

# Biomarkers for tuberculosis disease activity, cure, and relapse

Robert S Wallis, T Mark Doherty, Phillip Onyebujoh, Mahnaz Vahedi, Hannu Laang, Ole Olesen, Shreemanta Parida, Alimuddin Zumla

*Lancet Infect Dis* 2009;  
9: 162-72

See [Leading Edge](#) page 137

Pfizer, New London, USA (R S Wallis MD); Statens Serum Institute, Copenhagen, Denmark (T M Doherty PhD); World Health Organization-Tropical Disease Research, Geneva, Switzerland (P Onyebujoh FRCP, M Vahedi MD); European Commission, Brussels, Belgium (H Laang PhD, O Olesen PhD); Max Plank Institute for Infection Biology, Berlin, Germany (S Parida MD); and University College London Medical School, London, UK (Prof A Zumla FRCP)

Correspondence to: Prof Alimuddin Zumla, Centre for Infectious Diseases and International Health, University College London Medical School, Windeyer Institute Room 126, 46 Cleveland Street, London W1T 4JF, UK. [a.zumla@ucl.ac.uk](mailto:a.zumla@ucl.ac.uk)

New drugs, vaccines, and other therapies will be required to realise the goal of global tuberculosis elimination or control. This Review covers the important role biomarkers can have in accelerating drug development by providing validated surrogate endpoints that can bring enhanced statistical power to small short studies. Candidate biomarkers should differentiate people with active tuberculosis from healthy individuals, normalise with therapy, and reproducibly predict clinical outcomes in diverse patient populations. Although a large number of promising candidate biomarkers have been examined to date, few patients in these studies have reached clinically meaningful outcomes, and few of the studies have been conducted to international research standards. These markers must be further studied in tuberculosis treatment trials to evaluate the kinetics of the responses and their relation to long-term clinical outcomes. These studies will benefit from multidisciplinary collaborations including microbiologists, immunologists, clinicians, tuberculosis control personnel, and the pharmaceutical and biotechnology industry.

## Introduction

Despite the availability of an inexpensive, effective, and reasonably well-tolerated therapy, tuberculosis continues to be a major global health problem, causing an estimated 8.8 million new cases and 1.6 million deaths annually.<sup>1</sup> Efforts of the past decade to control tuberculosis by the consistent application of existing strategies have met with only limited success, slowing the rate of increase but failing to make substantial progress toward the goal of tuberculosis elimination. Efforts have been frustrated by the spread of HIV/AIDS in tuberculosis-endemic regions, and by the global emergence of strains of *Mycobacterium tuberculosis* resistant to present tuberculosis drugs (multidrug-resistant [MDR] and extensively drug-resistant [XDR-TB] tuberculosis).<sup>2</sup> It is now generally acknowledged that new approaches will be required to improve diagnosis, shorten treatment, improve outcomes (in MDR and XDR tuberculosis), and enhance protection afforded by vaccination, if the goal of tuberculosis elimination is ever to be realised. This Review focuses on the biomarkers (or biological markers) and surrogate endpoints that will be required to support the development of new drugs for tuberculosis; a companion review<sup>3</sup> focuses more specifically on host immune markers for protection.

Tuberculosis treatment trials have typically assessed the sum of the proportions of patients whose cultures never convert to negative (failures) and those that become positive again after completing therapy (relapses). Modern studies use genetic typing to confirm the identity of the initial and relapse isolates, because relapses must be distinguished from tuberculosis recurrence due to reinfection. Relapses are a key problem in tuberculosis drug development, for although they occur in only a small percentage of patients given optimal therapy, rates increase to unacceptable levels as treatment is shortened. Relapses can occur up to 2 years after completion of therapy, thus requiring large, lengthy trials to ensure adequate statistical power to demonstrate non-inferiority.

The requirement for biomarkers in tuberculosis stems from two critical features of human *M tuberculosis* infection: its long and variable natural history, and the essential role played by minority bacillary subpopulations. Non-replicating persisters are thought to be the main impediment to shortening therapy, because they are relatively unaffected by most tuberculosis drugs.<sup>4</sup> The size and nature of the bacterial subpopulations sharing this phenotype remain a matter of controversy, because they can be found both within latent foci contained in granulomas and in the sputum of large cavities.<sup>5</sup> Although many studies have established an association between cavitary disease and relapse,<sup>6-9</sup> the processes of granuloma formation, caseation, and cavitation are closely linked. Biomarker research therefore has the added task of determining the relative contributions of these two non-replicating subpopulations to relapse.

## Biomarker definitions, classifications, and characteristics

Biomarkers are measurable characteristics that indicate normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention.<sup>10</sup> In clinical trials biomarkers offer the possibility of a surrogate endpoint that can substitute for clinical endpoints. The most valuable biomarkers measure an event that is directly involved in pathogenesis or protection, and for which changes early during treatment can be related to the pharmacology and pharmacokinetics of the intervention. Experience in HIV indicates the potential of a biomarker such as plasma HIV RNA and a corresponding surrogate endpoint (eg, the proportion of patients with undetectable HIV RNA after 24 weeks of treatment) to accelerate research.<sup>11</sup> However, other research has indicated the ease with which apparently appropriate biomarkers may give totally misleading results, dissociated from clinically meaningful events.<sup>12,13</sup> Thus early markers of disease activity may not necessarily be satisfactory predictors of ultimate therapeutic success. A distinction is made between correlates of an outcome, for which a

relation has been observed, and surrogates for that outcome, which have been validated across multiple studies, sites, populations, and different types of interventions. The identification and validation of surrogate endpoints is critically important for the field to advance.

Biomarkers may be classified in several ways. 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, and will not be confounded by concomitant illnesses or therapies and may also serve as diagnostic tests. Tests may consist of a single analyte, or may be highly multiplexed, such as those from gene expression microarrays. Finally, analytes may be of either host or pathogen origin.

### Clinical biomarker applications

Multiple uses have been proposed for biomarkers in tuberculosis. During drug development, for example, biomarkers may assist in target selection and lead identification, optimisation, demonstrating proof of concept, selection of appropriate dose and dosing schedules, and selecting drug combinations with additive or synergistic interactions. Although some biomarkers may be more appropriate for specific stages of development, the continued use of a single marker from preclinical studies through dose selection phase IIb trials would be an important advantage.

Similar roles have been proposed for biomarkers in tuberculosis vaccine development. The ability to quantify T-cell reactivity to vaccine antigens, to correlate this activity with bactericidal activity against live mycobacteria, and to determine the durability of these responses over time in relation to vaccine dose, use of adjuvant, and dosing schedule will be critical to the acceleration of development of new live or subunit vaccines. These roles become increasingly important as the number of vaccine candidates grows and as vaccination strategies grow increasingly complex.

A potential role for biomarkers in routine clinical care has also been proposed. It has been noted, for example, that although rates of durable cure of 3 or 4 month tuberculosis-treatment regimens are inferior to those of standard 6 month therapy, biomarkers might facilitate the identification of early responders for whom shortened therapy might be appropriate.<sup>14,15</sup> The ability to classify tuberculosis patients at diagnosis or early during chemotherapy, according to relapse risk, might then allow resources to be focused on those patients with higher likelihood of poor outcome. Similarly, biomarkers that accurately indicate the risk of reactivation of latent tuberculosis infection in specific individuals might facilitate the targeted application of isoniazid preventive therapy in tuberculosis endemic regions.

Special roles may be considered for biomarkers in HIV/tuberculosis co-infected individuals. Although the general goal of antiretroviral therapy (ART) is

	Associated outcome	Patients (outcomes)*
<b>Sputum microbiology</b>		
Month 2 culture conversion	Recurrence	2450 (187) <sup>21</sup>
Serial sputum colony counts	Treatment effect	75 <sup>22</sup>
	Correlation with other markers	122 <sup>23</sup>
Serial MGIT or Bactec time to positivity	Treatment effect, failure, and recurrence	26 (13), <sup>24</sup> 42 (2) <sup>25</sup>
Early bactericidal activity (EBA)	Treatment effect	..
<b>Other tuberculosis-specific biomarkers</b>		
Sputum Ag85B-RNA	Treatment effect	18 <sup>26</sup>
Sputum Ag85	Recurrence	42 (2) <sup>25,27</sup>
Sputum Ag 85	Treatment effect; correlation with other markers	40 <sup>28</sup>
Urine tuberculosis DNA	Treatment effect	20 <sup>29</sup>
Anti-alanine dehydrogenase	Treatment failure	168 (10) <sup>30</sup>
Volatile organic compounds	Active tuberculosis	23 (19) <sup>31-33</sup>
<b>Non-specific immune activation markers</b>		
Neopterin	Treatment effect	39 (11) <sup>34</sup>
	Recurrence	31 <sup>35</sup>
sICAM1	Treatment effect	30 <sup>36</sup>
sIL2R	Treatment effect	44 <sup>37</sup>
sTNFR, granzyme B at diagnosis	Correlation with 2 month culture conversion	36 (18) <sup>38</sup>
Sputum interferon $\gamma$	Treatment effect	15 <sup>39</sup>
CRP	Treatment effect, death	105, <sup>40</sup> 100, <sup>41</sup> 18 <sup>42</sup>
suPAR	Death	47 (13) <sup>43</sup>
	Correlation with 2 month culture conversion	20 (12) <sup>44</sup>
Natural killer T cells at tuberculosis diagnosis	Correlation with 2 month culture conversion	21 (8) <sup>31</sup>
<b>Tuberculosis-induced IFN<math>\gamma</math> production (IGRA)</b>		
ELISPOT	Eradication of tuberculosis infection	14 (7) <sup>45</sup>
Whole blood culture	Predicting active tuberculosis	6 (5), <sup>46</sup> 6 (6) <sup>47</sup>
ELISPOT	LTBI treatment effect	38 <sup>45</sup>
ELISPOT	Tuberculosis treatment effect	18 (5) <sup>48</sup>
<b>Immune mediated tuberculosis killing</b>		
Whole blood culture	Tuberculin skin test status	12 <sup>49,50</sup>
	BCG vaccine effect	10, <sup>49-51</sup> 50 <sup>52</sup>
Whole blood culture	Effect of HIV/AIDS	22 <sup>53</sup>
Whole blood culture	Effect of antiretroviral therapy	15 <sup>54</sup>
Whole blood culture	Effect of anti-TNF treatment	20 <sup>55</sup>
Whole blood culture	Vitamin D effect	19 <sup>56</sup>
<b>Treatment mediated tuberculosis killing</b>		
Whole blood culture	Treatment effect	8 regimens, <sup>57</sup> 10 <sup>58</sup>
Whole blood culture	Correlation with other biomarkers	36 <sup>59</sup>
<b>Highly multiplexed assays</b>		
Transcriptomics	Active tuberculosis and LTBI	40 <sup>60</sup>
Proteomics	Active tuberculosis	60 <sup>61</sup>
Metabolomics (Kaufmann SHE and Parida S, personal communication)	Active tuberculosis and LTBI	300
<p>...=sample size not given. MGIT=mycobacterial growth indicator tube. ICAM=intercellular adhesion molecule. sILR2=soluble interleukin 2 receptor. TNFR=tumour necrosis factor receptor. CRP=C reactive protein. suPAR=soluble urokinase plasminogen activator receptor. IGRA=interferon gamma release assay. LTBI=latent tuberculosis infection. TNF=tumour necrosis factor. *Numbers in parentheses indicate the number of subjects reaching the designated endpoint.</p>		
<b>Table 1: Candidate biomarkers in tuberculosis</b>		

	Addition to regimen	2 month culture conversion			Relapse		
		Patients (N)	Rate (%)	Δ (%)	Patients (N)	Rate (%)	Δ (%)
<b>E Africa<sup>62,63</sup></b>							
6SH	..	154	49	NA	112	29	NA
6SHR	R	148	69	20	112	2	-27
6SHZ	Z	150	66	17	112	11	-18
<b>India<sup>64</sup></b>							
2SHZ/5S <sub>2</sub> H <sub>2</sub> Z <sub>2</sub>	..	129	72	NA	129	6	NA
2SHRZ/3 or 5S <sub>2</sub> H <sub>2</sub> Z <sub>2</sub>	R	261	92	20	269	2.6	-3.4
<b>E Africa<sup>65,66</sup></b>							
6SHR	..	169	70	NA	166	2	NA
2SHRZ/4TH or 4S <sub>2</sub> H <sub>2</sub> Z <sub>2</sub>	Z	347	82	12	338	6	4
<b>E Africa<sup>67,68</sup></b>							
2SHR/4 or 6TH	..	194	75	NA	159	13	NA
2SHRZ/4 or 6TH	Z	179	87	12	153	6	-7
<b>Hong Kong<sup>69,70</sup></b>							
6SHR	..	148	88	NA	143	6	NA
2SHRZ/4 or 6S <sub>2</sub> H <sub>2</sub> Z <sub>2</sub>	Z	167	95	7	174	5	-1
2SHRE/4 or 6S <sub>2</sub> H <sub>2</sub> E <sub>2</sub>	E	171	81	-7	168	16	10
<b>TBTC<sup>7</sup></b>							
2HREZ/4HR	..	415	81	NA	415	6.7	NA

Data are ranged by regimen, where numerals indicate months of treatment; subscripted numerals indicate number of weekly doses if treatment is not administered daily. S=streptomycin. H=isoniazid. NA=not applicable. R=rifampicin. E=ethambutol. Z=pyrazinamide. T=thiacetazone. TBTC=Centers for Disease Control Tuberculosis Trials Consortium.

Table 2: Relationship between two-month sputum culture conversion and relapse

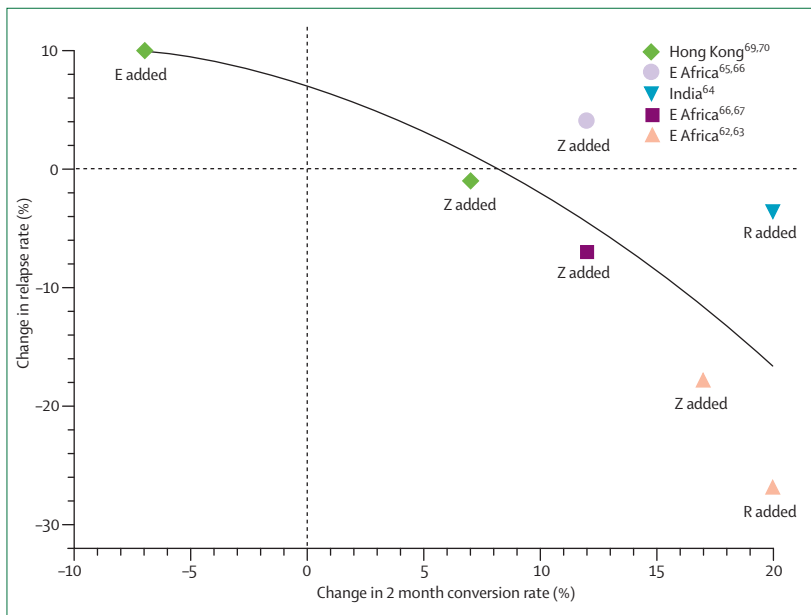


Figure 1: Relationship between change in 2-month sputum culture conversion and relapse rates due to new drug addition to tuberculosis regimen from table 2.

reconstitution of protective immunity, ART can have deleterious short-term consequences in HIV-infected patients by inducing immune reconstitution inflammatory syndrome (IRIS). At present, physicians

lack tools to predict, diagnose, and manage IRIS risks and optimise long-term outcomes in co-infected individuals. In some regions HIV infection has been associated with tuberculosis drug resistance,<sup>16</sup> although some of this risk represents enhanced susceptibility to recently transmitted resistant *M tuberculosis* strains. AIDS also seems to particularly predispose to the emergence of acquired rifamycin resistance during intermittent tuberculosis therapy.<sup>17</sup> The biological and pharmacological factors responsible for this resistance are inadequately understood.

The potential role for new vaccines and immunotherapeutics in the prevention and management of drug-resistant and HIV-associated tuberculosis remains largely untapped. Many of the biomarkers developed to facilitate tuberculosis drug and vaccine development will also be useful to help address these questions.

Children have historically posed unique challenges with regard to tuberculosis diagnosis and monitoring due to their inability to produce sputum. These problems have been amplified by the HIV/AIDS epidemic, which has increased the number of children with tuberculosis, and made its clinical and laboratory diagnosis more difficult.<sup>18–20</sup> It is anticipated that many of the tools developed in adults to detect specific and non-specific responses to *M tuberculosis* will facilitate work in children, but specific studies in this population will be required.

### Candidate biomarkers

#### Sputum microbiology

Table 1 summarises the available literature regarding biomarkers in tuberculosis. The marker with which there is greatest experience as a predictor of non-relapsing cure is sputum culture status after 2 months of tuberculosis therapy, for which the data are summarised in table 2.<sup>21,71</sup> This marker may be examined at three levels: across trials, within trials, and at the level of individual patients. Across trials, an inverse relation exists between 2 month conversion and relapse rates; however, this relation depends on a single study arm (6SH) with an atypically high relapse rate (29%). A more robust relation exists when the data are examined within studies as the increment afforded by the addition of a new drug (figure 1). Lastly, limited data indicate the marker may also be predictive for individual patients. In Study 22 of the TB Trials Consortium of the US Centers for Disease Control (TBTC),<sup>7</sup> for example, 2 month culture positivity was an independent predictor of relapse. However, the marker was relatively insensitive (identifying only half of all relapses) and lacked adequate positive predictive value for use as a guide to treatment of individual patients. The required 2-month interval also prohibits the study of individual drugs that generally cannot be studied safely as monotherapy for more than 10–14 days due to concerns regarding emergence of resistance. Combining a new drug with others may reduce the likelihood of detecting its effect, a potential disadvantage.

Two 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, using time to sputum culture conversion by Kaplan-Meier analysis as the outcome measure. In the second, sputum colony-forming unit (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.<sup>22,23</sup> The omission of days 0 to 2 removes effects on replicating rapidly killed bacteria (early bactericidal activity [EBA]) that are unrelated to treatment outcome. Other studies have substituted time to positivity in automated culture systems for colony counts on agar.<sup>24,25</sup> Since these systems are increasingly used globally for tuberculosis diagnosis, they may make it possible to increase the level of prognostic information afforded by routine medical testing in tuberculosis. Cultures in broth tend to remain positive longer during treatment than those on agar. The prognostic importance of occasional positive cultures in liquid medium, which represents low numbers of bacilli late during treatment, is not yet known.

#### Other tuberculosis-specific markers

Several studies have examined other microbial markers. Two studies have examined levels of *M tuberculosis* antigen 85 in sputum by ELISA. The magnitude and duration of increases in this protein during the first week of therapy predicted subsequent relapse in four of 42 patients.<sup>25</sup> Its induction was due to isoniazid, and was prevented by concomitant administration of rifampicin and by the higher of two doses of benzoxazinorifamycin, a long-acting rifamycin.<sup>28</sup> Induction of antigen 85 does not occur in isoniazid-resistant strains, potentially limiting the application of this marker in clinical trials.<sup>72</sup> One study has examined antigen 85B RNA, finding that it was cleared more rapidly than viable colony counts from sputum during therapy.<sup>26</sup> The 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. Similarly, lipid bodies, which are detected in sputum with increasing frequency as treatment progresses, have been identified as a distinctive morphologic feature of *M tuberculosis* after adaptation to hypoxia.<sup>5</sup> These may also serve as potential indicators of relapse risk.

There is growing interest in detection of pathogen markers that can be measured in urine, due to the ease and safety with which these specimens may be collected. One study has reported the presence of small fragments of *M tuberculosis* IS6110 DNA in urine of 79% (34/43) of patients with pulmonary tuberculosis but not in healthy controls.<sup>29</sup> These DNA fragments, termed transrenal (tr)DNA, are thought to arise due to apoptosis of host cells, because none of the patients in that study

had overt renal tuberculosis. None of 20 patients positive at diagnosis remained positive after 2 months of standard therapy. Responses have not yet been evaluated at earlier time points or in relation to sputum culture conversion. Other studies have shown reduced diagnostic sensitivity on urine PCR, except possibly in patients with AIDS and tuberculosis.<sup>73-77</sup> The method presently requires nested PCR amplification, without which assay sensitivity may not be sufficient for strains with low IS6110 copy numbers.<sup>77</sup> Monitoring of *M tuberculosis* trDNA 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.

Detection in urine of mycobacterial lipoarabinomannan and other antigens has been reported in some tuberculosis patients and in animals with experimental *M tuberculosis* infection.<sup>78-83</sup> No studies have yet examined the clearance of these antigens during treatment, or established a relation with clinical outcome or with another surrogate endpoint. 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.

Volatile organic compounds produced by *M tuberculosis* may be detected in the head space above cultures; some may also be detected in the exhaled breath of patients with active tuberculosis.<sup>33</sup> The potential of this approach for tuberculosis diagnosis is currently being explored. Its potential to indicate prognosis during treatment is not yet known.

Antibody levels to some mycobacterial antigens are raised at diagnosis and may be modulated by treatment. One study examined antibody levels to ten antigens in 168 patients before, during, and at the completion of treatment, and in additional household and endemic controls.<sup>30</sup> Ten patients failed therapy. Antibodies to early secreted antigenic target 6 kDa protein and Rv2626c were higher in patients than controls and decreased with treatment, but these did not distinguish failures from cures. Antibody levels to alanine dehydrogenase were higher in failures than cures. However, they were no higher in patients than controls, nor did they change during therapy.

#### Non-specific immune activation markers

Several studies have examined non-specific markers of immune activation as predictors of tuberculosis outcome. Neopterin is a soluble marker of macrophage activation that is a recognised prognostic indicator in HIV/AIDS.<sup>84</sup> Levels are increased in blood at tuberculosis diagnosis in proportion to the radiographic extent of disease and decline during treatment.<sup>34,85,86</sup> Levels are highest in patients with tuberculosis and HIV/AIDS, in whom they predict mortality; however, these deaths seemed to be related to HIV, and not active tuberculosis per se.<sup>85</sup> In HIV-uninfected tuberculosis patients matched for extent

of disease at baseline, raised concentrations of neopterin, after completion of tuberculosis treatment, were associated with relapse.<sup>35</sup> Larger studies are warranted to verify this finding, which may permit extended treatment or targeted follow-up for selected patients at higher risk of relapse after completion of treatment.

Other activation markers have also been shown to rise at baseline and to fall with treatment. Intercellular adhesion molecule (ICAM) 1, for example, is a ligand for leucocyte integrins that is mainly expressed by endothelial cells. A soluble form, sICAM, is released into the bloodstream. sICAM1 levels are elevated in tuberculosis patients at diagnosis in proportion to disease extent, and decrease in response to anti-tuberculosis treatment.<sup>36,87–89</sup> In one study, a model including change in sICAM1 during the 1st week of therapy predicted 2 month sputum culture conversion.<sup>44</sup> Early treatment effects have also been reported for  $\beta 2$  microglobulin, soluble interleukin 2 receptor (sIL2R), interferon  $\gamma$ , and tumour necrosis factor (TNF) and its receptors in blood, and TNF, interleukin 6, and interferon  $\gamma$  in sputum.<sup>37,39,85</sup> Analysis of interferon  $\gamma$  in this context may provide some level of specificity for tuberculosis, because levels of this cytokine are not raised in sputum of patients with bacterial pneumonia.<sup>39</sup>

C-reactive protein (CRP) is an acute phase protein produced by the liver that promotes phagocytosis. Serum CRP levels are increased in tuberculosis, particularly in patients with advanced disease, and they decline with therapy.<sup>41,90–92</sup> The urokinase plasminogen activator receptor (uPAR) is a cell surface receptor involved in cell adhesion and motility that is mainly expressed by macrophages and monocytes. The soluble form of this receptor, suPAR, is raised in patients with active tuberculosis and relates directly with the number of mycobacteria in sputum.<sup>43</sup> Levels were reported to fall by the end of treatment.

In other studies, baseline measurements of several activation markers have predicted 2 month sputum culture status or other outcomes, including serum CRP, suPAR, soluble tumour necrosis factor receptor (sTNFR) 1 and sTNFR2, and granzyme B.<sup>38,42,43,85</sup> Multivariate analyses may be helpful in future studies of these markers to determine the extent to which they are associated with other recognised baseline predictors of relapse, such as the bacterial burden and the presence of cavitory disease. Although baseline measurements cannot serve as an indicator of treatment effect, they may help ensure equality across study arms.

#### Tuberculosis-induced cytokine expression

Interferon  $\gamma$  is required for protection against mycobacterial infection.<sup>93</sup> Its production in response to mycobacterial antigens is increased in healthy tuberculin skin test reactors compared with unsensitised individuals, ostensibly indicating protection.<sup>94</sup> When the period of incubation is sufficiently long to permit

activation of otherwise resting memory T cells, interferon  $\gamma$  production is depressed in tuberculosis patients at diagnosis and recovers slowly with treatment.<sup>95</sup> However, assays with short incubation periods (termed interferon  $\gamma$  release assays [IGRAs]) have tended to show the reverse, with high levels at diagnosis that decline with treatment. Natural history studies of household tuberculosis contacts, have indicated that high levels of interferon  $\gamma$  production precede overt tuberculosis, an effect similar to that observed for large tuberculin skin test reactions.<sup>46,47,96,97</sup> In one study of tuberculosis patients, ELISPOT IGRA responses declined from baseline to 3 months in all of 13 patients with an adequate clinical response to treatment, but remained raised in five treatment failures.<sup>48</sup> In a similar study of recently tuberculosis-exposed British schoolchildren, isoniazid preventive therapy reduced the frequency of interferon- $\gamma$ -producing T cells by 68%.<sup>45</sup> Responses also declined in seven of 14 children with borderline tuberculin skin test and positive ELISPOT who did not receive isoniazid, a finding attributed to immune clearance of infection (ie, protection). It seems likely from these studies that recently acquired *M tuberculosis* infection or active or incipient tuberculosis gives rise to increased numbers of primed or partially activated T cells in the circulation, and that these cells are preferentially detected by short incubation IGRAs, which therefore indirectly indicate bacterial load. Further studies are warranted to determine the kinetics of the appearance and resolution of these cells to better understand the potential role of the assay as an indicator of incipient or resolved tuberculosis.

It seems from these studies that other approaches will be required to understand the cellular or cytokine basis of natural or vaccine-induced protection against tuberculosis if they are to be used as biomarkers. Relative mRNA expression levels of interferon  $\gamma$ , interleukin 4, and interleukin 4 $\delta$ 2 (a splice variant of interleukin 4) may serve as such an indicator, because ratios of interferon  $\gamma$  or interleukin 4 and interleukin 4 $\delta$ 2 decline as healthy contacts develop tuberculosis, and increase as tuberculosis cases are cured.<sup>98–101</sup> The ratio of interleukin 4 and interleukin 4 $\delta$ 2 has also been reported to be increased in longstanding latent tuberculosis infection, presumably indicating low risk of reactivation.<sup>102</sup> These cytokine ratios may serve as indicators of the ability to contain residual foci of latent tuberculosis infection after completion of therapy, and thereby indicating risk of relapse.

Studies in HIV indicate waning immunity is particularly associated with loss of multifunctional CD4+ T cells capable of producing interferon  $\gamma$ , TNF, and interleukin 2.<sup>103</sup> It also seems that certain subsets of interferon- $\gamma$ -producing CD8+ cells may also be important for protection.<sup>104</sup> Further studies are warranted to determine the potential applicability of these research tools to field trials in tuberculosis.

### Immune mediated *M tuberculosis* killing

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 *Mycobacterium microti* in mononuclear cell cultures served as an indicator of BCG vaccine effect.<sup>105</sup> 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).<sup>49,50</sup> Immune control of growth is inferior in skin-test-negative people and in young children, enhanced by BCG vaccination or vitamin D, impaired by T cell depletion or HIV infection, and restored by antiretroviral therapy.<sup>49–56</sup> As such, these assays may serve as a summary measure of anti-mycobacterial host defences and thus facilitate tuberculosis vaccine development. The extent to which these assays might indicate how well tuberculosis therapy works in the long term is not yet known.

### Treatment-mediated tuberculosis killing

Whole blood culture may also serve to study drug effects in treatment trials, because concentrations of administered drugs in cultures reflect those in vivo at the time of phlebotomy. The approach therefore can be used to examine the pharmacokinetic/pharmacodynamic relation and the combined effects of immunotherapy and chemotherapy on intracellular mycobacteria. One study found that whole blood bactericidal activity during tuberculosis therapy correlated with the decline in sputum colony counts and was superior in 2 month sputum culture converters.<sup>59</sup> Two studies reported that regimens for drug-sensitive tuberculosis were superior to those for MDR tuberculosis, consistent with required treatment durations and outcomes associated with these regimens.<sup>57,58</sup> Reduced activity of certain drugs may be demonstrated in the model when mycobacterial growth is restricted by immune pressure, similar to observations in vivo.<sup>106</sup> The whole blood models may be particularly suited to explore the dose–response relation of second-line tuberculosis drugs and the combined effects of drug and immunotherapies for MDR tuberculosis and XDR tuberculosis, in short, early phase II trials, together with assessment of EBA.

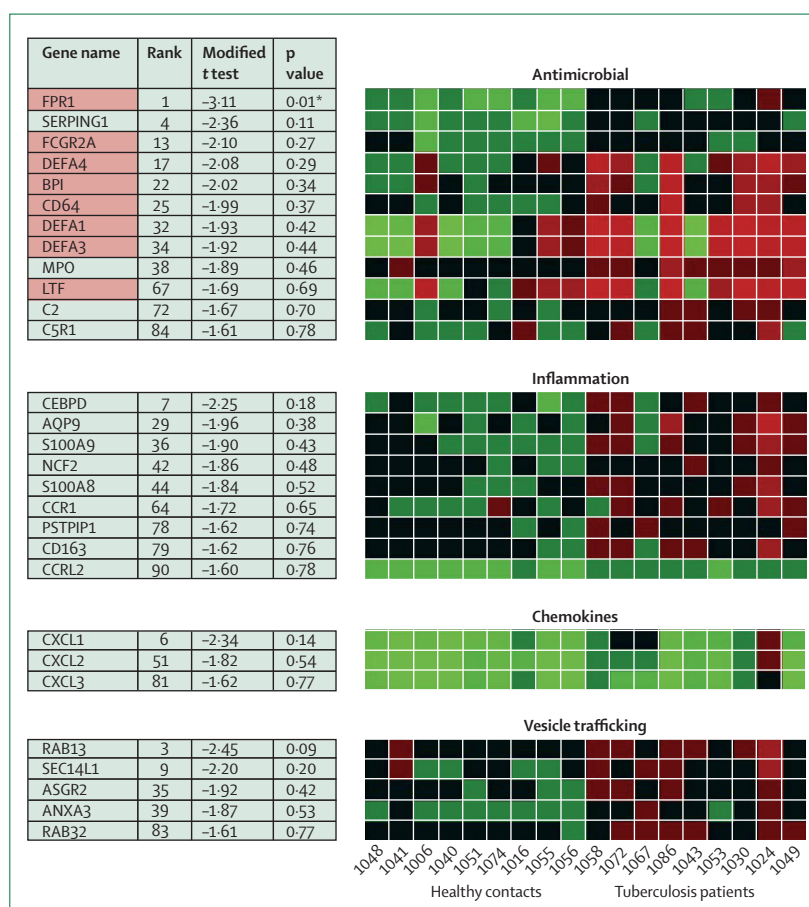
### Combinations of markers

A small number of studies have integrated combinations of markers to predict treatment outcome. One report used multiple regression analysis to identify combinations of early microbiological markers that predicted two relapses in a cohort of 43 patients.<sup>27</sup> 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. Two studies showed that combinations of immune parameters could be used to predict 2 month culture conversion.<sup>31,38</sup> Both studies examined numbers of CD3<sup>dim</sup>/CD56<sup>+</sup> natural killer

T cells, which were more prominent in tuberculosis patients compared with controls. One ongoing study in BCG-vaccinated infants has used multiplex ELISA and flow cytometry to identify profiles associated with protection from tuberculosis.<sup>107</sup> The study lacked an unvaccinated control arm, however, and seemed to identify innate rather than acquired (ie, BCG attributable) immune factors.

### Highly multiplexed assays

A small number of studies suggest that specificity and higher predictive value can be achieved for otherwise non-specific tests by measuring multiple parameters. These advanced technological platforms include proteomics, transcriptomics, and metabolomics. Agranoff and colleagues,<sup>61</sup> for example, found that tuberculosis could be differentiated from other infectious



**Figure 2: Predominant upregulation of innate immunity genes in peripheral blood mononuclear cells from patients with tuberculosis**

Increased gene expression is marked in red, decreased gene expression in green; intensities depict the gradient. Numbers on the left (1048 to 1056) represent *M tuberculosis*-infected healthy donors and latent tuberculosis individuals (LTBI). Numbers on the right (1058 to 1049) represent tuberculosis patients. Functional classification of genes revealed four major groups (antimicrobial, inflammation, chemokines, and vesicle trafficking). Ranking positions, test statistics (modified t test), and p values adjusted for multiple testing are given. Test statistics indicate the direction of effect—ie, relative-up (positive sign of test statistics) or downregulation (negative sign of test statistics) of transcription in tuberculosis patients relative to LTBI. \*Statistical significance. Reproduced from Jacobsen et al,<sup>109</sup> with permission from Springer.

and inflammatory conditions based on proteomic fingerprinting study of serum by surface enhanced laser desorption/ionisation time-of-flight mass spectrometry. Serum amyloid A and transthyretin were among the candidate biomarkers. A single analytic method can detect a very large number of peptides, although the technique is relatively insensitive, it can be supplemented by highly sensitive assays detecting a much smaller panel of candidate markers. A related approach reduces the potential number of candidate molecules to a set of small molecules termed the metabolome, representing metabolic intermediates, hormones and other signalling molecules, and secondary metabolites. The potential advantage of this approach is the reduced number of potential analytes in a single biological specimen. The main disadvantage is that multiple analytical methods seem to be necessary to complete their characterisation.

Two recent reports indicate the feasibility of distinguishing various stages of *M tuberculosis* infection by gene expression microarray. One study found a large number of candidate genes that were differentially expressed by mononuclear cells of three groups of people: tuberculosis patients, people with latent tuberculosis infection, and uninfected people.<sup>108</sup> A minimal group of genes was identified, comprised of lactoferrin, CD64, and the Ras-associated GTPase 33A, that was sufficient for classification of the three groups of people (figure 2). A second report identified signatures involving expression profiles of nine genes in blood cells that could distinguish active tuberculosis, latent tuberculosis infection, cure, and tuberculosis recurrence.<sup>60</sup> Tuberculosis patients were evaluated in this study at the time of diagnosis, presumably by identifying baseline parameters of relapse risk. It seems likely that similar studies done early during therapy could identify treatment effects associated with future durable cure or relapse.

### Clinical strategies for biomarker validation

It is evident from this Review that despite a large body of work tuberculosis biomarker research remains far from its goal of validated surrogate endpoints, with few patients reaching clinically meaningful outcomes, and few studies being done to international research standards. Moreover, the route to marker validation remains relatively uncertain. Whereas drug and vaccine development proceeds through phase I, II, and III trials, each with distinct objectives and requirements, no corresponding guidance exists for biomarkers. This section outlines some of the issues that may arise in the design and execution of such studies.

It is logical that as minimum starting requirements, candidate tuberculosis biomarkers be capable of detecting differences between tuberculosis patients and controls, and that values in patients normalise with successful therapy. Biomarkers that normalise sooner are not necessarily preferred, since early normalisation does not imply superior linkage to clinical outcome, and since

equally important roles may exist for biomarkers early and late during treatment. The requirements for early stage biomarker studies are similar to those for diagnostic tests. Tuberculosis cases should be confirmed microbiologically. Tuberculosis control programme standards for clinical monitoring, treatment, and outcome (cured or failed) are appropriate. People used as controls should be recruited from the same research site as patients. The most rigorous criterion for control patients is that they be tuberculosis suspects for whom the diagnosis has been excluded. However, this strategy may cause some potentially valuable candidate markers to fail unnecessarily, due to the inevitable inclusion among the controls of some patients whose tuberculosis diagnosis was missed, or who will subsequently have that diagnosis established. This is potentially important, because most tuberculosis clinics do not follow up suspects in whom tuberculosis has been excluded, even if no alternative diagnosis has been established. Healthy controls who are not tuberculosis suspects may be recruited as an alternative. The HIV status of all patients and controls should be known. These issues will be familiar to developers of tuberculosis diagnostic tests.

After this stage, the development paths of diagnostics and biomarkers diverge, due to the increased study requirements regarding the time course of response and ultimate outcome of treatment for biomarker development. The similarity between these studies and tuberculosis treatment trials makes it reasonable to consider doing these “phase II” biomarker studies in conjunction with, or as substudies of, existing or planned trials of new tuberculosis drugs. These trials fall into the following categories.

Initial trials of new tuberculosis drugs in patients (phase IIa) often take the form of EBA trials. Such trials are short (up to 1 week drug exposure) and small (15 patients per group), and have quantitative sputum microbiology and pharmacokinetics as main endpoints. They provide an opportunity to assess other microbiological biomarkers, such as sputum and urine molecular studies, and whole blood bactericidal activity. These studies are best done in patients with presumed drug-sensitive infections without prior treatment.

Two divergent strategies have been adopted by the pharmaceutical industry, government organisations, and non-governmental organisations to advance new tuberculosis drugs through later phase II clinical trials. The first has as its goal improving the outcome of MDR and XDR tuberculosis treatment. Patients are required to have infections resistant to isoniazid and rifampicin, and all will have received some prior treatment. Patients are randomised to an optimised background regimen plus placebo or study drug, with the main endpoint being sputum culture conversion at 2–4 months. Ethical considerations would likely require an open roll-over trial, to which all patients would be eligible, and in which all patients would receive active drug. Patients whose

### Search strategy and selection criteria

Articles cited in this Review were obtained through searches of Medline, meeting abstract databases, and reference lists from key reviews. Search terms included “tuberculosis”, “biomarker”, “surrogate endpoints”, “relapse”, and “clinical trial”. Priority was given to primary research publications. The search was limited to English, but was not restricted by date.

cultures had not converted would have their regimens re-optimised, whereas those with an apparent response to therapy would continue on their present background regimen, thus maintaining blinding. In this setting, substudies could readily examine the relation of selected biomarkers to sputum culture conversion. Statistical power for the endpoint of culture conversion will be high, due to the high proportion of treatment failures in MDR and XDR tuberculosis patients. However, statistical power will be low with regard to later outcomes such as durable cure, since the treatment of only a small proportion of patients will continue unchanged throughout the study.

An alternate strategy has been advanced in studies of moxifloxacin, with the goal of shortening the duration of therapy. Patients in these studies have drug sensitive infection, have had no prior therapy, and are randomly assigned to a standard or experimental regimen. The main endpoint has been sputum culture conversion at 2 months, after which all patients continue on standard therapy. These studies are valuable to establish the relation of biomarkers to 2 month conversion. As in MDR and XDR tuberculosis patients, the statistical power to study long-term outcomes is limited, because although the proportion of patients whose therapy continues unchanged is increased to half, the proportion with relapse or failure is greatly reduced.

Phase III trials will be needed to establish definitively the relation of a biomarker to relapse. Such studies must be of sufficient size to collect adequate numbers of tuberculosis recurrences; the trials that are likely to be most productive are those with higher relapse rates—ie, unsuccessfully shortened regimens. Initial and recurrent strains must be tested by RFLP or other methods to distinguish disease due to reinfection from true relapse. Studies need to be conducted in contrasting settings, for example, Africa and either Asia or South America, to ensure that findings are not limited by the genetic backgrounds of the host or pathogen, or influenced by concomitant parasitic infections or other regionally common conditions. This is a particular concern for the highly multiplexed assays. These studies will be large and costly, and best conducted with the assistance of research organisations familiar with the requirements for international registrational clinical trials. Separate studies may be required to evaluate biomarkers in people with HIV infection. Such trials will be additionally

complex due to the requirement to include patients receiving antiretroviral therapy. Finally, studies of selected biomarkers under tuberculosis programme conditions will be needed to examine their impact on the delivery and outcome of routine care.

### Conflicts of interest

We declare that we have no conflicts of interest.

### Acknowledgments

The following individuals participated in the WHO-TDR/EC Expert Meeting on TB Biomarkers held in Geneva on July 2–4, 2008, and assisted in development of this manuscript: Tom Ottenhoff, Dick van Soellingen, Andrew Nunn, Camille Loch, Denny Mitchison, Nigel Klein, Paranj Narayanan, Abraham Aseffa, and Hanna Akuffo.

### References

- 1 WHO. Global tuberculosis control – surveillance, planning, financing. WHO/HTM/TB 2008.393. Geneva: World Health Organization, 2008.
- 2 WHO. Anti-tuberculosis drug resistance in the world: report no. 4. WHO/HTM/TB/2008.394. Geneva: World Health Organization, 2008.
- 3 Doherty TM, Wallis RS, Zumla A. Biomarkers for tuberculosis disease status and diagnosis. *Curr Opin Pulm Med* (in press).
- 4 Rao SP, Alonso S, Rand L, Dick T, Pethe K. The protonmotive force is required for maintaining ATP homeostasis and viability of hypoxic, nonreplicating *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* 2008; **105**: 11945–50.
- 5 Garton NJ, Waddell SJ, Sherratt AL, et al. Cytological and transcript analyses reveal fat and lazy persister-like bacilli in tuberculous sputum. *PLoS Med* 2008; **5**: e75.
- 6 Mallory KF, Churchyard GJ, Kleinschmidt I, De Cock KM, Corbett EL. The impact of HIV infection on recurrence of tuberculosis in South African gold miners. *Int J Tuberc Lung Dis* 2000; **4**: 455–62.
- 7 Benator D, Bhattacharya M, Bozeman L, et al. Rifampentine and isoniazid once a week versus rifampicin and isoniazid twice a week for treatment of drug-susceptible pulmonary tuberculosis in HIV-negative patients: a randomised clinical trial. *Lancet* 2002; **360**: 528–34.
- 8 Nettles RE, Mazo D, Alwood K, et al. Risk factors for relapse and acquired rifamycin resistance after directly observed tuberculosis treatment: a comparison by HIV serostatus and rifamycin use. *Clin Infect Dis* 2004; **38**: 731–36.
- 9 Sonnenberg P, Murray J, Glynn JR, Shearer S, Kambashi B, Godfrey-Faussett P. HIV-1 and recurrence, relapse, and reinfection of tuberculosis after cure: a cohort study in South African mineworkers. *Lancet* 2001; **358**: 1687–93.
- 10 Biomarkers working group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther* 2001; **69**: 89–95.
- 11 Holodniy M. HIV-1 load quantitation: a 17-year perspective. *J Infect Dis* 2006; **194** (suppl 1): S38–44.
- 12 Echt DS, Liebson PR, Mitchell LB, et al. Mortality and morbidity in patients receiving encainide, flecainide, or placebo. The cardiac arrhythmia suppression trial. *N Engl J Med* 1991; **324**: 781–88.
- 13 CAST II investigators. Effect of the antiarrhythmic agent moricizine on survival after myocardial infarction. *N Engl J Med* 1992; **327**: 227–33.
- 14 Balasubramanian R, Sivasubramanian S, Vijayan VK, et al. Five year results of a 3-month and two 5-month regimens for the treatment of sputum-positive pulmonary tuberculosis in south India. *Tubercle* 1990; **71**: 253–58.
- 15 Hong Kong Chest Service/British Medical Research Council. 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. *Am Rev Respir Dis* 1991; **143**: 700–06.
- 16 Gandhi NR, Moll A, Sturm AW, et al. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet* 2006; **368**: 1575–80.

- 17 Wallis RS, Weyer K, Fourie PB. Acquired rifamycin resistance: pharmacology and biology. *Expert Rev Anti Infect Ther* 2008; **6**: 223–30.
- 18 Coulter JB. Diagnosis of pulmonary tuberculosis in young children. *Ann Trop Paediatr* 2008; **28**: 3–12.
- 19 Chintu C, Mudenda V, Lucas S, et al. Lung diseases at necropsy in African children dying from respiratory illnesses: a descriptive necropsy study. *Lancet* 2002; **360**: 985–90.
- 20 Ansari NA, Kombe AH, Kenyon TA, et al. Pathology and causes of death in a series of human immunodeficiency virus-positive and -negative pediatric referral hospital admissions in Botswana. *Pediatr Infect Dis J* 2003; **22**: 43–47.
- 21 Mitchison DA. Assessment of new sterilizing drugs for treating pulmonary tuberculosis by culture at 2 months [letter]. *Am Rev Respir Dis* 1993; **147**: 1062–63.
- 22 Rustomjee R, Diacon AH, Allen J, et al. Early bactericidal activity and pharmacokinetics of the diarylquinoline TMC 207 in pulmonary tuberculosis. *Antimicrob Agents Chemother* 2008; **52**: 2831–35.
- 23 Davies GR, Brindle R, Khoo SH, Aarons LJ. Use of nonlinear mixed-effects analysis for improved precision of early pharmacodynamic measures in tuberculosis treatment. *Antimicrob Agents Chemother* 2006; **50**: 3154–56.
- 24 Epstein MD, Schluger NW, Davidow AL, Bonk S, Rom WN, Hanna B. Time to detection of *Mycobacterium tuberculosis* in sputum culture correlates with outcome in patients receiving treatment for pulmonary tuberculosis. *Chest* 1998; **113**: 379–86.
- 25 Wallis RS, Perkins M, Phillips M, et al. Induction of the antigen 85 complex of *M. tuberculosis* in sputum: A determinant of outcome in pulmonary tuberculosis. *J Infect Dis* 1998; **178**: 1115–21.
- 26 Desjardin LE, Perkins M, Wolski K, et al. Measurement of sputum *M. tuberculosis* messenger RNA as a surrogate for response to chemotherapy. *Am J Respir Crit Care Med* 1999; **160**: 203–10.
- 27 Wallis RS, Perkins M, Phillips M, et al. Predicting the outcome of therapy for pulmonary tuberculosis. *Am J Respir Crit Care Med* 2000; **161**: 1076–80.
- 28 Wallis RS, Phillips M, Johnson JL, et al. Inhibition of INH-induced expression of *M. tuberculosis* antigen 85 in sputum: a potential surrogate marker in TB chemotherapy trials. *Antimicrob Agents Chemother* 2001; **45**: 1302–04.
- 29 Cannas A, Goletti D, Girardi E, et al. *Mycobacterium tuberculosis* DNA detection in soluble fraction of urine from pulmonary tuberculosis patients. *Int J Tuberc Lung Dis* 2008; **12**: 146–51.
- 30 Azzurri A, Kanaujia GV, Sow OY, et al. Serological markers of pulmonary tuberculosis and of response to anti-tuberculosis treatment in a patient population in Guinea. *Int J Immunopathol Pharmacol* 2006; **19**: 199–208.
- 31 Veenstra H, Baumann R, Carroll NM, et al. Changes in leucocyte and lymphocyte subsets during tuberculosis treatment; prominence of CD3dimCD56+ natural killer T cells in fast treatment responders. *Clin Exp Immunol* 2006; **145**: 252–60.
- 32 Syhre M, Chambers ST. The scent of *Mycobacterium tuberculosis*. *Tuberculosis (Edinb)* 2008; **88**: 317–23.
- 33 Phillips M, Cataneo RN, Condos R, et al. Volatile biomarkers of pulmonary tuberculosis in the breath. *Tuberculosis (Edinb)* 2007; **87**: 44–52.
- 34 Immanuel C, Rajeswari R, Rahman F, Kumaran PP, Chandrasekaran V, Swamy R. Serial evaluation of serum neopterin in HIV seronegative patients treated for tuberculosis. *Int J Tuberc Lung Dis* 2001; **5**: 185–90.
- 35 Hosp M, Elliott AM, Raynes JG, et al. Neopterin, beta 2-microglobulin, and acute phase proteins in HIV-1-seropositive and -seronegative Zambian patients with tuberculosis. *Lung* 1997; **175**: 265–75.
- 36 Demir T, Yalcinoz C, Keskinel I, Demiroz F, Yildirim N. sICAM-1 as a serum marker in the diagnosis and follow-up of treatment of pulmonary tuberculosis. *Int J Tuberc Lung Dis* 2002; **6**: 155–59.
- 37 Chan CH, Lai CK, Leung JC, Ho AS, Lai KN. Elevated interleukin-2 receptor level in patients with active pulmonary tuberculosis and the changes following anti-tuberculosis chemotherapy. *Eur Respir J* 1995; **8**: 70–73.
- 38 Brahmabhatt S, Black GF, Carroll NM, et al. Immune markers measured before treatment predict outcome of intensive phase tuberculosis therapy. *Clin Exp Immunol* 2006; **146**: 243–52.
- 39 Ribeiro-Rodrigues R, Resende CT, Johnson JL, et al. Sputum cytokine levels in patients with pulmonary tuberculosis as early markers of mycobacterial clearance. *Clin Diagn Lab Immunol* 2002; **9**: 818–23.
- 40 Lawn SD, Wiktor S, Coulibaly D, Ackah AN, Lal RB. Serum C-reactive protein and detection of tuberculosis in persons co-infected with the human immunodeficiency virus. *Trans R Soc Trop Med Hyg* 2001; **95**: 41–42.
- 41 Bajaj G, Rattan A, Ahmad P. Prognostic value of 'C' reactive protein in tuberculosis. *Indian Pediatr* 1989; **26**: 1010–13.
- 42 Scott GM, Murphy PG, Gemidjioglu ME. Predicting deterioration of treated tuberculosis by corticosteroid reserve and C-reactive protein. *J Infect* 1990; **21**: 61–69.
- 43 Eugen-Olsen J, Gustafson P, Sidenius N, et al. The serum level of soluble urokinase receptor is elevated in tuberculosis patients and predicts mortality during treatment: a community study from Guinea-Bissau. *Int J Tuberc Lung Dis* 2002; **6**: 686–92.
- 44 Djoba Siawaya JF, Bapela NB, Ronacher K, et al. Immune parameters as markers of tuberculosis extent of disease and early prediction of anti-tuberculosis chemotherapy response. *J Infect* 2008; **56**: 340–47.
- 45 Ewer K, Millington KA, Deeks JJ, Alvarez L, Bryant G, Lalvani A. Dynamic antigen-specific T-cell responses after point-source exposure to *Mycobacterium tuberculosis*. *Am J Respir Crit Care Med* 2006; **174**: 831–39.
- 46 Doherty TM, Demissie A, Olobo J, et al. Immune responses to the *Mycobacterium tuberculosis*-specific antigen ESAT-6 signal subclinical infection among contacts of tuberculosis patients. *J Clin Microbiol* 2002; **40**: 704–06.
- 47 Diel R, Lodenkemper R, Meywald-Walter K, Niemann S, Nienhaus A. Predictive value of a whole blood IFN-gamma assay for the development of active tuberculosis disease after recent infection with *Mycobacterium tuberculosis*. *Am J Respir Crit Care Med* 2008; **177**: 1164–70.
- 48 Carrara S, Vincenti D, Petrosillo N, Amicosante M, Girardi E, Goletti D. Use of a T cell-based assay for monitoring efficacy of antituberculosis therapy. *Clin Infect Dis* 2004; **38**: 754–56.
- 49 Kampmann B, Gaora PO, Snewin VA, Gares MP, Young DB, Levin M. Evaluation of human antimycobacterial immunity using recombinant reporter mycobacteria. *J Infect Dis* 2000; **182**: 895–901.
- 50 Cheon SH, Kampmann B, Hise AG, et al. Bactericidal activity in whole blood as a potential surrogate marker of immunity after vaccination against tuberculosis. *Clin Diagn Lab Immunol* 2002; **9**: 901–07.
- 51 Hoft DF, Worku S, Kampmann B, et al. Investigation of the relationships between immune-mediated inhibition of mycobacterial growth and other potential surrogate markers of protective mycobacterium tuberculosis immunity. *J Infect Dis* 2002; **186**: 1448–57.
- 52 Kampmann B, Tena GN, Mazazi S, Young D, Eley B, Levin M. A novel human in vitro system to evaluate antimycobacterial vaccines. *Infect Immun* 2004; **72**: 6401–07.
- 53 Tena GN, Young DB, Eley B, et al. Failure to control growth of mycobacteria in blood from children infected with human immunodeficiency virus, and its relationship to T cell function. *J Infect Dis* 2003; **187**: 1544–51.
- 54 Kampmann B, Tena-Coki GN, Nicol M, Levin M, Eley B. Reconstitution of antimycobacterial immune responses in HIV-infected children receiving HAART. *AIDS* 2006; **20**: 1011–18.
- 55 Saliu O, Sofer C, Stein DS, Schwander SK, Wallis RS. Tumor Necrosis Factor Blockers: differential effects on mycobacterial immunity. *J Infect Dis* 2006; **194**: 486–92.
- 56 Martineau AR, Wilkinson RJ, Wilkinson KA, et al. A single dose of vitamin D enhances immunity to mycobacteria. *Am J Respir Crit Care Med* 2007; **176**: 208–13.
- 57 Wallis RS, Palaci M, Vinhas S, et al. A whole blood bactericidal assay for tuberculosis. *J Infect Dis* 2001; **183**: 1300–03.
- 58 Janulionis E, Sofer C, Song HY, Wallis RS. Lack of activity of oral clofazimine against intracellular *M. tuberculosis* in whole blood culture. *Antimicrob Agents Chemother* 2004; **48**: 3133–35.

- 59 Wallis RS, Vinhas SA, Johnson JL, et al. Whole blood bactericidal activity during treatment of pulmonary tuberculosis. *J Infect Dis* 2003; **187**: 270–78.
- 60 Mistry R, Cliff JM, Clayton C, et al. Gene expression patterns in whole blood identify subjects at risk for recurrent tuberculosis. *J Infect Dis* 2007; **195**: 357–65.
- 61 Agranoff D, Fernandez-Reyes D, Papadopoulos MC, et al. Identification of diagnostic markers for tuberculosis by proteomic fingerprinting of serum. *Lancet* 2006; **368**: 1012–21.
- 62 East African-British Medical Research Councils. Controlled clinical trial of four short-course (6-month) regimens of chemotherapy for treatment of pulmonary tuberculosis. Second report. *Lancet* 1973; **1**: 1331–38.
- 63 East African-British Medical Research Councils. Controlled clinical trial of four short-course (6-month) regimens of chemotherapy for treatment of pulmonary TB. Third report. *Lancet* 1974; **2**: 237–40.
- 64 Tripathy SP. [Controlled clinical trial of a 3-month regimen and 2 5-month regimens in the treatment of pulmonary tuberculosis. 2d study of the short-term treatment administered in Madras]. *Bull Int Union Tuberc* 1983; **58**: 97–101 (in French).
- 65 East African-British Medical Research Councils. Controlled clinical trial of four 6-month regimens of chemotherapy for pulmonary tuberculosis. Second report. *Am Rev Respir Dis* 1976; **114**: 471–75.
- 66 East African-British Medical Research Councils. Controlled clinical trial of four short-course (6-month) regimens of chemotherapy for treatment of pulmonary tuberculosis. *Lancet* 1974; **2**: 1100–06.
- 67 East African-British Medical Research Councils. Controlled clinical trial of four short-course regimens of chemotherapy for two durations in the treatment of pulmonary tuberculosis. Second report. *Tubercle* 1980; **61**: 59–69.
- 68 East African-British Medical Research Councils. Controlled clinical trial of four short-course regimens of chemotherapy for two durations in the treatment of pulmonary tuberculosis: first report. *Am Rev Respir Dis* 1978; **118**: 39–48.
- 69 Hong Kong Chest Service-British Medical Research Council. Controlled trial of 6-month and 8-month regimens in the treatment of pulmonary tuberculosis. First report. *Am Rev Respir Dis* 1978; **118**: 219–28.
- 70 Hong Kong Chest Service-British Medical Research Council. Controlled trial of 6-month and 8-month regimens in the treatment of pulmonary tuberculosis: the results up to 24 months. *Tubercle* 1979; **60**: 201–10.
- 71 Wallis RS, Johnson JL. Surrogate markers to assess clinical efficacy of new antituberculous drugs. In: Yew WW, ed. *The development of new antituberculosis drugs*. Hauppauge, NY: Nova Science Publishers; 2006: 95–113.
- 72 Garbe TR, Hibler NS, Deretic V. Isoniazid induces expression of the antigen 85 complex in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 1996; **40**: 1754–56.
- 73 Rebollo MJ, San Juan GR, Folgueira D, et al. Blood and urine samples as useful sources for the direct detection of tuberculosis by polymerase chain reaction. *Diagn Microbiol Infect Dis* 2006; **56**: 141–46.
- 74 Torrea G, Van de Perre P, Ouedraogo M, et al. PCR-based detection of the *Mycobacterium tuberculosis* complex in urine of HIV-infected and uninfected pulmonary and extrapulmonary tuberculosis patients in Burkina Faso. *J Med Microbiol* 2005; **54**: 39–44.
- 75 Kafwabulula M, Ahmed K, Nagatake T, et al. Evaluation of PCR-based methods for the diagnosis of tuberculosis by identification of mycobacterial DNA in urine samples. *Int J Tuberc Lung Dis* 2002; **6**: 732–37.
- 76 Aceti A, Zanetti S, Mura MS, et al. Identification of HIV patients with active pulmonary tuberculosis using urine based polymerase chain reaction assay. *Thorax* 1999; **54**: 145–46.
- 77 Lok KH, Benjamin WH Jr, Kimerling ME, et al. Molecular differentiation of *Mycobacterium tuberculosis* strains without IS6110 insertions. *Emerg Infect Dis* 2002; **8**: 1310–13.
- 78 Boehme C, Molokova E, Minja F, et al. Detection of mycobacterial lipoarabinomannan with an antigen-capture ELISA in unprocessed urine of Tanzanian patients with suspected tuberculosis. *Trans R Soc Trop Med Hyg* 2005; **99**: 893–900.
- 79 Tessema TA, Bjune G, Assefa G, Svenson S, Hamasur B, Bjorvatn B. Clinical and radiological features in relation to urinary excretion of lipoarabinomannan in Ethiopian tuberculosis patients. *Scand J Infect Dis* 2002; **34**: 167–71.
- 80 Choudhry V, Saxena RK. Detection of *Mycobacterium tuberculosis* antigens in urinary proteins of tuberculosis patients. *Eur J Clin Microbiol Infect Dis* 2002; **21**: 1–5.
- 81 Singh KK, Dong Y, Hinds L, et al. Combined use of serum and urinary antibody for diagnosis of tuberculosis. *J Infect Dis* 2003; **188**: 371–77.
- 82 Kashino SS, Pollock N, Napolitano DR, Rodrigues V Jr, Campos-Neto A. Identification and characterization of *Mycobacterium tuberculosis* antigens in urine of patients with active pulmonary tuberculosis: an innovative and alternative approach of antigen discovery of useful microbial molecules. *Clin Exp Immunol* 2008; **153**: 56–62.
- 83 Napolitano DR, Pollock N, Kashino SS, Rodrigues V Jr, Campos-Neto A. Identification of *Mycobacterium tuberculosis* ornithine carboxyltransferase in urine as a possible molecular marker of active pulmonary tuberculosis. *Clin Vaccine Immunol* 2008; **15**: 638–43.
- 84 Fahey JL, Taylor JM, Detels R, et al. The prognostic value of cellular and serologic markers in infection with human immunodeficiency virus type 1. *N Engl J Med* 1990; **322**: 166–72.
- 85 Wallis RS, Helfand MS, Whalen C, et al. Immune activation, allergic drug toxicity, and mortality in HIV-positive tuberculosis. *Tuber Lung Dis* 1996; **77**: 516–23.
- 86 Turgut T, Akbulut H, Deveci F, Kacar C, Muz MH. Serum interleukin-2 and neopterin levels as useful markers for treatment of active pulmonary tuberculosis. *Tohoku J Exp Med* 2006; **209**: 321–28.
- 87 Walzl G, Ronacher K, Djoba Siawaya JF, Dockrell HM. Biomarkers for tuberculosis treatment response: challenges and future strategies. *J Infect* 2008; **57**: 103–09.
- 88 Lai CK, Wong KC, Chan CH, et al. Circulating adhesion molecules in tuberculosis. *Clin Exp Immunol* 1993; **94**: 522–26.
- 89 Mukae H, Ashitani J, Tokojima M, Ihi T, Kohno S, Matsukura S. Elevated levels of circulating adhesion molecules in patients with active pulmonary tuberculosis. *Respirology* 2003; **8**: 326–31.
- 90 Plit ML, Theron AJ, Fickl H, Van Rensburg CE, Pendel S, Anderson R. Influence of antimicrobial chemotherapy and smoking status on the plasma concentrations of vitamin C, vitamin E, beta-carotene, acute phase reactants, iron and lipid peroxides in patients with pulmonary tuberculosis. *Int J Tuberc Lung Dis* 1998; **2**: 590–96.
- 91 Lee JH, Chang JH. Changes of plasma interleukin-1 receptor antagonist, interleukin-8 and other serologic markers during chemotherapy in patients with active pulmonary tuberculosis. *Korean J Intern Med* 2003; **18**: 138–45.
- 92 Baynes R, Bezwoda W, Bothwell T, Khan Q, Mansoor N. The non-immune inflammatory response: serial changes in plasma iron, iron-binding capacity, lactoferrin, ferritin and C-reactive protein. *Scand J Clin Lab Invest* 1986; **46**: 695–704.
- 93 Dorman SE, Holland SM. Interferon-gamma and interleukin-12 pathway defects and human disease. *Cytokine Growth Factor Rev* 2000; **11**: 321–33.
- 94 Ellner JJ, Hirsch CS, Whalen CC. Correlates of protective immunity to *Mycobacterium tuberculosis* in humans. *Clin Infect Dis* 2000; **30** (suppl 3): S279–82.
- 95 Hirsch CS, Toossi Z, Othieno C, et al. Depressed T-Cell interferon-gamma responses in pulmonary tuberculosis: analysis of underlying mechanisms and modulation with therapy. *J Infect Dis* 1999; **180**: 2069–73.
- 96 Higuchi K, Harada N, Fukazawa K, Mori T. Relationship between whole-blood interferon-gamma responses and the risk of active tuberculosis. *Tuberculosis (Edinb)* 2008; **88**: 244–48.
- 97 Edwards LB, Acquaviva FA, Livesay VT. Identification of tuberculosis infected: dual tests and density of reaction. *Am Rev Respir Dis* 1973; **108**: 1334–39.
- 98 Wassie L, Demissie A, Aseffa A, et al. Ex vivo cytokine mRNA levels correlate with changing clinical status of Ethiopian TB patients and their contacts over time. *PLoS ONE* 2008; **3**: e1522.

- 99 Demissie A, Wassie L, Abebe M, et al. 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. *Infect Immun* 2006; **74**: 2817–22.
- 100 Dheda K, Chang JS, Breen RA, et al. In vivo and in vitro studies of a novel cytokine, interleukin 4delta2, in pulmonary tuberculosis. *Am J Respir Crit Care Med* 2005; **172**: 501–08.
- 101 Siawaya JF, Bapela NB, Ronacher K, Beyers N, van Helden P, Walzl G. Differential expression of interleukin-4 (IL-4) and IL-4 delta 2 mRNA, but not transforming growth factor beta (TGF-beta), TGF-beta RII, Foxp3, gamma interferon, T-bet, or GATA-3 mRNA, in patients with fast and slow responses to antituberculosis treatment. *Clin Vaccine Immunol* 2008; **15**: 1165–70.
- 102 Demissie A, Abebe M, Aseffa A, et al. Healthy individuals that control a latent infection with *Mycobacterium tuberculosis* express high levels of Th1 cytokines and the IL-4 antagonist IL-4delta2. *J Immunol* 2004; **172**: 6938–43.
- 103 Darrah PA, Patel DT, De Luca PM, et al. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against *Leishmania major*. *Nat Med* 2007; **13**: 843–50.
- 104 Mittrucker HW, Steinhoff U, Kohler A, et al. Poor correlation between BCG vaccination-induced T cell responses and protection against tuberculosis. *Proc Natl Acad Sci USA* 2007; **104**: 12434–39.
- 105 Cheng SH, Walker L, Poole J, et al. Demonstration of increased anti-mycobacterial activity in peripheral blood monocytes after BCG vaccination in British school children. *Clin Exp Immunol* 1988; **74**: 20–25.
- 106 Wallis RS, Song HY, Whalen C, Okwera A. TB chemotherapy: Antagonism between immunity and sterilization. *Am J Respir Crit Care Med* 2004; **169**: 771–72.
- 107 Soares AP, Scriba TJ, Joseph S, et al. Bacillus Calmette-Guérin vaccination of human newborns induces T cells with complex cytokine and phenotypic profiles. *J Immunol* 2008; **180**: 3569–77.
- 108 Jacobsen M, Repsilber D, Gutschmidt A, et al. Candidate biomarkers for discrimination between infection and disease caused by *Mycobacterium tuberculosis*. *J Mol Med* 2007; **85**: 613–21.