylamine (nHBHA)-induced IFN-γ secretion by PBMCs.
- Gene expression pattern that allows discrimination between LTBI and TB disease, e.g. Rab33a expression (assay development in progress).
- Granulysin serum levels increased during TB cure.
- Mono mycolate glycerol-specific T cells releasing IFN-γ in latently infected individuals (not TB patients).
- High Mtb-specific IFN-γ/IL-10 ratio during TB cure.
- High IL-4/IL-44α ratio (identified outside TBVAC, but considered an important candidate).
- MIG (CXCL9, monokine induced by IFN-γ) as a new biomarker that can assess IFN-γ-induced
downstream responses in TB (rather than assessing only IFN-γ production).

- Focused antigen discovery (to be included):
  - phase-specific *Mtb* gene expression patterns and immune recognition
  - identification of the *Mtb* peptidome expressed on *Mtb*-infected human cells.

**Markers of disease**

- Local nHBHA/ESAT-6-CFP-10-specific IFN-γ-producing T cells.
- IL-4-producing T cell receptor (TCR)γδ cells and CD8+ T cells increased in TB patients.
- Chemokine receptor (CXCR) 5 + inducible costimulator (ICOS) + CD40 ligand (CD40L) + Temra Vγ9Vδ2 T cells increased in TB patients.
- Reduced frequency of antigen-specific effector T cells and Temra in TB patients.

**Immune assays**

- ELISPOT assays harmonized and protocols optimized (standard operating procedures). Earlier versions of these protocols were successfully used in immunological monitoring of the *Mtb* 72F and HYB1 trials in Lausanne, Switzerland, and Leiden, the Netherlands, respectively (manuscripts submitted).
- Six-day lymphocyte stimulation test for immune profiling (memory and functions).

**Immune assays to be developed with high priority**

**Intracellular cytokine staining: multicolour flow cytometry panels (including multiple cytokines)**

Optimization and testing of multicolour panels is extremely important. When validated, these assays have a very low false-positive rate, a very low limit of detection, and high sensitivity, reproducibility and linearity, making it suitable for qualitative and quantitative analysis of cellular immune responses in clinical trials of candidate vaccines in selected settings. The quantitative and qualitative data produced from these assays should enable more in-depth characterization of vaccine-induced T-cell responses, and aid in determining how cellular immunity contributes to vaccine efficacy.

**Mtb killing assays**

The functional capacity to inhibit bacterial outgrowth (CFU) or viability are important measures of the (in vitro) capacity of immune cells to control bacterial survival. However, many of these assays are not yet robust, which is why the Stop TB Partnership is initiating international efforts to improve the various assays around. Future biomarker projects we hope will use the assays recommended by the Stop TB Partnership’s working group. Robust *Mtb* killing assays could have an important impact on the identification of surrogate markers of protection.

**Track 2**

Markers of protection

- *Mtb*-specific CD8+ T cells identified by HLA tetramers for different epitopes.
- Multifunctional *Mtb*-reactive T cells identified.
- PPD-induced IL-17, IP-10, following vaccination.
- New Th17 markers identified (CCR6, IL-22; other marker profile combinations by intracellular

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1 CFP, culture filtrate protein.
staining).
- *Mtb* lipid-specific T cells have antimycobacterial effector activity.
- *Mtb* antigen-induced granulysin production from cells.
- Ratio of latency antigen/ESAT-6 specific T cells.
- TCRγδ T cells have adjuvant activity.

**Markers of disease**
- DC-SIGN expression on alveolar myofibroblasts discriminates between TB patients.
- CD64 expression and lactoferrin expression.
- Regulatory T cells among PBMC/local lymphocytes (e.g. BCG-induced CD8+ regulatory T cells suppress via CCL4; HBHA induces CD4+ regulatory T cells).

**Immune assays**
- List of genes and sets of probes for multiplex expression profiling (multiplex ligation-dependent probe amplification).
- Large sets of new genome-wide expression data from human myofibroblasts and dendritic cells in TB.

**Important goals for future TB biomarker research (discovery, translational and clinical)**
1. To identify novel biomarkers or biomarker signatures that are associated with protection against TB disease, and/or markers associated with (early) disease activity and lack of protection/susceptibility.
2. To prioritize and validate current biomarkers as well as new markers from point 1 above in small cohorts (TB patients, BCG vaccinees, LTBI and healthy controls).
3. To adapt current biomarkers to assays that are simple, robust and reproducible and can preferably be used in TB-endemic settings.
4. To harmonize, standardize and apply assays for clinical trial monitoring.
5. To advise on the selection of tailor-made sets of biomarkers for clinical trial monitoring based on vaccine type, antigen expression and trial setting. Decision-making matrices for prioritization of tailor-made biomarker sets can be developed for different vaccine- and drug-interventional or observational (longitudinal) studies.
Surrogate endpoints (correlates): a European (TBVAC) experience

by

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Department of Infectious Diseases
Leiden University Medical Centre
Leiden, Netherlands

Correlates can demonstrate immunogenicity and identify the most effective vaccine antigens, vaccine formulations and strategies (dose, vehicle, adjuvant, immunisation schedule etc) in an early stage.

Correlates will impact on the time and resources needed for evaluation of (long and costly) efficacy trials (vaccines, drugs, ...).

Correlates can identify essential pathways and mechanisms of protection or disease in human infectious disease -- better tools to combat disease.

Surrogate endpoints must reasonably likely predict clinical endpoint or long term benefit

Only a subset of biomarkers are surrogate endpoints.
Limitations of biomarkers

- Biomarkers need to be evaluated and refined continuously, and ultimately must be “validated” in relation to clinical efficiency / benefit.

- Prematurely accepted biomarkers may:
  - Fail to show true benefit of intervention (insufficient sensitivity & specificity)
  - Erroneously suggest benefit of intervention (false positive)

- In complex diseases (autoimmunity, cancer, chronic infections), single biomarkers may capture only a portion of intervention’s effects

  ⇒ Need for comprehensive and multiplex profiles with enhanced sensitivity (and specificity)

Biomarkers in TB: measuring what and in whom?

- Exposure → Infection → Progressive infection → Active disease
- Infection → Latent infection → Nativly protected 80%
- Infection → Reactivation (5-10%)
- Infection → Developing severe disease
- Infection → Developing early disease
- Infection → Developing late disease (reactivation)

Markers or correlates of protection from:
- Infection
- Developing latent infection
- Developing early disease
- Developing late disease (reactivation)
- Developing severe disease
- Disease activity
- Protection from TB
- Prox. cure & protection from relapse

Objectives TBVAC “Correlates” WP(4)

I. To identify and test biomarkers of
   - Protection
   - Disease / pathology in human tuberculosis

   For monitoring vaccine-induced immunity and protection

II. To compare, evaluate, optimise and harmonise assays for immunological monitoring;
    - To help implementing these as well as to develop new assays (based on I) for monitoring of vaccine-induced immunity & memory in clinical trials
**Correlate WP: interactions outside TBVAC**

- **TBVAC**
- **Correlate-WP**
- **FP6 vaccine consortia assays**
- **BMGF GC6.12 Biomarkers.Ag**
- **Aeras Biomarkers.Ag assays**
- **WHO Biomarkers assays**
- **STOP-TB immunisation.dgs and HIV killing assays**
- **MUVAPEPRED**
- **NIH Biomarkers.Ag assays**
- **Others: RDCT, NACCAP / AFEEROL**

**Human correlates in TBVAC**

- **Track 1:** biomarker characterization and validation
  - Priority
  - Validates biomarkers identified in TBVAC1 and focuses on testing, comparing and prioritising markers and assays in humans
  - Develops and refines assays

- **Track 2:** new biomarker discovery track
  - Identifies new biomarkers by genome-wide expression profiling and various innovative molecular approaches -> fills pipeline with new candidates

**A major activity in the TBVAC1 correlates-WP has been assay development, optimization and harmonization**

- Newly implemented in TBVAC1 WP4:
  - Antigen batch standardization for testing in comparative fashion
  - HLA tetramers produced and distributed
  - Assay protocol standardization (using NIBSC rIFNγ standards):
    - **WB4:** robust protocol (GC6)
    - **PBMC:** 6d LST assay robust protocol
    - **ELISPOT:** converged on basic protocols after extensive discussion, testing, optimisation and harmonisation (incl. off-A tuning, AIM-V,...)
    - **ICS:** still exploratory (track #2), but high potential
      - Interactions with GOs, Aeras, HIV consortia
  - Divided tasks for head-to-head comparison of at least two assays per lab, using reference standards
  - Consensus gene list drafted for multiplex assays (MLPA, arrays, other)
Example 2: LUMC & ULB

In TST+ donors,
- 6 d LST strongly correlates with TST
- QFT strongly correlates with direct Elispot
- 6d LST correlates well with short term cultured Elispot
- long term assays correlate and short term assays correlate, but long and short assays only moderately correlate

Short and long-term assays thus measure different aspects of the immune response (T_{low} vs. T_{high}). Short term assays can detect recent but often not more remote infection.

⇒ Relying only on QFT or direct Elispot may miss latent infection!

Biomarkers of protection, immunity or disease in TBVAC1. Status track #1, to be built upon in TBVAC2 and TBV1

Protection:
• WBA FPD induced IFNγ following BCG vaccination
• rHHA induced IFNγ responses by IFNγ-γ
• gene expression pattern that allows discrimination between latent Mtb infection and TB disease, e.g. Th1/Th2 expression
• assay development in progress
• granuloma/skin levels increase during TB-tube
• tumor necrosis factor specific T cells releasing IFNγ in latent infected individuals (not TB patients)
• high Mtb-specific IFNγ/IL-10 ratio
• high IL16/CIL ratio discovered outside TBVAC1, but considered promising marker
• MIG (CXCL9) monoclonal induced by IFNγ is a new biomarker that can assess IFNγ induced downregulation responses in TB (rather than assessing only IFNγ production)

Immune response:
• Elispot assays harmonized & protocols optimized ⇒ 50%
• successfully used in both M88/22 and H1C3 trials at Innsbruck
• 2-day lymphocyte stimulation test for immune profiling (memory & function)
• Develop ICS protocols to measure correlates / markers in M0 activated T-cell subsets
• Develop ICS protocols to measure memory T-cell subsets
• Develop MTE killing assays in relation to correlate identification and validation

Disease:
• local mHHA/EAT-En-GFP specific IFNγ producing T cells
• IL-2 producing T-effector cells and CD8 T cells increased in TB patients
• COX2+CD8+CD45RO+ Tumors Vs. WWYNT cells increased in TB patients
• reduced frequency of T effector and T tumors in TB patients

Importance of T cell memory subsets, multifunctional T-cells and protective immunity

• Memory formation and longevity is imprinted during T-cell priming
• Recent insights from immunology: "More is not better":
  - low antigen dose induces better T-cell memory
  - high Ag = T-cell exhaustion and replicative senescence
  - lack of overt inflammation likely induce better memory responses (strong inflammation blocks memory formation)
• Central memory (T_cm) and multifunctional T-cells correlate well with protection
• Implications for vaccination of the antigen naive as well as antigen experienced host (pre- and post-exposure vaccination)
• Measuring IFNγ does not equal protective immunity!!
Potential biomarkers of protection or disease: current status track

2 (priorities in blue)

Protection
• MTB-specific CD8 T cells identified by HLA tetramers for different epitopes
• Multifunctional MTB reactive T cells identified
• FPD-induced IL-17, IFN-γ, following vaccination
• New DB17 markers identified (CC16, IL-22, other marker profile combinations by R2)
• Memory T cell subset activation in response to MTB antigens
• MBT lipid-specific T cells have anti-erythocytosis effector activity
• MBT antigen-induced granuloyxin production from cells
• Resting Ag+ SAT-6-specific T-cells
• rHBA induced IFNγ / perinatal cells
• mHBA-specific IFNγ producing CD4 and CD8 T cells frequency
• TCRβ6 T cells have adjuvant activity

Disease
• DC-SIGN expression on alveolar Mφs discriminates TB patients
• CD68 + Lactate production
• Treg cells among PBMCs / local lymphocytes
  → e.g. SCG induced CD4 Tregs suppresses CD8, HBA induces CD4 Tregs

Immune assays
• List of genes and sets of probes for multiplex (MLPA, array) genetic profiling
• Large sets of new genome wide expression data from human Mφ / DC in TB

Subunit and live vaccines require different biomarkers

Example 1: HBBHA-induced IFN-γ secretion
In case of a subunit vaccine:
Only if HBBHA is part of the subunit vaccine, it can be a biomarker of vaccine induced immunity. If it is not part of the subunit vaccine, it can then be a potential biomarker of infection or disease activity post vaccination.
In case of live vaccine:
HBBHA can likely be used as a biomarker of vaccine induced immunity but not of infection / disease activity post vaccination.

Example 2: MTB lipid specific anti- mycobacterial T cell activity
In case of protein-based subunit vaccine:
MTB lipid specific T cell activity can be a biomarker of infection / disease activity post vaccination, but not in this case of vaccine induced immunity.
In case of live vaccine:
MTB lipid specific T cell activity can now be a biomarker of vaccine induced immunity.

Subunit and live vaccines require different biomarkers depending on vaccine context.

Biomarkers can serve different purposes depending on vaccine context. For each trial potential biomarkers will have to be ranked and selected on a tailor made basis.

Correlates in TBVAC

• Track 1:
  (i) validation of identified candidate biomarkers, and
  (ii) assay development, optimization & harmonization for monitoring clinical trials
  The increasing number of vaccines going into clinical trials (phase 1, 2 & 3) urge for consistent immunological monitoring tools.
  This requires testing in larger cohorts at relevant TB endemic sites.

• Track 2:
  Continued identification and prioritization of novel biomarkers / correlates
  and biomarker profiles / signatures.
  • IFNγ is an essential component but not a sensitive biomarker of protection on its own.
  Other markers need to be identified and tested (in large cohorts -- Africa & Asia).
  • TBVAC WP 4 has identified and prioritised a promising set of markers, which need further validation.
  • Correlate identification activities need to involve & access novel technologies and knowledge platforms (open the window...)
  • Can MBT killing assays be developed to read out "protective efficacy"? (STOC-1TB)
  • Can focussed forward Ag discovery disentangle phase specific Mtb gene expression patterns and immune recognition?
  • Can we identify the Mtb peptides expressed on Mtb infected human cells (pHIA elution) -- vaccine targets, correlates of infection, correlates of immunity

  ✓ Multiples / multicomponent biomarker signatures will be needed.
  ✓ Different vaccines will need different, tailor made sets of biomarkers.
  ✓ A given biomarker / profile may serve different purposes depending on the context.
Multiplex host response profiling in correlate discovery research: possibilities and challenges

- Transcriptomic host response profiling approaches
  - MLPA and related dedicated platforms
  - Genome or (innate-adaptive) immunome wide arrays (data available)

- Multiplex protein host response profiling approaches
  - Cytokines and chemokines
  - Effector molecules
  - Relate to MTB killing / growth inhibition

- Need for sophisticated data collection, management and analysis....

Towards consensus prioritization matrices

| Correlate of |
|---|---|---|---|
| Infection | "A" / innate response | "B" / adaptive response | "C" / systemic response |
| Marker / t assay 1 | 12/34 | 13/45 |
| Marker / t assay 2 | 23/45 | 34/56 |
| Marker / t assay 3 | 34/56 | 45/67 |
| Marker / t assay 4 | 45/67 | 56/78 |
| Marker / t assay 5 | 56/78 | 67/89 |
| Marker / t assay 6 | 67/89 | 78/90 |
| Marker / t assay 7 | 78/90 | 89/01 |
| Marker / t assay 8 | 89/01 | 01/12 |

Incorporate variables for new vaccines

Feasibility | Priority (0-1) | Combine with |
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WP4 participants TBVAC1

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A4.7. New insights into promising biomarkers in TB: challenges, prospects and interventions

Dr Shreemanta Parida and Professor Dr Dr h. c. Stefan H. E. Kaufmann, Max-Planck Institute for Infection Biology, Berlin, Germany

Natural history of TB disease and opportunities for biomarkers discoveries

Infected aerosols coughed up by active pulmonary TB patients with open cavitary diseases are the most common source of the infection through inhalation. Fortunately, in general, 70% of individuals exposed to these infected aerosols are able to deal with the encounter thereby preventing the infection from establishing itself, but the actual pathophysiology of this process is still unknown and should be a priority on the research agenda. *Mtb* establishes itself successfully in about 30% of immunocompetent, exposed individuals and about 40% of these (a total of 10–12% of those exposed) fall victim to the primary disease while the rest are able to contain the infection with an effective immune response. In this situation, *Mtb* is thought to reside in the human subjects in a non-replicating persistent stage. These individuals can remain latently infected and live their life without any sign of TB. Their lifetime risk of reactivating the disease and developing secondary clinical TB is estimated to be from 2% to about 23% (Parrish, Dick & Bishai, 1998). It is further estimated that one third of the world’s population are latently infected and the risk of developing secondary clinical TB has been compounded at least about 100-fold by the HIV epidemic (Kaufmann, 2007). There is thus an absolute need to find biomarkers that can distinguish between individuals infected having the highest risk of developing secondary TB (susceptibility) and those who have the highest probability of resisting development of the disease (protection). This would significantly help the control programme to break the chain of transmission of the disease in the long run. We are also in need of validated biomarkers that can be translated as point-of-care assays for early diagnosis and treatment of active TB patients in high-incidence countries.

Definitions of terms in biomarker research

Biomarker: characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathological processes or physiological/pharmacological responses to an intervention.

Correlate of protection: measurable sign in a host in response to an infectious agent indicating whether the individual is being protected against infection and/or developing disease.

Surrogate of protection: validated marker of correlate of protection.

Clinical end-point: characteristic or variable that reflects the final outcome of disease in terms of function, effect, progress, recovery, survival or death.

Surrogate end-point: biomarker that is intended to substitute for a clinical end-point, predicting clinical outcome in terms of benefit, or harm or lack of benefit or harm.

Biosignatures: a set of biomarkers that provide a portrait of the biological condition.

New advanced technology: global “-omics” approach

Recent developments in technology have resulted in different “-omics” platforms such as transcriptomics, proteomics, lipidomics, glycomics and metabolomics, which use global approaches in determining relevant genes, proteins or molecules involved in the pathophysiology of the disease. Technological advancements in this decade have allowed us to use this approach on an explorative basis to narrow down our focus on relevant candidates and processes connecting each another in an integrated manner, which is typically not possible with classical reductionist models.

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2 Source: Kaufmann & Parida, 2008; Biomarkers working group, 2001; Colburn, 2000.
Systems biology is a relatively new biological study field that focuses on the systematic study of complex interactions in biological systems, thus using a new perspective (integration instead of reduction) to study them. Particularly from 2000 onwards, the term has become widely used in the biosciences, and in a variety of contexts. One of the goals of systems biology is to discover new emergent properties that may arise from the interaction of multiple processes in order to understand better the entirety of processes that happen in a biological system. This also gives us the scope to understand the complex interactions of persistent infection such as TB that range from molecular and cellular to the population level, occurring over disparate timescales ranging from seconds to years (Young, Stark & Kirschner, 2008).

Immunology

Since the time of Robert Koch, the scientific community has struggled to understand the disease from an immunological viewpoint, so as to design effective interventions for controlling the disease. This started with use of crude preparations of antigens and has led to the current use of subunit antigens, either purified proteins or from recombinant expression systems. We have also investigated the role of many host molecules in pathogenesis and protection.

Transcriptomics

Transcriptome analysis evaluates global changes in gene transcript (mRNA: protein-coding part) expression profiles in a cell, tissue, organ or whole organism, providing a dynamic link between the genome, the proteome and the cellular levels. The field has evolved rapidly over the last five years, with several significant advances in technologies and data analysis for interpretation as well as its applications to drug discovery and development. Due to its accessibility, peripheral blood is the most feasible tissue source in clinical assessment of differential gene expression between diseases and drug treatment. Lymphocyte gene expression links to clinical, biochemical and pathological changes in the human body. This can be effectively exploited to investigate the pharmacodynamic effects of drugs at the genomic level in order to predict efficacy, side-effects of drugs and especially clinical outcomes. This approach has been successfully used in the field of oncology and currently is a major focus in infectious diseases.

Validity and results of gene expression studies heavily rely on the appropriate choice of the study groups. Therefore, the so-called principles of comparability, which are well known from both clinical and epidemiological studies, need to be applied to microarray experiments and their validity should not be compromised by selection, confounding or information bias (Repsilber et al., 2005). Rab33A – a member of Ras-associated small GTPases – a specific regulator of intracellular vesicle trafficking, was found to be differentially expressed in the studies conducted by our group on PMBCs from patients with TB and healthy control subjects, suggesting its involvement in the disease processes (Jacobsen, 2005). In a subsequent study, candidate biomarkers were assessed for optimal study group discrimination using a linear discriminant analysis that could cluster TB patients, Mtb-infected healthy subjects and noninfected healthy subjects using a minimal group of genes comprising lactoferrin, CD64 and the Ras-associated GTPase 33A (Jacobsen et al., 2006). Heterogeneity in the cellular composition of tissue specimens frequently confounds data analysis in microarray studies and we have demonstrated that deconfounding of transcriptome analyses for cellular heterogeneity greatly improves interpretability, and hence the validity of transcriptome profiling results (Jacobsen et al., 2007).

Proteomics

Proteomics is the study of the full complement of proteins expressed by a cell at a point of time. Alterations in protein abundance, function, and structure can serve to indicate the pathological abnormalities even before the onset of clinical symptoms. Hence these can be used as potential diagnostic or prognostic biomarkers (Issaq, Xiao & Veenstra, 2007). Serum proteome contains a treasure trove of protein biomarkers. More and more proteins in different biofluids are being identified with better analytical tools. However, the ability to deplete abundant proteins and to separate scanty amounts of target antigens by effective chromatography from sample preparations still poses a major challenge. Proteome analysis of anti-TB drug responses in *M. smegmatis* model system shows that translation, cell cycle control, and energy production are downregulated with drug treatment. In contrast, systems related to the drugs’ targets, such as lipid, amino acid and nucleotide metabolism, show specific protein expression changes associated with a particular drug treatment (Wang & Marcotte, 2008). This approach can be used to elucidate novel targets for new drug developments.

A proteome approach, combining high-resolution two-dimensional electrophoresis with mass spectrometry, was used to compare the cellular protein composition of two virulent strains of *Mtb* with two attenuated strains of *M. bovis* BCG, in order to identify unique proteins of these strains. From the 4000 open reading frames in the *Mtb* genome, the separation of proteins from whole mycobacterial cells by two-dimensional electrophoresis resulted in silver-stained patterns comprising about 1800 distinct protein spots. Of these, 96 were exclusively detected either in the virulent (56 spots) or in the attenuated (40 spots) mycobacterial strains. A total of 53 of these spots were analysed by mass spectrometry, of which 41 were identified, including 32 *Mtb*-specific spots. Of these, 12 *Mtb*-specific spots were identified as proteins encoded by genes previously reported to be deleted in *M. bovis* BCG. The remaining 20 spots unique to *Mtb* were identified as proteins encoded by genes that are not known to be missing in *M. bovis* BCG (Mattow et al., 2001).

Metabolomics

Metabolomics is the systematic study of the unique chemical fingerprints that specific cellular processes leave behind – specifically, the study of their small-molecule metabolite profiles. In host–pathogen interactions, metabolites can be derived from the host or the pathogen, or even the environment. Thus, while transcriptomic (mRNA gene expression data) and proteomic analyses do not tell the whole story of what might be happening in a cell, metabolic profiling can give an instantaneous snapshot of the physiology of that cell.

Metabolome refers to the complete set of small-molecule metabolites (such as metabolic intermediates, hormones and other signalling molecules, and secondary metabolites) to be found within a biological sample, such as a single organism (Glassbrook & Ryals, 2001). The word was coined in analogy with transcriptomics and proteomics; like the transcriptome and the proteome, the metabolome is dynamic, changing from second to second. Although the metabolome can be defined readily enough, it is not currently possible to analyse the entire range of metabolites by a single analytical method. In January 2007, scientists at the University of Alberta and the University of Calgary, Canada, completed the first draft of the human metabolome. They catalogued and characterized 2500 metabolites, 1200 drugs and 3500 food components that can be found in the human body. Metabolomics can be applied to any body fluid or tissue and its approach is an emerging field, which is proving superior to any other postgenomic technology for pattern-recognition analyses of biological samples.

4 http://www.metabolomics.ca
Efforts are under way to monitor the metabolic state of the host and \textit{Mtb}, depicting the interplay of primary and intermediate metabolites from biochemical pathways (e.g. compounds like glucose, cholesterol, ATP and lipid signalling molecules) involving genes, proteins and lipids during the molecular crosstalk over the course of infection in macrophage culture. High information densities generated by these techniques and their inherent multivariate profiling capabilities usually necessitate the use of combinations of chemometric and mathematical modelling methods to classify biochemical perturbation reflecting specific physiological or pathological states. These would then be integrated with the profiles of transcriptomics, proteomics, lipidomics and glycomics for resolving the complexities of the network.

The first proof-of-principle study conducted by our group in collaboration with the leading industrial partner Metabolon Inc., based in Durham, USA, along with our academic collaborators at Stellenbosch University, South Africa, using Metabolon’s platform using sera samples from 38 age- and sex-matched individuals with newly diagnosed pulmonary TB patients and latently infected (TST-positive) and clinically healthy contacts from a TB-endemic population in South Africa. Initial results are convincing enough to further explore the potential of metabolomics to identify novel biomarkers that can distinguish the state of TB infection, and be used for monitoring patients during chemotherapy, for identification of drug-resistance markers, for predicting potential non-responders (to manage these groups of patients effectively with other drug regimens) as well as potential markers to screen candidate novel drugs in development. An integrated approach is needed, to examine the evidence and to put this together with results of prospective studies in other “-omics”.

The study has been repeated in 48 age- and sex-matched individuals with newly diagnosed pulmonary TB and latently infected (TST-positive) and clinically healthy contacts from the endemic population in South Africa. Analysing these two studies together, there were about 365 metabolites found to be either up- or down-regulated between these two different groups, out of which about 130 could be identified and named, whereas about another 235 were unnamed metabolites. Taking all of these factors into account, both studies were consistent and substantiate the proof-of-principle of this approach. Currently, different purified small molecules of \textit{Mtb} are being analysed by liquid chromatography-mass spectrometry and gas chromatography-mass spectrometry platforms to investigate if metabolites of \textit{Mtb} can be detected among the unnamed metabolites that were never run on these analytical platforms, to be curated in a database.

\textbf{Types of biomarkers}

Using various technology platforms, either alone or in combination, biomarkers can be studied across the biological system using DNA/RNA for transcriptomics, proteins for proteomics, and enzymes and intermediate small molecules by metabolomics profiling. T-cell responses are studied to examine the immune response in TB, using a battery of assays in combination with specific \textit{Mtb} antigens.

Biosignatures represent a set of biomarkers in combinations of either multiple markers using single or multiple platforms: these would be more appropriate for TB than a single biomarker (Jacobsen et al., 2008).

\textbf{Why develop biomarkers for TB?}

We have argued that the vast majority (approximately 80% in sub-Saharan Africa) of all those who become infected with \textit{Mtb} will control the pathogen throughout their life without developing clinical signs of disease. Thus, the host immune system has mechanisms at hand to control the pathogen efficaciously although it does not achieve sterile eradication of \textit{Mtb}. Will it be possible to learn from this
naturally induced host response and better understand those mechanisms that control infection and exploit their value as potential indicators of protection? If so, then a biomarker of protection against TB could be defined and serve as a guideline for monitoring vaccine-induced immunity. Such a biomarker of protection against disease could provide valuable information about vaccine efficacy prior to the clinical end-point of TB disease outbreak.

In the area of clinical trials, a biomarker is defined as a characteristic feature that is objectively measured and evaluated as an indicator of a normal or a pathological process or of the response to an intervention (Biomarkers working group, 2001). Thus, the biomarkers we aim to identify – biomarkers of protection and susceptibility to natural infection with \textit{Mtb}, leading to the adoption of a marker of vaccine-induced immunity against TB – fall into this definition (Box 2).

With support from the Bill & Melinda Gates Foundation under the Grand Challenges in Global Health programme, we are attempting to define biomarkers for TB in the context of HIV/AIDS in five African nations, namely, Ethiopia, the Gambia, Malawi, South Africa and Uganda. This collaborative project falls under the broader thematic goal “To Create New Vaccines” among seven major goal-oriented themes and specifically under Grand Challenge 6 (out of the 14 defined Grand Challenges topics): “Learn which immunological responses provide protective immunity”. The countries involved are all hard hit by TB and HIV/AIDS. TB incidences range from 940 per 100 000 in South Africa as the highest to 257 per 100 000 in the Gambia as the lowest, and from 70% HIV infection in TB patients in Malawi as the highest to 6.3% in Ethiopia (WHO, 2008).

A major goal of our strategy is the definition of a surrogate end-point for a phase II/III clinical trial with novel TB vaccines and perhaps also with new drugs against TB. Several TB vaccines are due to enter, or have already completed, clinical phase I trials and a few candidates have reached phase II trials. Some are expected to enter phase III clinical trials in the near future. The greatest obstacle will thus come at the end of the research and development pipeline with a phase III clinical trial aimed at definitely determining protective vaccine efficacy in adults. Currently, the success of a vaccine in a phase III clinical trial is measured by the prevention of active TB outbreak in study group participants. In other words, clinical diagnosis of TB disease serves as clinical end-point of the trial. Even with a TB incidence rate of 1 in 100, as seen in the worst-hit parts of South Africa and Swaziland, large groups of individuals would need to be enrolled to get adequate power in the study to obtain conclusive evidence. Added to this is the long incubation time of latent \textit{Mtb} infection, ranging from months to lifelong. About 10–20% of those who become infected with \textit{Mtb} during a phase III trial in Africa will develop active TB at a later time point, with 5–10% probably occurring within the first two years. Thus, it has been estimated that clinical trials for TB vaccines may last years (possibly a decade, if not longer) comprising thousands of study subjects. In view of this grave situation, biomarkers could help to accelerate the development process at different stages of clinical trials.

Even before a phase III clinical trial, biomarkers that provide robust information for educated decisions about entry into, or termination of, phase II clinical vaccine trials as well as information for decisions on combinations of different vaccine candidates in heterologous prime–boost schemes would be extraordinarily helpful.

As an added value, information on biomarkers for TB can provide the basis for: (i) novel diagnostic tools to more reliably diagnose active TB; (ii) prognostic markers of infection outcome, i.e. TB disease reactivation or latent infection; and (iii) predicting drug treatment outcome, i.e. treatment success, drug failure or relapse.

\textsuperscript{5} http://www.gcgh.org/NewVaccines/Challenges/LearnaboutImmunologicalResponses/Pages/default.aspx

\textsuperscript{6} http://www.biomarkers-for-tb.net/
Biomarkers need in the context of TB

- Surrogate markers of immune protection – for assessing potential vaccine candidates.
- Surrogate markers of bacterial clearance (clinical end-point):
  1. markers for assessing potential drug candidates
  2. markers of relapse
  3. markers of treatment failure (drug resistance).
- Diagnostic markers:
  1. markers of infection
  2. prognostic markers of reactivation/disease.

Identification/differentiation of disease state

- Infected:
  1. with high risk of disease (susceptibility)
  2. with prognosis of protection
  3. special subsets – extrapulmonary TB, paediatric populations, with HIV coinfection.
- Treatment response (lack of bacterial clearance with drugs):
  1. persisters with risk of relapse
  2. drug resistance.

Required performance characteristics of biomarkers

- Immune correlates of protection
- High degree of specificities and sensitivities
- Easy to perform and can be translated at field sites/peripheral clinics
- Simple and quicker results
- Cost–effectiveness
- Sensitive disease marker for assisting diagnosis and management

Challenges and way forward

Despite efforts over a century, we still do not have an overall basic understanding of protective immunity. This needs to be addressed using a holistic approach, by concerted efforts to collate initiatives, expertise and experiences (synergies) within the scientific community. This also demands synergies and interactions with similar initiatives for different diseases or platforms (horizontal and vertical). We ought to work cohesively, with open and honest interaction. It is essential to solve the intellectual property issues at the outset in clear and stringent terms to move forward progressively.

The present failure to adequately control TB is the result of lost opportunities in funding research and development over the last few decades (Kaufmann & Parida, 2007). According to the latest report of the Treatment Action Group, TB research and development investments need to increase fivefold to US$ 2 billion per year in order to support the basic, applied, and operational research necessary to develop new tools to ultimately eliminate TB (Feuer, 2007). The scientific community has the responsibility to increase awareness among donors and to steer the process to collectively and individually contribute to stop this resilient Mtb from causing a public health menace.
A4.8. Designing studies for TB and TB/HIV biomarkers: challenges and opportunities

Professor Andrew Nunn, Medical Research Council Clinical Trials Unit, London, England

Although surrogate markers offer the opportunity to identify candidate drugs with the potential for shortening TB chemotherapy, they have their limitations as has been shown in trials of antiretroviral drugs. Whereas there is a clear inverse association between in HIV RNA reduction and disease progression, the Delta trial – comparing zidovudine monotherapy with combination therapy – gave rise to some quite unexpected results (Delta Coordinating Committee and Virology Group, 1999). Although the decline in HIV RNA was greater in the combined arms, the proportion with disease progression was higher in the combined treatment arms than in the monotherapy arm. A perfect surrogate would require that it be not only a good predictor of treatment outcome but also explain the treatment effect. Its predictive ability should not depend on the treatment being assessed (Prentice, 1989).

In the context of anti-TB treatment trials, bacteriological results during the early stages of treatment – whether microscopy, culture or the rate of decline of CFU – all suffer from the obvious limitation that although they may be good predictors of outcome, if there is variation in the drugs given subsequently or in the duration of treatment, these markers may have no predictive value at all. This is well illustrated in the Fourth East African Short course study, where patients in four of the regimens received exactly the same drugs in the intensive phase but relapse rates ranged from 11% to 32% (East African/British Medical Research Councils, 1981) and in the Union Study A where a common intensive phase was followed by a continuation phase with different drugs and different durations (Jindani, Nunn & Enarson, 2004).

It is essential that markers that appear to distinguish between regimens during treatment are validated with respect to subsequent failure and relapse rates. The impressive differences in serial sputum colony counts results in various regimens found in the OFLOTUB trial – a multicentre, randomized, control trial of ofloxacin-containing, short-course regimen for the treatment of pulmonary tuberculosis – (Rustomjee et al., 2008b) need to be validated in a trial with adequate follow-up for relapse. End-points must be objectively measured and reinfections need to be clearly distinguished from relapses.

There has been a change of approach in the conduct of some recent cancer trials due to the substantial increase in the number of new agents available for testing. The intermediate end-point of tumour progression has been used to select the candidates to take forward to longer-term trials of survival. This strategy reduces the number of trials needed but runs the risk of rejecting an effective drug. As already indicated, culture conversion rates do differentiate between treatment regimens but may not necessarily provide a substitute for long-term relapse rates.

In the evaluation of a surrogate, the choice of populations will be important, as results may be population dependent. Results from British Medical Research Council studies indicated that whereas higher culture conversion rates were observed in Hong Kong compared with East Africa with the same regimen, relapse rates were lower in the African population (Fox, Ellard & Mitchison, 1999). Highly effective regimens will not yield useful results when validating a surrogate marker: more useful results will be obtained from an unsuccessful shortened regimen.

Mistry et al. (2007) used a different cross-sectional design to distinguish between relapses and non-relapses by gene expression. They did not, however, distinguish between relapses and reinfections. The authors propose to test the predictive value of the markers they identified in a larger population.

Although sensitivity and specificity are useful markers of the value of a test, they do not tell us the probability that the test will give the correct diagnosis: this requires the positive and negative predictive
values of the test. These are critically dependent on the prevalence of the disease (or abnormality, such as relapse) within the chosen population. To take an extreme case, a test with 100% sensitivity and 95% specificity in the context of a low prevalence of 1 in 1000 will have a positive predictive value of only 2%. With a prevalence of 3%, the predictive value is 38% and with a prevalence of 5%, it increases to 51%. In the context of a treatment with a 5% relapse rate, all true relapses would be predicted but half of those predicted to be relapses would be non-relapses.

If a biomarker is to be used in patient management to identify those needing an extension of their treatment, it must have a very high, close to 100%, sensitivity to avoid missing relapses. If a four-month regimen required 15% of patients to have extended treatment, a test with 100% sensitivity but only 80% specificity would mean that, in addition to the 15% requiring extra treatment, a further 17% would receive an unnecessary extension of their regimen.
Bio-markers: Challenges & Opportunities

Andrew Nunn
MRC Clinical Trials Unit
London

Surrogate markers

- Surrogate markers offer the opportunity to reduce the need for long term trials of new interventions, enabling the selection of only the most promising ones for longer term assessment.

- However, as has been demonstrated in the field of HIV, unexpected results may be obtained and there are risks in over reliance on results from these studies.

Evaluation of CD4 and HIV RNA in the Delta trial

- In the Delta trial combination therapy with AZT and ddI or ddC was found to be significantly better than AZT alone in reducing the probability of disease progression or death.

- Comparative assessments of HIV RNA and CD4 results at week 8 resulted in unexpected findings.

- HIV RNA considerably overestimated and CD4 underestimated the benefit from combination therapy.

- Even when used together RNA and CD4 proved to be imperfect surrogates

- However, these markers have been used extensively to assess new therapies.
### Disease progression or death by HIV RNA at 8 weeks (Delta trial)

<table>
<thead>
<tr>
<th>RNA at 8 weeks*</th>
<th>ZDV</th>
<th>ZDV + ddI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Events N</td>
</tr>
<tr>
<td>&lt; 9</td>
<td>15</td>
<td>0%</td>
</tr>
<tr>
<td>9-70</td>
<td>57</td>
<td>11%</td>
</tr>
<tr>
<td>70-380</td>
<td>106</td>
<td>40%</td>
</tr>
<tr>
<td>&gt; 380</td>
<td>155</td>
<td>58%</td>
</tr>
<tr>
<td>Total</td>
<td>333</td>
<td>41%</td>
</tr>
</tbody>
</table>

* x 100 copies/ml.

### What do we require of a surrogate?

- A perfect surrogate should not only be a good predictor of treatment outcome but should also completely explain the treatment effect.
- This means the predictive ability should be the same regardless of the treatment being assessed – i.e. there should be no interaction between the marker level and the treatment.


### Limitations of surrogates measured during treatment

- Surrogates such as culture results during the initial intensive phase, the rate of sputum conversion, decline in bacterial load, etc suffer from the obvious limitation that although they may be good predictors of outcome if the subsequent continuation phase varies either in the drugs given or the duration they may have no predictive value at all.
## Limitations

<table>
<thead>
<tr>
<th>Regimen</th>
<th>N</th>
<th>Relapses</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>104</td>
<td>17</td>
<td>16%</td>
</tr>
<tr>
<td>B</td>
<td>104</td>
<td>11</td>
<td>11%</td>
</tr>
<tr>
<td>C</td>
<td>98</td>
<td>32</td>
<td>32%</td>
</tr>
<tr>
<td>D</td>
<td>105</td>
<td>30</td>
<td>30%</td>
</tr>
</tbody>
</table>

In all four regimens the 2 month culture results were negative in ~85% of patients.

## Limitations

<table>
<thead>
<tr>
<th>Regimen</th>
<th>N</th>
<th>Relapses</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2SHRZ/2HRZ</td>
<td>104</td>
<td>17</td>
<td>16%</td>
</tr>
<tr>
<td>2SHRZ/2HR</td>
<td>104</td>
<td>11</td>
<td>11%</td>
</tr>
<tr>
<td>2SHRZ/2HZ</td>
<td>98</td>
<td>32</td>
<td>32%</td>
</tr>
<tr>
<td>2SHRZ/2H</td>
<td>105</td>
<td>30</td>
<td>30%</td>
</tr>
</tbody>
</table>

4th East African Short Course Study.

## Limitations

In the IUATLD Study A two regimens had identical culture conversion rates of ~85% but very different failure/relapse rates.

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>2EHRZ/4HR</td>
<td>4%</td>
</tr>
<tr>
<td>2EHRZ/6HE</td>
<td>10%</td>
</tr>
</tbody>
</table>
**The marker and the endpoint**

- A marker that can distinguish well between alternative regimens needs to be validated.

- The Phase II SSCC OFLOTUB trial distinguished very effectively between the four regimens wrt rate of decline in CFUs but how well would this predict long term outcome?

- We don’t know but the likelihood is it would only be useful in regimens of shorter than 6 months duration.

---

**The marker and the endpoint**

- A marker and the true endpoint need to be studied together.

- A second best alternative would be to perform a Phase trial II followed by a Phase III trial – as in the OFLOTUB project.

- The true endpoint needs to be objectively measured so we can be confident we are sure of it. Reinfestations need to be distinguished from true relapses.

---

**An example from cancer**

- Because of the dramatic increase in potential new anti-cancer drugs it has become impossible to test them all in convention long term trials of survival.

- Multiple agents are being compared in a two stage process using an intermediate surrogate of tumour progression to select the best candidates to compare against the control.

- There are risks – a good candidate could be rejected in error.
Evaluating a surrogate

- An existing Phase III trial has its advantages but also limitations.
  - Population selection
  - Highly effective regimens unlikely to yield useful information.
  - Results from one study need to be validated in another – they are unlikely to be as good predicting the second time round.
- Needs to be done in different studies and differing populations.

An alternative approach

- Mistry et al (JID 2007) used an alternative approach of a matched cross-sectional design in which they used gene expression patterns to identify those at risk for recurrent tuberculosis.
- They propose to test the predictive value of these markers in a larger population of treated patients and suggest that other researchers should do the same in different geographical locations.

Indices to assess diagnostic markers
**Sensitivity & Specificity**

- Sensitivity – the proportion of true positives correctly identified by the marker.
- Specificity – the proportion of true negatives correctly identified by the marker.

**Example**

<table>
<thead>
<tr>
<th>Disease present</th>
<th>Yes</th>
<th>No</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnostic test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>231</td>
<td>32</td>
<td>263</td>
</tr>
<tr>
<td>Negative</td>
<td>27</td>
<td>54</td>
<td>81</td>
</tr>
<tr>
<td>Total</td>
<td>258</td>
<td>86</td>
<td>344</td>
</tr>
</tbody>
</table>

Sensitivity = 231/258 = 90%
Specificity = 54/86 = 63%

Sensitivity and specificity are not influenced by the population prevalence.

**Positive & negative predictive values**

The sensitivity and specificity of a test will not tell us the probability that test will give us the correct diagnosis.

The positive predictive value is the proportion of patients with positive results who are correctly diagnosed.

The negative predictive value is the proportion of patients with negative results who are correctly diagnosed.
PPV & NPV

<table>
<thead>
<tr>
<th>Diagnostic test</th>
<th>Disease present</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>Positive</td>
<td>231</td>
</tr>
<tr>
<td>Negative</td>
<td>27</td>
</tr>
<tr>
<td>Total</td>
<td>258</td>
</tr>
</tbody>
</table>

Positive predictive value = 231/263 = 88%
Negative predictive value = 54/81 = 59%

In contrast to sensitivity & specificity the PPV & NPV depend critically on the prevalence of the disease or abnormality.

PPV in low prevalence setting

Suppose we decrease the prevalence from 75% to 25%; how does this affect the PPV?

<table>
<thead>
<tr>
<th>Diagnostic test</th>
<th>Disease present</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>Positive</td>
<td>77</td>
</tr>
<tr>
<td>Negative</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>86</td>
</tr>
</tbody>
</table>

Positive predictive value = 77/172 = 45%
Negative predictive value = 163/172 = 95%

PPV in a very low prevalence setting

If we have a very good test,
sensitivity = 100%, specificity = 95%
but prevalence is very low, say 1 in 1000
the positive predictive value is only 2%

\[
PPV = \frac{sens \times prev}{(sens \times prev + (1 \times spec) \times (1 \times prev))}
\]

\[
= \frac{1 \times (0.001)}{(1 \times 0.001) + (0.05) \times (0.999)} = 0.02
\]
A test for patients needing extended treatment

- Suppose we had a test which enabled us to determine who needed >4 months treatment.

- If 15% of patients were in that category and our test was 100% sensitive but only 95% specific.

- In every 1000 patients we would correctly select 150 needing extra treatment but we would unnecessarily treat a further 42 patients.

- With 90% specificity we would treat 85 patients unnecessarily.
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Lok KH et al. (2002). Molecular differentiation of Mycobacterium tuberculosis strains without IS6110 insertions. Emerging Infectious Diseases, 8(11):1310–1313.


Wedlock DN et al. (2007). Vaccination of cattle with Danish and Pasteur strains of *Mycobacterium bovis* BCG induce different levels of IFN gamma post-vaccination, but induce similar levels of protection against bovine tuberculosis. *Veterinary Immunology and Immunopathology*, 118(1–2):50–58.


Bibliography for Annex 4


ANNEX 5. PAEDIATRIC TB: DIAGNOSTICS AND BIOMARKERS

Professor Nigel Klein, Great Ormond Street Hospital for Sick Children, Institute of Child Health, London, England, and Professor Alimuddin Zumla, Centre for Infectious Diseases and International Health, University College London, London, England

A5.1. Introduction

Although cases among children account for a relatively small proportion of all TB cases worldwide, children with TB represent a sentinel event in the community indicating recent transmission from an infectious individual, commonly an adult from their household. With the advent of the HIV/AIDS epidemic and the associated rise in the number of HIV-infected people with TB, especially in sub-Saharan Africa, TB in children has now become a major clinical problem (Chintu et al., 1993; Marais, 2007; Marais et al., 2007; Rekha & Swaminathan, 2007). A landmark necropsy study of 164 children dying of respiratory illnesses in Lusaka, Zambia, found that 24% of these children had died of TB (Chintu et al., 1993). These results were confirmed by other autopsy studies of children from Botswana (Ansari et al. 2002; Ansari et al., 2003). The perception that TB in children was not a public health problem was dismissed by these studies, which were used by the WHO Integrated Management of Childhood Illness group, and TB in children, as in adults, is currently considered a global priority (WHO, 2006; WHO, 2008).

A5.2. Diagnosis of paediatric TB

The diagnosis of TB in children has historically proved very difficult (Coulter, 2008; Marais, 2007; Marais et al., 2007). Coinfection with HIV has changed the clinical picture and presentation of TB in children and the clinical diagnostic criteria are no longer applicable since opportunistic lung infections confuse the clinical picture (Ansari et al., 2003; Chintu et al., 2002). The diagnosis of TB in children is traditionally based on chest radiography, tuberculin skin testing and microbiological testing, and usually follows discovery of an infectious case within that child’s household (Marais, 2007; Marais et al., 2007). However, compared with adults, these investigations are less likely to be positive in children and in most cases a clinical diagnosis is made without microbiological evidence. Obtaining sputum samples from children is problematic. Although newer more rapid and sensitive diagnostic tests, which take into account recent advances in molecular biology, immunology and chromatography are being developed, the data for children are even more limited than in adults (Cannas et al., 2008; Connell et al., 2008; Kafwabulula et al., 2002). Better diagnostic techniques are therefore also needed to make an early diagnosis of TB in a child and thereby prevent progression of TB disease. While a resurgence of interest in childhood TB over the past decade has occurred, very little investment has been made in research on diagnostics, drugs, vaccines and biomarkers studies. Newer anti-TB drugs are being tested in clinical trials in adults but not in children (Coulter, 2008; Donald & Schaaf, 2007).

There is a growing need to conduct clinical trials of new TB drugs in HIV-infected and non-infected children. All biomarker studies described in this meeting report are applicable to children as well.

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7 Supplementary contribution from discussions at the meeting.
A5.3. Special clinical situations in paediatric TB

In addition to the low diagnostic yield in children, there are some particular areas that need to be explored specifically in children. Biomarker studies should be applied to these special situations.

1. TB in children who are HIV-infected.
2. Extrapulmonary disease is more common in children than adults, occurring in approximately 25% of infants and children aged less than four years.
3. Central nervous system disease, especially TB meningitis, is the most serious complication of TB in children and occurs in about 4% of children with TB. The overall mortality has been reported to be 13%, with approximately half of survivors developing permanent neurological sequelae. Tuberculomas of central nervous system infection may occur and are usually characterized by solitary brain lesions, sometimes occurring after commencement of anti-TB treatment.
4. Musculoskeletal disease, which primarily affects weight-bearing bones and joints, particularly the vertebrae, is very debilitating in children.
5. The combination of extrapulmonary disease in combination with HIV appears to have a devastating effect in many cases (Lishimpi et al., 2002; Luo et al., 1994; Rekha & Swaminathan, 2007; WHO, 2006; Chintu et al., 1995; Chintu et al., 2004; Chintu, 2007; Oeltmann et al., 2008).

A5.4. Diagnostics and clinical biomarkers

Young children and infants may be unable to produce sputum and, when they do, microscopic examination is often negative because they have progressive primary disease. As an alternative to obtaining sputum, early morning gastric aspirate samples are often collected by aspiration of overnight gastric contents via a nasogastric tube. This takes advantage of the fact that infants and young children will often swallow respiratory secretions, which are pooled in the stomach overnight and which can be collected prior to ingestion of food in the morning. The yield from microscopy of gastric aspirate samples in children with proven pulmonary TB is less than 20%, compared with 75% in adults. The rates of detection in microscopy from other extrapulmonary samples, such as cerebrospinal fluid, are even lower mainly because of the paucibacillary nature of disease at these sites.

Culture of gastric aspirates has provided a more useful method of diagnosis in children with suspected pulmonary TB. Three consecutive morning gastric aspirates yield Mtb in 30–50% of cases and may be as high as 70% in infants. The culture yield from other body fluids or tissues from children with extrapulmonary TB is usually less than 50% due to lower numbers of mycobacteria in these sites of disease.

TST suffers from both poor sensitivity (false-negative results) and specificity (false-positive results). The lowest sensitivity is found in younger children. Up to 10% of otherwise normal children with culture-proven TB do not react to tuberculin initially. Most of these children will become reactive during treatment, suggesting that TB disease may itself contribute to immunosuppression. False-negative TST may also occur in children with severe TB disease, those with debilitating or immunosuppressive illnesses, malnutrition or other severe infections. The rate of false-negative TST in children with TB who are infected with HIV is unknown, but it is certainly higher than 10% and is dependent on the degree of immunosuppression, particularly the CD4 count. False-positive TST results may also occur.

The utility of the newer diagnostic tests is even less clear in children than in adults, although some of the data look promising. It is as yet unclear if either the QuantiFERON or ELISPOT assays will really offer major advances over the TST (Connell et al., 2008). As with adults, there is a need to clarify if and when these new assays should be used for children. Furthermore, there is a desperate
need to identify new methods of diagnosing TB in children. Urine-based tests for the detection of mycobacterial DNA, LAM and proteins are under way and, if successful, would provide a non-invasive method of diagnosing TB. The EU, TDR, the United States Agency for International Development, United Kingdom’s Department for International Development and donor agencies must now recognize the importance of the growing problem of paediatric TB, especially in sub-Saharan Africa, and must make a concerted effort to invest funds on this area.
References for Annex 5


The Special Programme for Research and Training in Tropical Diseases (TDR) is a global programme of scientific collaboration established in 1975. Its focus is research into neglected diseases of the poor, with the goal of improving existing approaches and developing new ways to prevent, diagnose, treat and control these diseases. TDR is sponsored by the following organizations:

UNICEF
UNDP
World Bank
WHO