UTILITY OF NEWER TECHNOLOGIES FOR THE DIAGNOSIS OF ACTIVE AND LATENT TUBERCULOSIS

by

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DECLARATION

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  T. J. Cashmore, J.G. Peter, R.N. van Zyl-Smit, P.L. Semple, A. Maredza, R. Meldau *et al*., 2010

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- **The Comparison of Same Day Versus Delayed Enumeration of TB-specific T Cell Responses**

  L.M. Lenders, R. Meldau, R.N. van Zyl-Smit, V. Woodburne, A. Maredza, T.J. Cashmore *et al.,* 2010

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- **Feasibility and Diagnostic Utility of Antigen-Specific Interferon-γ Responses for the Rapid Immunodiagnosis of Tuberculosis Using Induced Sputum**

  Cashmore T.J, Nurse, B, Meldau, R, van Zyl-Smit, R.N, Lenders, L, Dheda, K

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ABBREVIATIONS

AFB	acid-fast bacilli
AIDS	acquired immunodeficiency syndrome
AraLAM	non-capped LAM
BAL	broncho-alveolar lavage
BCG	Bacille Calmette-Guérin
CDC	Centre for Disease Control
CFP-10	culture filtrate protein-10
CMI	cell mediated immune
CSF	cerebro-spinal fluid
DC	dendritic cell
DNA	di-ribonucleic acid
DOTS	directly observed short-course
DTT	dithiothreitrol
ELISPOT	enzyme-linked immunospot assay
ELISA	enzyme-linked immunosorbent assay
ESAT-6	early secretory antigenic target-6
FoxP3	forkhead box P3
HIV	human immunodeficiency virus
IFN-γ	interferon-gamma
IGRA	interferon-gamma release assay
IL-	interleukin
IQR	inter-quartile range
IU	international unit
kDa	kiloDalton
LAM	lipoarabinomannan
LM	lipomannan
LTBI	latent tuberculosis infection
ManLAM	mannose-capped LAM
MDR-TB	multi-drug resistant TB
MGIT	mycobacterial growth indicator tube
MHC	major histocompatibility complex
MCP-2	monocyte chemotactic protein-2

MR	mannose receptor
M.tb	*M.tuberculosis*
NHLS	National Health Laboratory Services
NAAT	nucleic acid amplification test
NICE	National Institute of Health and Clinical Excellence
NK	natural killer
NKT	natural killer T-cell
NTM	non-tuberculous mycobacteria
OCT	optimal cutting temperature
OD	optical density
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHA	phytohaemaglutinin
PILAM	phospho-myo-inositol-capped LAM
POC	point of care
PPD	protein-purified derivative
PTB	pulmonary tuberculosis
QFT	QuantiFERON®TB Gold
QFT-GIT	QuantiFERON®TB Gold-in-Tube
RD-1	region of difference-1
RNA	ribonucleic acid
ROC	receiver operating characteristic
rpm	revolutions per minute
SD	standard deviation
SEB	staphylococcal enterotoxin B
SFC	spot-forming cell
SFU	spot forming unit
SI	sputum induction
TB	tuberculosis
Th1	T helper-1
Th2	T helper-2
Th17	T helper-17

TLR	Toll-like receptors
TMB	tetramethylbenzidine
TNF-α	tissue necrosis factor-alpha
Treg	regulatory T cell
TST	tuberculin skin test
UCL	University College London
UCLH	University College London Hospital
WHO	World Health Organisation
XDR-TB	extensively drug resistant TB

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ABSTRACT

Background:

More than a third of the world's population is affected by latent tuberculosis (LTBI). Since the 1800s the tuberculin skin test (TST) has been the only available test for LTBI. Recently, interferon-gamma release assays (IGRAs) have been developed which are based upon the responses of peripheral blood effector cells to *M.tb*-specific antigens [early secretory antigenic target - 6 (ESAT-6) and culture filtrate protein (CFP10)]. Discordance between the TST and IGRAs has been well documented but remains largely unexplained. By contrast, active tuberculosis (TB) results in approximately 10 million cases per year and approximately 2 million deaths. However, control efforts are hampered by suboptimal diagnostic tools and techniques

Methods:

Quantitative PCR of tissue from TST skin biopsies was used to investigate the immunopathogenesis of TST-positive, IGRA-negative discordance in the setting of LTBI. In smear negative and sputum scarce individuals, antigen-specific responses in cells from induced sputum were evaluated in a standardized ELISPOT assay incorporating ESAT-6 and CFP10. Additionally, LAM, a heat stable lipopolysaccharide antigenic marker of the genus *Mycobacterium*, was evaluated as a diagnostic marker in induced sputum.

Results/ Conclusions:

It was found that regulatory T cells (Tregs) and cytokines such as IL-4 were expressed at a higher level in TST-positive, IGRA-negative compared to TST-positive, IGRA-positive subjects. In induced sputum, whilst the ELISPOT assay showed that rapid immunodiagnosis is indeed possible, its clinical feasibility is limited as only 11% of the results were evaluable. Finally, the non-cellular fraction of induced sputum was evaluated using a lipoarabinomannan (LAM) ELISA antigen-detection assay. Whilst the assay

was feasible when using induced sputum, the sensitivity and specificity of the assay was suboptimal and not clinically useful as a diagnostic test for TB.

CHAPTER 1

INTRODUCTION

1.1 EPIDEMIOLOGY

In 2003 the World Health Organisation declared tuberculosis (TB) a global health emergency (World Health Organisation, 2009). TB remains a major health problem worldwide, with one third of the world's population thought to be infected with the disease. The most recent WHO report estimates that 13.7 million individuals had active TB in 2007 (206 per 100 000 population), and 9.27 million people (139 per 100 000) developed TB in that year alone (WHO, 2009).

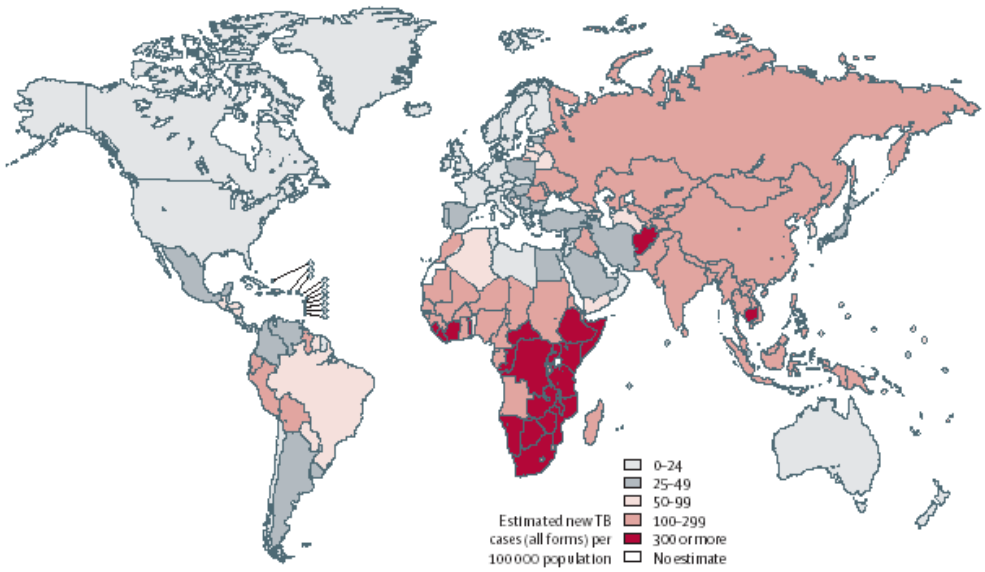

Figure 1.1 Estimated number of new TB cases per 100 000 population. (World Health Organisation, 2009)

An estimated 15% of all incident TB cases were HIV-positive; 79% of these were in the African region and 11% were in the South-East Asia region.

Most of the TB cases recorded in 2007 were in Asia (55%) and Africa (31%). The five countries that ranked first to fifth in terms of total number of cases in 2007 were India (2 million), China (1.3 million), Indonesia (0.5 million), Nigeria (0.46 million) and South Africa (0.46 million) (World Health Organisation, 2009). The extremely high prevalence of TB, especially in sub-Saharan Africa, (Corbett *et al.*, 2006) could be attributed to several factors. These include the HIV pandemic, poverty, lack of an effective vaccine, non-availability of a healthcare infrastructure in many poor areas and importantly, a lack of a rapid and accurate TB-specific diagnostic method.

1.2 HISTORY & PATHOGENESIS

The causative agent for tuberculosis was identified by Robert Koch in 1881 although evidence of TB dates back, according to one report, to 1000-400 BC (Zimmerman, 1979). Koch identified the bacillus as a slender, straight or slightly curved organism which is non-motile and does not form spores (Figure 1.2).

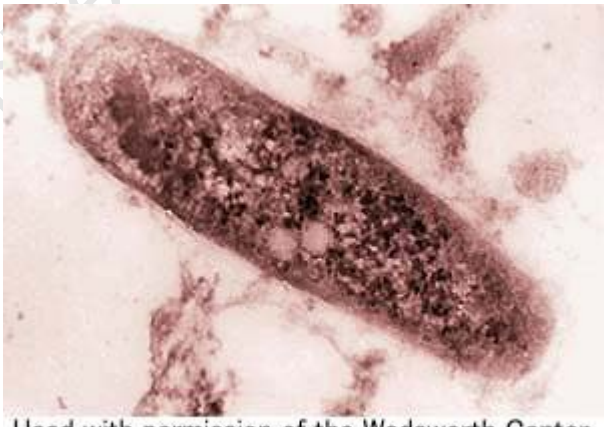

Figure 1.2 Electron microscope view of a “Koch’s bacillus”

The causative agent of tuberculosis is *Mycobacterium tuberculosis (M.tb)* – a bacillus with specific and typical pathogenetic traits in the human host. It is spread primarily by droplet inhalation of only 1-10 bacteria, usually via a cough of an infected tuberculous patient (Sundaramurthy and Pieters, 2007). Most people exposed to *M.tb* will not become infected and of those infected, a further 90% will remain asymptomatic as the mycobacteria can remain dormant as latent TB infection (LTBI) (Russell, 2007). LTBI describes a clinically asymptomatic state when the host shows no signs of active disease yet has viable, non-replicating *M.tb* organisms within tissues (Dheda *et al*., 2010).

Once infection has occurred alveolar macrophages and dendritic cells (DCs) in the distal airways are the first to be exposed to the droplets containing the *M.tb* pathogens (Meya and McAdam, 2007). Phagocytosis is facilitated by Toll-like receptors (TLR), macrophage mannose receptors and complement receptor 3 (Ernst, 1998). Although the bacilli are engulfed, many of these virulent bacteria resist phagocytosis and intracellular killing by interfering with host trafficking pathways. They inhibit the maturation of the bacillus-containing phagosome and fusion with the lysosome (Sturgill-Koszycki *et al*., 1996). In addition, the bacilli can modulate the membrane proton-pump inhibitor, thereby preventing acidification of the phagolysosome. The bacilli have molecules in their cell walls which may be cytotoxic, such as LAM, which inhibit the cell's response to interferon-γ (IFN-γ), and the ability to present antigen (Rook and Hernandez-Pando, 1996). Thus, mycobacteria manage to create a safe environment within the macrophage where they can survive and even replicate (Houben *et al*., 2006).

In the early stages of infection, only the lung compartments are involved so no naïve CD4+ T cells are exposed to foreign antigens. The bacterial population are hereby allowed time to grow and resist the host adaptive immune response (Wolf *et al*., 2008). New macrophages and DCs are continually attracted to the site of

infection as *M.tb*-infected macrophages are lysed and bacilli are released. Thus, there are no associated signs or symptoms of disease in the early stages of infection.

The adaptive immune response is primarily initiated upon stimulation in the lymph nodes (Salgame, 2005). Here, infected DCs upregulate major histocompatibility (MHC) Class II and co-stimulatory molecules and present captured *M.tb* antigens to naïve T cells (Alaniz *et al*., 2004). Approximately 2-6 weeks after initial infection (Edwards and Kirkpatrick, 1986), activated T-cells migrate to inflammatory sites in the lungs to initiate an ongoing interaction between T-lymphocytes and macrophages in the form of a cellular mass called a granuloma, or tubercle, hence the name of the disease (Russell, 2007).

The granuloma consists of a caseating centre of degenerating infected macrophages and multi-nucleated foamy giant cells (Langhans), surrounded by peripheral mononuclear cells. During this phase of disease, the mycobacteria are contained. There are still no signs of disease and the individual is not infectious. The tubercle may become surrounded by fibroblasts and then calcify, forming the typical x-ray picture seen in TB patients. Alternatively, a lowering in immune status, e.g. HIV, or malnutrition, may result in the bacilli growing exponentially thereby leaving dead and dying macrophages to form the characteristic caseous, necrotic intragranulomatous centre. The bacilli continue to increase in number, causing further necrosis which finally results in bronchial erosion and drainage of the liquefied material. The individual may now expectorate infectious sputum which can result in perpetuating disease (Cardona, 2010).

Extrapulmonary TB may occur early in infection when mycobacteria spread distally either indirectly via the lymphatic system or through direct erosion into one of the pulmonary blood vessels. In approximately 15% of patients, further

extrapulmonary sites may include the bones, pleura, genito-urinary system, meninges, peritoneum and skin (Raja, 2004). Persistence of viable extrapulmonary tubercle bacilli may result in secondary lung lesions causing reactivation of clinical disease years or even decades later. Primary disease is typified by a single granulomatous lesion in the middle or lower lobes with enlarged draining lymph nodes, while endogenous reactivation is often recognised by a single or multiple cavitations in the apical lung area with multiple secondary tubercles (Baron, 1996).

1.3 HOST IMMUNE RESPONSE

TB causes a large number of deaths, more than from any single infectious disease. Co-infection with HIV, especially in sub-Saharan Africa, causes further complications, morbidity and mortality. The host immune response can be subdivided into two interactive categories:

1.3.1 Innate Immune Response

Cells of the innate host immune response act as the initial, non-specific protection against invading pathogens. Alveolar macrophages perform a variety of key functions in the host response. These include:

1. phagosome-lysosome fusion with *M.tb* after surface-molecule-binding,
2. inhibition of mycobacterial growth and killing through free radical based or cytokine-mediated mechanisms,
3. recruitment of specific immune cells for local inflammation, and
4. presentation of antigens to T-cells to invoke acquired immunity.

Macrophages interact with other effector cells via chemokines and cytokines, which serve to attract and activate inflammatory cells such as lymphocytes. It is these contact mechanisms between the host and *M. tuberculosis* which may confer either host or bacillus protection.

Cell surface receptor molecules, such as mannose receptors, play an important role e.g. mannose receptor and mycobacterial binding appears to be mediated by the mycobacterial surface glycoprotein lipoarabinomannan (LAM). Bacilli utilise LAM to resist phago-lysosomal formation via mycobacterial sulphatides to produce ammonia. This continual macrophage necrosis leads to bacillary entrance into the extracellular milieu where the cellular immune response is evoked (Kaufmann and McMichael, 2005). Neutrophils also play a role in the innate immune response and are responsible for the recruitment of inflammatory cells for a local response. DCs transport mycobacterial antigen into the lymphoid tissue where the adaptive immune response can be initiated. Natural killer (NK) cells, natural killer T-cells (NKT) cells, and $\gamma\delta$ cells also play a role defending the host against invading pathogens. Innate immunity may explain the likely clearance of the organism with lack of a tuberculin skin test (TST) or interferon-gamma release assay (IGRA) reaction in a large population of exposed individuals (Figure 1.3).

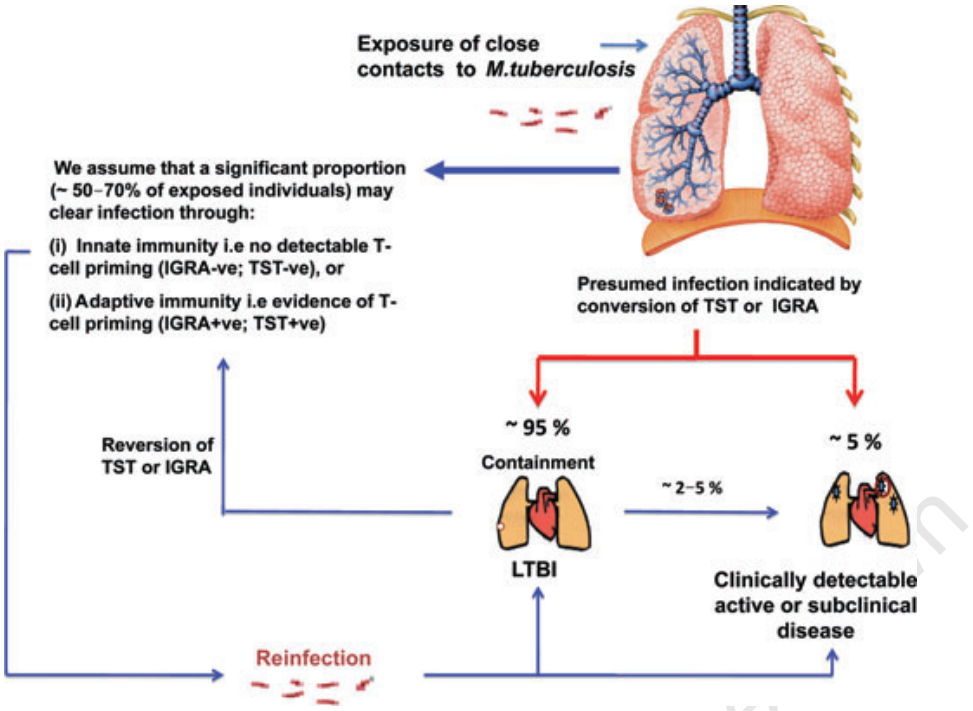

Figure 1 The spectrum of *Mycobacterium tuberculosis* infection and the life cycle of *M. tuberculosis*. Many exposed individuals (~30–50%) have no immunodiagnostic evidence of *M. tuberculosis* infection or T-cell priming despite heavy exposure to *M. tuberculosis*. These individuals, although unproven, may be innately resistant to *M. tuberculosis* infection. Of those who have immunodiagnostic evidence of T-cell priming (+ve TST or IGRA) it is likely that a substantial proportion have LTBI (clinically asymptomatic *M. tuberculosis* infection during which organisms are in a state of non-replicating persistence). Some individuals have transiently positive responses, and may subsequently 'revert' their reactions—they may have 'acute resolving infection' or clear their infection. These individuals may become reinfected and progress to active tuberculosis. A proportion of patients with LTBI may also progress to active disease. Given the lack of a gold diagnostic standard for LTBI some of these postulates are based on circumstantial evidence and remain unproven. A multitude of clinical conditions (HIV-1 infection, diabetes mellitus, malnutrition, tobacco smoking, TNF-α inhibitor therapy, helminth coinfection) may permit tipping of the immunological balance and promote transition from latent infection to active disease. IGRA, interferon-γ release assay; LTBI, latent tuberculosis infection; TNF-α, tumour necrosis factor-α; TST, tuberculin skin test.

Figure 1.3 The spectrum of Mycobacterium tuberculosis infection and the life cycle of M. tuberculosis. (Dheda *et al*., 2010)

1.3.2 Adaptive Immune Response

Adaptive immunity involves all immune responses evoked for specific antigens, and includes the development of immunological memory. CD4+ T-cells, CD8+ T-cells, and CD1-restricted T-cells are involved with DCs playing a vital role in both

the initiation and control of the adaptive immune response. The CD4+ T cells can be divided into T helper-1(Th1), T helper-2 (Th2), T helper-17 (Th17) and regulatory T cells (Tregs) (Table 1.1).

T-helper cell type	Effector Cytokines	Function
Th1	IFNγ, TNF-α, IL-2	Maturation and activation of granulocytes; pro-inflammatory
Th2	IL-4, IL-5, IL-9, IL-10, IL-4δ2	Promotion of antibody production by B cells; suppression of Th1
Th17	IL-17	Recruitment of neutrophils to site of inflammation; suppression of Th1 and Th2
Treg	TGF-β, IL-10	Modulation of Th1, Th2, Th17

Table 1.1 Characteristics of the 4 main types of T helper cell types

M.tb evokes a classical Th1-type cellular immune response in the host. Indeed, it is this response that is believed to be primarily responsible for much of the pathology associated with clinical TB.

During infection, MHC Class II molecules present large peptides from *M.tb* residing within the macrophage vacuoles to the CD4+ T-cells, resulting in their activation. Interleukin-12 is induced following phagocytosis of *M.tb* bacilli by DCs and macrophages, which polarizes cells to a Th1-type immune response (Ladel *et al*., 1997). $CD4^+$ T-cells are of primary importance in the cell mediated immune

(CMI) response and in the control of TB infection. Their primary role is the production of IFN-γ, and possibly other cytokines, involved in macrophage activation, and enhanced mycobacteriostatic or mycobacteriocidal capabilities. Cytokines, such as IFN-γ, decrease phagocytic receptor expression resulting in diminished mycobacterial adhesion (Barnes, 1994). IFN-γ is a key cytokine in the control of *M.tb* infection and is produced by $CD4^+$ T cells, $CD8^+$ T cells and NK cells. Tregs are simultaneously activated and, although their role has not been definitively elucidated, they have been reported to be involved in immunosuppression of the effector cells (Askenasy *et al*., 2008).

Studies in murine models confirm that activated T cells migrate to the site of infection where they form typical tuberculous granulomas. Here both $CD4^+$ and $CD8^+$ T cells attempt to contain the infection within the granuloma and prevent disease reactivation. $CD8^+$ T cells attack infected macrophages causing lysis and release of bacilli from their protected environment. They are then exposed to activated macrophages for renewed uptake and killing. $CD8^+$ T-cells also play a significant role as they produce IFN-γ and IL-4. Thus, they may play a role in regulating the immune system balance between Th1- and Th2-type cells in the lungs of patients with pulmonary TB (PTB).

The manner by which mycobacterial proteins gain access to MHC Class I for presentation to the $CD8^+$ T cells is still poorly understood. It has been found that slow regression of disease while receiving therapy was associated with an increase in $CD8^+$ T cells in broncho-alveolar lavage (BAL) fluid (Yu *et al*., 1995). In addition, killing of intracellular bacteria was dependent on the cytolytic mediators perforin and granulysin (Stenger *et al.*, 1998). Some CD8+ T cells have an alternative, yet critical role in detecting infected cells where the pathogen is in the cytoplasm. CD1 molecules transport lipid antigens via the endoplasm for presentation at the cell surface to CD8+ T cells (Stenger *et al*., 1997).

1.4 DIAGNOSIS

After exposure to *M.tb*, 30-50% of all individuals show no immunodiagnostic signs of infection (tuberculin skin test reactivity). Of the remainder, who probably develop LTBI, approximately 5% develop active TB within the next 2-5 years of initial infection. The remaining 95% are not likely to develop disease unless they develop an immuno-compromised state such as HIV, diabetes, malnutrition (Dheda *et al*., 2010). Concurrent symptoms of active TB disease include a prolonged cough, at first non-productive then mucoid and purulent, with low grade remittent fever, night sweats, chills, weight loss, anorexia, and fatigue, chest pain and haemoptysis later in disease (Kumar, 2007).

Timeous and accurate diagnosis of TB depends upon clinical recognition of typical symptoms together with a medical history. Ideally this diagnosis should be confirmed microbiologically. Other modalities may also facilitate the diagnosis such as radiology, immunological and molecular-biological assays or where available, histology (Lange and Mori, 2010).

1.4.1 Smear & culture

In order to confirm diagnosis of *M.tb* disease, culture of acid fast bacilli (AFB) in sputum is the “gold standard” test. A drawback of microbiological smear is the number of false negative results, most likely due to the need for a minimum of 5 000 bacilli/ml of sputum (Teixeira *et al.*, 2007). The preferred method of staining for *M.tb* is auramine-rhodamine for fluorescence microscopy, which is more sensitive than conventional Ziehl-Neelsen staining for light microscopy (Steingart *et al*., 2006). Culture of *M.tb* organisms from a specimen [sputum, pus, cerebro-spinal fluid (CSF), BAL, etc.] is the most common way to make a definitive diagnosis. However, results may take four to eight weeks, and expenses are often prohibitive in resource-poor, high TB-burden settings.

Culture is far more sensitive than smear requiring only 10-100 viable bacilli per ml of sputum (Colebunders and Bastian, 2000). Indeed, while smear is inexpensive, rapid and relatively simple, making it the preferred diagnostic technique for low-income high TB-burden areas, its low sensitivity remains a serious diagnostic limitation. It has been shown to have a sensitivity of 20-80% (Steingart *et al.*, 2007). Smear negative TB occurs more frequently in HIV-positive than in HIV-negative individuals (Johnson *et al*., 1998). Only 44% of all new cases are identified by the presence of AFB on sputum smears (WHO, 2009).

1.4.2 Immunological tests

There are several other techniques currently employed to aid the diagnosis of TB (latent infection or active disease). These include the tuberculin skin test (TST), interferon-gamma release assays [(IGRAs) including T-SPOT®.*TB,* QuantiFERON® TB Gold in Tube], LAM ELISA, and nucleic acid amplification tests (NAATs). A short summary of each of these follows.

1.4.2.1 Tuberculin Skin Test (TST)

The TST remains the standard for immunodiagnosis of latent TB since the beginning of the 20th century. Until recently, it was the only means of diagnosis in LTBI. A meta-analysis by Pai *et al.* in 2008 showed the overall sensitivity of the TST to be approximately 77% (Pai *et al.*, 2008). The TST involves an intradermal injection of an extract of sterile supernatant of *M.tb*-cultured filtrate called purified protein derivative (PPD) (Sarrazin *et al*., 2009). In individuals previously exposed to TB, 48-72 hours after PPD administration a palpable raised induration of the skin develops. The extent of induration is measured and a cut off of 5-15 mm determined by patient risk-factors such as immune status, HIV-status, high-risk exposure to mycobacteria, diabetes mellitus, etc is used to define a subject with LTBI.

The TST reaction is based upon a delayed type hypersensitivity response to PPD, a crude mixture of approximately 200 *M.tb* antigens (Lee and Holzman, 2002). As this mixture contains antigens common to *Mycobacterium bovis* and other non-tuberculous mycobacteria (NTM), it's specificity in Bacille Calmette-Guérin (BCG)-vaccinated populations may be unreliable. The "boosting" phenomenon results in complicated interpretation of serial TST testing (van Zyl-Smit *et al.*, 2009). Positivity of the TST may also be associated with age, foreign birth immigrants in low burden, first world settings) and increased time as a healthcare worker – all indicators of possible remote exposure to *M.tb* (Alvarez-Leon *et al.*, 2009). In immunocompromised individuals such as sufferers of HIV/AIDS, advanced TB, malnutrition, poverty or malignancy, sensitivity may be low. (Andersen *et al.*, 2000). Additionally, Lawn *et al.* reported that patients with a low CD4+ cell count have a high rate of false negative results (Lawn *et al.*, 2009).

It is not an expensive test requiring a specialised laboratory, and can be performed in the field. Specific training is required to apply the test accurately but no other medical expertise is required. Thus, it is ideal for resource-poor settings. However, one of the biggest drawbacks of the TST is that it is unable to distinguish between active TB and LTBI (Lange *et al*., 2009).

1.4.2.2 Interferon gamma release assay (IGRA)

A new *in vitro* blood test which measures the Th1-type immune response has been developed namely the interferon gamma release assay (IGRA). The UK National Institute of Health and Clinical Excellence (NICE) now recommends the use of IGRAs in i) individuals who are at risk of LTBI and have tested positive by TST, and ii) individuals in whom the TST would be unreliable e.g. immunosuppressive conditions, who would often give a false negative result. In contrast, the guidelines of the United States of America recommend the use of IGRAs in all groups as a replacement of the TST, if available (Mazurek *et al*., 2010).

There are currently two types of commercially available IGRAs, namely i) QuantiFERON®-TB Gold and Gold-in-Tube (QFT and QFT-IT respectively) (Cellestis Ltd, Carnegie, Australia) and ii) T-SPOT®.*TB* (Oxford Immunotec, Oxford, United Kingdom). IGRAs have been shown to be highly sensitive and specific tests for the diagnosis of latent *M.tb* infection (LTBI) (Dheda *et al*., 2005; Pai *et al*., 2008).

Both types of assays determine the *ex vivo* release of interferon-γ (IFN-γ) from pre-sensitized host effector (memory) T-cells after overnight stimulation with *M.tb*-specific antigens. These antigens are early secreted antigenic target 6kDa (ESAT-6), and culture filtrate protein 10 (CFP-10) and additionally TB 7.7 in the QFT-GIT assay. They are encoded on the region of difference (RD-1) in the *M.tb* genome, a region associated with virulence and attenuation of the BCG vaccine. This domain is absent from BCG substrains and many non-tuberculous mycobacteria (NTM) except *M. marinarum*, *M.szulgai* and *M.kansasii* (Arend *et al*., 2000; Dheda *et al*., 2009; Lalvani *et al*., 2001). There are, however, homologues of these proteins in the genome of *M.leprae* (Gey van Pittius *et al*., 2002).

IGRAs show less cross-reactivity with the BCG vaccine strain (Dheda *et al*., 2005; Pai *et al*., 2007) and with most NTM (Andersen *et al*., 2000), thereby showing improved specificity than the TST. This could be explained by the fact that PPD stimulation in the TST measures a variety of protein antigens, whereas IGRAs measure IFN-γ responses to only two or three antigenic stimuli encoded on the RD-1 of *M.tb.* The approximate specificity of the IGRAs is 95% (Lewis *et al*., 2003). A further advantage of the IGRAs is that a single visit by the patient may be adequate in appropriate circumstances. In addition, should serial testing be necessary, such as in immuno-compromised patients or health care workers, this is possible as there is no induction of the "boosting" phenomenon (van Zyl-Smit *et al*., 2009).

IGRAs, unlike the TST, have an internal positive control to indicate whether test results are indeterminate or evaluable (Dheda *et al*., 2005). The validation of IGRAs to detect LTBI suffers from the lack of a gold standard, so active TB disease has often been used as a proxy marker for LTBI. Studies of IGRA responses in asymptomatic first-world subjects at low risk of LTBI were performed to determine specificity (Dheda *et al*., 2005; Lalvani and Pareek). IGRAs may be more reactive than TSTs in certain settings; they may detect up to 70-80% of individuals with LTBI (Mori, 2009; Pai *et al.*, 2004).

The use of IGRAs to diagnose LTBI is not commonly used in the developing world. Although a substantial proportion of the population has LTBI, the lifetime chance of developing active TB may be as low as 5% (Falk and Fuchs, 1978). Yet, in the developing world IGRAs probably have especially great importance as compulsory BCG vaccine administered at birth may increase the risk of a false-positive TST result. The high annual risk of TB transmission results in healthcare facilities being overly stretched to handle the high numbers of active TB cases, especially in children. In addition, technical problems such as the requirement for cold rooms to store reagents and incubators to process samples, highly qualified trained laboratory personnel in equipped laboratories, and the high cost pose even greater problems. Consequently, the use of IGRAs in the developing world would probably be restricted to the following situations: epidemiologic surveillance, children with TB, malnourished TB patients and HIV-TB co-infection (Dheda *et al*., 2009).

Sensitivity of the IGRAs for diagnosis of active TB is suboptimal, with false-negative results in both HIV-negative and HIV-positive individuals (Menzies *et al.*, 2007). This is explained by the fact that IGRAs rely on CD4+ cells and their performance will be influenced by conditions with dysfunctional CD4+ cells. In addition, the underlying immunosuppressive mechanisms of cytokines such as

IL4, IL9 and IL10, and the action of Tregs can result in false negative IGRAs. These will be discussed in Chapter 3.

T-SPOT®.TB

The T-SPOT®.*TB* test measures spots formed by purified IFN-γ-producing T cells in an enzyme-linked immunospot (ELISPOT) technique [spot forming cells/well (SFCs/well)].

Figure 1.4 T-SPOT®.*TB* test kit

For the T-SPOT®.*TB* assay, peripheral blood mononuclear cells (PBMCs) are incubated with ESAT-6, CFP-10 and PHA in a 96-well microtiter ELISPOT plate for 16-20 hours. If the patient is infected with *M.tb*, the T-cells may recognise the antigens and secrete IFN-γ which is captured by antibody in the bottom of each well. The cytokine-bound antibodies are detected and represented by spots in the well, where each spot corresponds to one reactive T-cell. These SFCs are counted to indicate the frequency of *M.tb*-specific T cells (Lalvani, 2007). A negative control well may contain up to 5 spots whilst a positive result is defined as ≥6 spots in either ESAT-6 or CFP-10 wells after subtracting the number of spots in the negative control well. For > 6spots in the negative control well: ESAT-6 or CFP-10 should have at least twice the number of spots as found in the negative control well to define a positive result. An indeterminate result defines a negative control well showing > 10 spots, or positive control well < 20 spots. A recent report by van-Zyl Smit *et al.* has suggested a "borderline zone" of

4-8 spots inclusive wherein samples should be cautiously interpreted depending on clinical characteristics (van Zyl-Smit *et al.*, 2009).

QuantiFERON®-TB Gold and QuantiFERON®-TB Gold-in-Tube

The QuantiFERON (QFT) contains an extra *M.tb*-specific antigen, TB7.7 in vacutainer tubes pre-coated with peptides, thereby ensuring immediate exposure to the specimen of whole blood.

Figure 1.5 QuantiFERON ®-TB Gold test kit

After overnight exposure to *M.tb* antigens, the QFT assay uses an enzyme-linked immunosorbent assay (ELISA) technique to measure an absolute quantity of IFN-γ (IU/ml) in the supernatants of whole blood.

The sensitivity and specificity of the T-SPOT®.*TB* and QFT were examined in an in-depth analysis by the Centres for Disease Control and Prevention, updated guidelines for using IGRAs (Mazurek *et al.*, 2010). The pooled results of the studies were as follows (Table 1.2).

Table 1.2 Pooled sensitivity and specificity rates of the immunological tests for TB infection

TB test	Sensitivity	Specificity
TST	89%	86%
T-SPOT	91%	88%
QFT-GIT	81%	99%

Lee *et al.* found that the sensitivity in QFT ranges from 74-83% (Lee *et al.*, 2006). Ferrara *et al.* evaluated this assay in a hospital setting and found the specificity to be 92.5% (95% CI 86-99%) (Ferrara *et al.*, 2005). Pai *et al.* conducted a meta-analysis of studies discussing sensitivity and specificity of IGRAs in various populations and specific influential factors (Pai *et al.*, 2004).

1.4.2.3 Discordance between the TST and IGRA

While both the TST and IGRA may be useful diagnostic tests, a meta-analysis of 17 studies by Pai *et al..* of 17 studies showed that there is only a 60-80% agreement between the two tests and kappa (k) statistics were highly inconsistent ranging from 0.03 to 0.87. (Pai *et al*., 2004). Similarly, Menzies *et al.* reported a discordance of 29% between the TST and QFT (Menzies *et al.*, 2007) and Soborg *et al..* reported a kappa value of 0.2 due to discordant positive TST and QFT tests (Soborg *et al*., 2009). Ponce de Leon *et al.* reported that discordance is also evident in patients with inflammatory disease (Ponce de Leon *et al*., 2008).

In determining whether the TST or IGRA test reading is diagnostic, the test cut-off is pivotal. The manufacturer's guidelines of the commercial IGRAs are pre-determined. Van Zyl-Smit *et al.* have recently described a "borderline zone" where within-person variability may be seen (van Zyl-Smit *et al.*, 2009). The TST cut-off is determined by specific host conditions. A cut-off of ≥5mm is used in immunosuppressed individuals such as HIV/AIDS or patients receiving immunosuppressive treatment. For high risk individuals such as prison workers, nurses, employees in homeless shelters, hypodermic drug users or in people arriving less than 5 years previously from a high burden area, a cut-off ≥ 10mm is used. In persons with no known risk for TB infection, an induration cut-off of ≥ 15mm is used (Duke, 2001).

A false positive TST result may result from cross-reactivity with NTM as well as previous BCG vaccination (Lee and Holzman, 2002). BCG uses a mixture of approx 200 non-specific antigens, some of which are in common with NTM as well as *Mycobacterium bovis* BCG, while IGRAs use only a few *M.tuberculosis*-specific antigens (Nahid *et al*., 2006; Pai *et al*., 2004). Additionally, recent studies are suggesting a "boosting" phenomenon whereby the body experiences a "vaccination" type effect which may affect any subsequent tests readouts (Mawa *et al.*, 2004; van Zyl-Smit *et al.*, 2009). So too, the reading of the TST may be somewhat variable when interpreting the induration width. In active TB the TST may be false negative in 10-25% of cases (Nash and Douglass, 1980). The latter result occurs from anergy or a similar immunocompromised condition as the TST is a delayed type hypersensitivity reaction.

The IGRA and TST do not measure the same components of the cellular immune response. Central memory T-cells persist long after an infection has been cleared, so may only produce IFN-γ during the longer incubation period associated with the TST, thus showing TST positivity but false IGRA negativity (Pai *et al.*, 2007). The production of and response to chemokines involved in the

TST response may have little influence on the antigen specific T cells responsible for IGRA readings (Orme *et al*., 1991).

1.4.3 LAM detection

An alternative TB-detection method is based upon direct ELISA detection of LAM, a 17.5kDa heat-stable lipopolysaccharide antigenic marker of the genus *Mycobacterium*. LAM is a major and structurally vital glycolipid component of the mycobacterial cell wall, accounting for up to 15% of the total bacterial weight (Hunter *et al*., 1986). The mycobacterial envelope consists of the plasma membrane, the wall and the capsule; it is this membrane to which LAM and it's precursors lipomannan (LM) and phosphatidylinositol mannosides attach (Besra *et al*., 1997). The antigenic glycolipid permeates beyond the capsule (Besra and Brennan, 1997; Nigou *et al*., 2003). The wall resembles a gram-positive wall but has lipid mycolate esters probably arranged to form a permeability barrier to polar molecules (Daffe and Draper, 1998) (Figure 1.6).

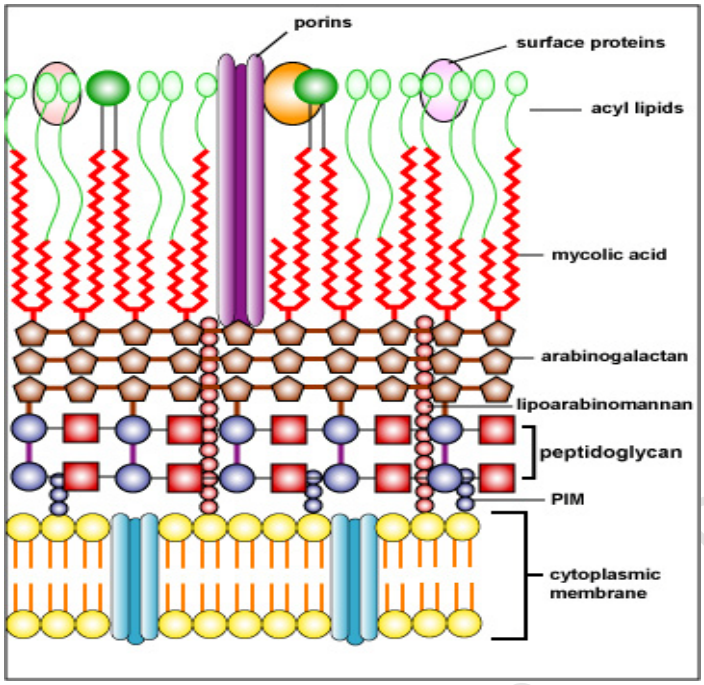

Figure 1.6 Cross-section of the mycobacterial envelope showing the position of LAM

There are 3 types of LAM structure according to the mycobacterial species: mannose-capped LAM (ManLAM), phospho-myo-inositol-capped LAM (PILAM) and non-capped LAM (AraLAM). The capping-type is an extremely important determinant in the host immune response. ManLAM, which is specific to metabolically active, slow growing, or degrading *Mycobacterium tuberculosis* (Chan *et al*., 1991), has been shown to have a powerful anti-inflammatory effect and functions as a key virulence factor. Thus, LAM is a crucial factor in determining the outcome of infection (Briken *et al.*, 2004).

Cell surface receptor molecules such as mannose receptors (MRs) are expressed on macrophages and monocytes and mediate binding of *M.tuberculosis* via the latter's surface glycoprotein, LAM. It is this coupling in addition to apoptosis, and phagosome maturation which LAM inhibits. LAM further disables the host immune response by interrupting IFN-γ signaling in

macrophages and IL-12 secretion by dendritic cells. Such mechanisms allow mycobacterial survival and dissemination to other parts of the body. Results from in vitro assays have shown that LAM mediates its biological activity via Toll-like receptor 2 (Briken *et al*., 2004).

Being independent of the host immunological system, these assays, such as the LAM antigen-capture ELISA assay, are especially beneficial in patients with compromised immunological systems e.g. HIV, malnutrition, cancer. TB-HIV co-infection is particularly prevalent in poverty stricken areas making this type of assay more accurate.

1.4.4 NAATs and culture techniques

More recently, newer diagnostic technologies with higher sensitivity and specificity are emerging. However, expertise and costly equipment make them somewhat prohibitive for poor-resource areas. They include *M.tb*-specific nucleic acid amplification tests (NAATs), biochemical tests used to detect mycobacteria. They were developed in order to shorten the time needed to obtain a diagnostic test result. NAATs include any test that directly detects bacterial genetic material such as PCR and DNA probes.

Further diagnostic options include colorimetric redox indicator methods (Martin *et al.*, 2008), and BACTEC460 and Mycobacterial Growth Indicator Tube (MGIT) culture systems. Studies have shown that liquid cultures are more rapid and sensitive than solid medium cultures (Cruciani *et al*., 2004). Liquid cultures detect bacterial growth in novel and unique ways such as radioactivity and oxygen concentration changes (Reisner *et al.*, 1995).

1.4.5 Sample acquisition

Research into any pathological mechanisms is dependent upon the sample quality. Sputum is the most commonly used sample for TB diagnosis. Apart from expectoration, gastric washings in children, bronchoscopy, laryngeal swab, or even fine needle aspiration or tissue biopsy can be performed. The sensitivity of routine smear-microscopy ranges from 20-80% (Steingart *et al*., 2007), culture takes several weeks to yield results, and suitable representative biological samples are frequently unobtainable either due to lack of sputum production or poor sample quality. This problem is increased by the HIV pandemic which increases the incidence of smear negative and sputum-scarce TB (Moore and Roper, 2007). Sputum induction is far less invasive, allows sampling of the lower airways in a non-invasive way (Vignola *et al.*, 2002) and is equal in yield to bronchoscopy. In addition, it does not require substantial medical expertise or great expense. Sputum induction is especially useful in sputum-scarce or smear-negative patients e.g. HIV-positive patients, as the saline irritates the airways forcing expectoration. In 1992 Pin *et al.* described the first standardised method for sputum induction using 3-5% hypertonic saline via a hypertonic nebulizer, to study inflammation in asthmatic patients (Pin *et al.*, 1992).

However despite all these methods, an effective and accurate rapid diagnostic tool is greatly lacking as TB still remains one of the leading global causes of mortality and morbidity.

1.5 TREATMENT

Anti-TB treatment involves the use of a combination of drugs for 6 months, often following the directly observed treatment short course (DOTS) program. Multiple drugs are used in order to prevent resistance to one drug and also to maximize the complimentary effects of the drugs e.g. isoniazid is bactericidal against

replicating bacteria while pyrazinamide is effective against bacteria located in acidic environments, inside macrophages or in areas of acute inflammation. Isoniazid, rifampicin, pyrazinamide and ethambutol are normally prescribed for two months, followed by isoniazid and rifampicin alone for a further four months. When the DOTS program is properly implemented, the success rate normally exceeds 95% and prevents the emergence of new multi-drug resistant TB strains. If no treatment is given fatality rates exceed 50% (Onyebujoh and Rook, 2004).

1.6 MULTI-DRUG RESISTANT TB (MDR-TB)

In every country surveyed, MDR-TB has been documented. It is defined as TB which is resistant to the most powerful TB drugs (Rook *et al*., 2004), namely rifampicin and isoniazid. Resistance to all major TB drugs has emerged, however any combination not including the above-mentioned drugs is not classed as MDR. More recently in South Africa, “extensively drug-resistant tuberculosis“ has emerged. This is defined as MDR-TB that is resistant to quinolones and also to any one of kanamycin, capreomycin, or amikacin (WHO, 2006). This strain of TB does not respond to any treatment currently available.

1.7 RATIONALE & OBJECTIVES

Every year TB claims the lives of more than 2 million individuals worldwide. Rapid, accurate, and field-friendly diagnostic tools would contribute to quicker disease intervention and treatment. Indeed, deficiencies in current TB diagnostic tools may largely explain the low global case detection rate of about 62% (World Health Organisation, 2009). The development and evaluation of new diagnostic tools is a priority for the Global Stop TB Working Group on New Diagnostics. To prevent transmission of *M.tb*, prompt and accurate diagnosis is a matter of urgency. Keeler *et al.* estimated that 400 000 lives could be saved annually (Keeler *et al.*, 2006).

It is estimated that a third of the world's population is infected with latent tuberculosis infection (LTBI). Although the TST is currently the only validated tool for diagnosis of LTBI its specificity is poor due to cross-reactivity with the BCG vaccination and non-tuberculous mycobacteria (NTM), and sensitivity is suboptimal in immuno-compromised individuals. Recently RD-1 antigen specific interferon-gamma release assays (IGRAs), namely T-SPOT®.*TB* and QFT-GIT have been developed. However, there is discordance both between the two commercial IGRAs and between the IGRAs and the TST which needs to be further explored.

In addition to the difficulty of diagnosing LTBI, sputum scarce and smear-negative TB poses great difficulty, which is exacerbated by the HIV pandemic. Sensitivity of smear microscopy is low, and culture takes several weeks. The yield of diagnostic methods is low and obtaining representative samples is often challenging (Dheda *et al*., 2009). Sputum induction is far less invasive than bronchoscopy and offers an equal if not higher microbiological yield. Additionally, it is far more patient-friendly and less expensive making it more suitable for resource-poor settings where sputum microscopy is often the most important

diagnostic test. Although IGRAs have been designed for use with blood, the feasibility and diagnostic utility of this rapid immunonodiagnostic method using induced sputum in a high burden setting is unknown and needs to be investigated.

More recently antigen-based diagnostic assays for TB have been developed. An ELISA-based assay using LAM has been tested in urine, cerebrospinal fluid, pleural fluid and expectorated sputum with moderate to poor success. The advantages of induced sputum may make it a more feasible sample for this immunodiagnostic assay.

1.8 AIMS OF THE STUDY

The aims of this study were:

1. to investigate the discordance between the antigen-specific interferon-γ release assay (IGRA) and tuberculin skin test (TST) with quantitative PCR using skin biopsy samples,
2. to evaluate the performance outcome of an RD-1 antigen-specific IGRA using cells from induced sputum for the diagnosis of *M.tb* infection,
3. to evaluate the utility of lipoaraminomannan (LAM) detection via enzyme-linked immunosorbent assays (ELISA) as a diagnostic test for *M.tb* infection using induced sputum.

All of the above were investigated in a high burden TB setting.

CHAPTER 2

GENERAL MATERIALS & METHODS

2.1 Discordance study

2.1.1 Subject recruitment

Individuals in whom the tuberculin skin test (TST) was indicated were recruited in Cape Town, South Africa and in London, UK between May 2009 and March 2010. Only individuals with no report of recent or current TB, or known immunosuppressive illness such as diabetes, cancer, hypertension, malnutrition or HIV/AIDS were included in the study. All recruits were over the age of 18 years. Human participation was approved by the University of Cape Town Health Sciences Faculty Research Ethics Committee (REC reference number 192/2008), and the research ethics committee for University College London Hospital (UCLH). Written informed consent was obtained from each participant.

This project formed the beginning of a larger collaboration between the Infection & Immunity Department, University College London (Dr. M. Noursadeghi, UCL) and our Lung Infection and Immunity Unit, Department of Medicine, University of Cape Town (UCT). I visited there for two months to carry out the necessary laboratory work.

2.1.2 Sample acquisition

The TST was administered intradermally by the Mantoux method using 0.1ml (2U) of PPD RT23 (2-TU, Statens Serum Institut, Copenhagen, Denmark) into both forearms. Each injection site was marked with indelible ink. At the same time, 10ml of peripheral blood was drawn to perform the T-SPOT®.*TB* assay. 4-6 hours later the injection site on the 1st forearm was anaesthetized with lignocaine,

before taking 2 x 3mm punch skin biopsies. The biopsies were symmetrically situated on either side of the marked injection site as close to each other as possible. 48 hours after initially administering the TST, 2 further skin biopsies were taken from the second forearm.

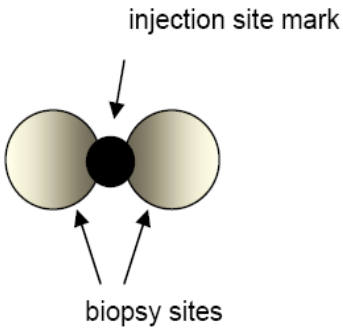

Figure 2.1 Situation of the 2 x skin punch biopsies on either side of the TST intradermal injection on each forearm

2.1.3 Sample processing

2.1.3.1 Skin biopsies

Two biopsies were taken on each forearm. The first biopsy was placed into an Eppendorf containing 1ml RNAlater buffer. The 2nd biopsy was embedded in Tissue-TEK® optimal cutting temperature (OCT) compound 4583 on a 20mm cork disc, then immediately frozen with cryospray, placed in a small durable plastic container and stored at -80ºC for later processing.

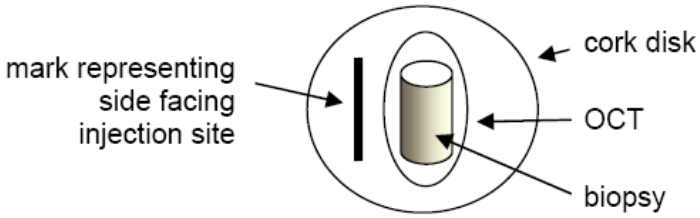

Figure 2.2 Mounting of the skin biopsy on a cork disk

All skin biopsies were stored at -80ºC until batched transport to UCL for further polymerase chain reaction (PCR).

For PCR, RNA was purified from skin biopsies using the RNeasy lipid tissue kit (Qiagen). Quantitative expression profiling was performed using the RT² Profiler™ PCR Array system with customised primer sets (http://www.superarray.com). The following genes were included in the analysis:

1. Markers of innate dendritic cell/ macrophage activation e.g. TNFα, IL-1β, IL-8, IL-12;
2. Markers of T-cell activation and proliferation e.g. IL-2;
3. Markers of T-helper1 (Th1)-type response e.g. IFN-γ, IL-12, Tbet
4. Markers of T-helper2 (Th2)-type response e.g. IL-4, GATA3
5. Markers of T-helper17 (Th17)-type response e.g. IL-17A, RORc
6. IFN-γ response genes in dendritic cells and macrophages e.g. IRF1

As part of this collaborative project, I worked on this PCR analysis at University College London.

2.1.3.2 T-SPOT®.*TB*-assay

The blood was transported immediately to the Lung infection & Immunity Unit, Groote Schuur Hospital where the T-SPOT®.*TB* assay was performed as per the manufacturer's instructions:

50ul of antigen (ESAT-6 and CFP-10) and phytohaemaglutinin (PHA) was added to duplicate wells followed by 250 000 cells made up in 100ul media (wherever possible). All samples underwent overnight stimulation at 37°C in a humidified 5% CO_2 incubator. Spot-forming cells (SFCs), representing IFN-γ release by peptide-specific T cells, were counted with an automated AID-ELISPOT reader system (Autoimmun Diagnostika, Strassberg, Germany). Simultaneously SFCs were counted manually to confirm the accuracy of the machine as induced sputum SFCs have an irregular appearance (see Figure 4.3). The mean of duplicate numbers of SFCs determined the reactivity of the assay. The results were classified as either positive or negative as per the pre-defined guidelines described in chapter 1.

2.1 Induced sputum study

2.2.1 Subject recruitment

TB suspects were recruited from Langa and Chapel Street Primary Health Care Clinics, and the Respiratory Clinic at Groote Schuur Hospital in Cape Town between March 2008 and April 2009. Study inclusion criteria included possible TB based on clinical examination in subjects who had not commenced TB treatment and were over 18 years of age. All the enrolled patients had a chest x-ray, which was reported by at least one pulmonologist, and subsequent treatment was continued independently of the research laboratory findings. Human participation was approved by the University of Cape Town Health Sciences Faculty Research Ethics Committee (REC number 421/2006), and written informed consent was obtained from each participant.

2.2.2 Sample acquisition

Individuals were requested to rinse their mouth in order to reduce the risk of contamination with salivary squamous epithelial cells and mouth flora. Strict infection control measures were followed during all acquisition and processing of sputum samples. The patient was then seated in an enclosed, negative-pressure sputum induction booth. Approximately 20ml of sterile 5% hypertonic saline (Sabax, Adcock Ingram Critical Care (PTY) LTD, Johannesburg, South Africa) was delivered via a Wilson's 402A ultrasonic nebuliser (Medimark, South Africa) over a period of 15 minutes or until 4-12ml of induced sputum could be collected in a sterile container. No pro-expectorating manoeuvres, such as chest percussion, were employed. Induction was immediately terminated if side effects such as dyspnoea, chest discomfort or nausea were reported. After sputum induction, the samples were transported to the Lung Infection & Immunity Laboratory, Groote Schuur Hospital and processed within 2 hours. An aliquot of each sample was processed at the National Health Laboratory Services (NHLS) microbiology laboratory, at Groote Schuur Hospital. Smear microscopy was performed by auromine-O staining for acid-fast bacilli (AFB). All samples were cultured for mycobacterium and other pathogens (MGIT 960).

After the results were obtained from the NHLS, the following criteria were used for diagnostic classification:

1. **Definite TB**: A clinical presentation compatible with TB with at least 1 positive culture (from any specimen) for *M.tuberculosis* with response to anti-TB therapy (Wilson *et al*., 2006).
2. **Probable TB**: A clinical-radiological picture highly suggestive of TB and/or anti-TB treatment initiated by the attending clinician based on clinical suspension, but not meeting the above-mentioned criteria for definite TB.

3. **Non-TB**: no evidence of TB based on smear microscopy and culture, and no radiological evidence to support the diagnosis of active TB, with or without an alternative diagnosis being established on patient follow-up.
4. **Indeterminate TB**: either the culture or chest x-ray results (or both) were unavailable, and the patient was lost to follow-up or transferred to another centre, thus making it impossible to confidently rule-out or rule-in TB. These patients were excluded from the analysis.

2.2.3 Sample processing

2.2.3.1 Processing of induced sputum

An optimization phase was first completed given the paucity of established processing methods of induced sputum for ELISPOT assays.

All induced sputum samples were processed using a protocol based on that used by Bhowmik *et al.* (1998) with minor modifications to isolate mononuclear cells which could be plated in the T-SPOT.®*TB* wells and developed as per the manufacturer's instructions.

Homogenization with 0.1% dithiothreitrol (DTT, Sigma, UK) was performed upon arrival of the sample in the laboratory. The optimal volume and rolling time for solution, dilution and homogenization was determined by the sample's final macroscopic appearance, viability of the induced sputum mononuclear cells and the degree of background discoloration in the T-SPOT®.*TB* (Oxford Immunotec).

Immediately after homogenization, the sample was either filtered through 2-ply sterile gauze, a 100µm cell strainer, or separated using Histopaque-1077 (Sigma). Sputum mononuclear cells were isolated by centrifugation at 1200rpm for 10 minutes and then counted using a Neubauer Haemacytometer chamber.

The highest viable cell count with the least particulate and unwanted matter retained determined the optimal filtration method.

To determine the optimal number of cells to be used per ELISPOT well, mononuclear induced sputum cells from 3 subjects were plated in duplicate wells at concentrations of $1x10^4$, $2.5x10^4$, $5x10^4$, $1x10^5$, $2.5x10^5$ and $5x10^5$ cells/well. The mean number of spot-forming cells (SFCs) for each concentration was calculated, and the optimal number of cells per well was determined.

The final method used was dilution of the induced sputum with 2 x volume of 0.1% DTT. After rolling for 20 minutes, the sample was filtered and washed twice with PBS. An aliquot was sent to the NHLS for microbiological testing (Gram and acid fast staining as well as TB culture). The non-cellular fraction was drawn off, while the remaining mononuclear cells were resuspended in 1-2 ml AIM-V® medium (Gibco, Invitrogen) and counted using a Neubauer haemacytometer chamber.

2.2.3.2 T-SPOT®.*TB*-assay

Induced sputum mononuclear cells were used in the T-SPOT®.*TB* assay as explained above in section 2.1.3.2.

2.2.3.3 LAM-specific techniques

4-10ml of the non-cellular fraction of the induced sputum was aliquoted into a 15ml tube and incubated in a waterbath at 95ºC for 30 minutes. After cooling to room temperature, the sample was centrifuged at 300g for 15 minutes to remove any solid particles and unwanted matter. 2 x 1.5mls aliquots of supernatant were stored at -20ºC until further processing.

To process, the samples were thawed at room temperature. In order to detect and measure the LAM amount in each sample, the Clearview® TB ELISA (Inverness Medical Innovations Inc, Scarborough, ME, USA) point-of-care ELISA strip kit was used. 100µl of processed sputum was added to the pre-coated wells including the relevant positive and negative controls. All controls, standards and samples were added in duplicate. The plate was then sealed with the self-adhesive plastic film provided in the kit and incubated at 24ºC in an air-conditioned room for 60 minutes.

The well contents were decanted into a wash-basin and the plate firmly tapped over a paper towel to remove any residual liquid. The wells were then manually washed 4 times with 250µl of prepared wash solution. 100µl of LAM-specific polyclonal antibody conjugated with horseradish peroxidase was added to the wells, at the same rate and sequence as the initial loading of the samples. The incubation and wash steps, as described above, were repeated. After washing a further 4 times, 100µl of tetramethylbenzidine (TMB) single component chromogenic substrate solution was added to the wells at the same initial rate and sequence. The plate was re-sealed and incubated at room temperature for 15 minutes. The reaction was stopped by adding 100µl of 1 molar sulphuric acid (in-house) to the plate at the same rate and in the same sequence as the TMB solution. Gentle shaking of the plate at each stage ensured there were no air bubbles and that the well contents were properly mixed.

Absorbance was measured at 450nm, single wavelength. Based on the standard curve established for each experimental plate, the OD reading was used to calculate the sample's LAM concentration. The assay results are calculated as per the manufacturer's instructions, the cut-off value was determined by the sum of 0.1 and the mean OD value of the negative control well. All samples with a mean OD above the cut-off value were defined as positive for LAM. Interpretation of LAM results was blinded and independent of laboratory or clinical diagnoses.

2.2.3.4 Protein quantification in induced sputum

The LAM-containing non-cellular fractions were isolated from varying volumes of induced sputum so had to be normalised for volume, total number of cells and protein concentration. In order to measure the protein content of the samples the Bio-Rad Protein Assay Kit II, 500-0002 (Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, California) was used.

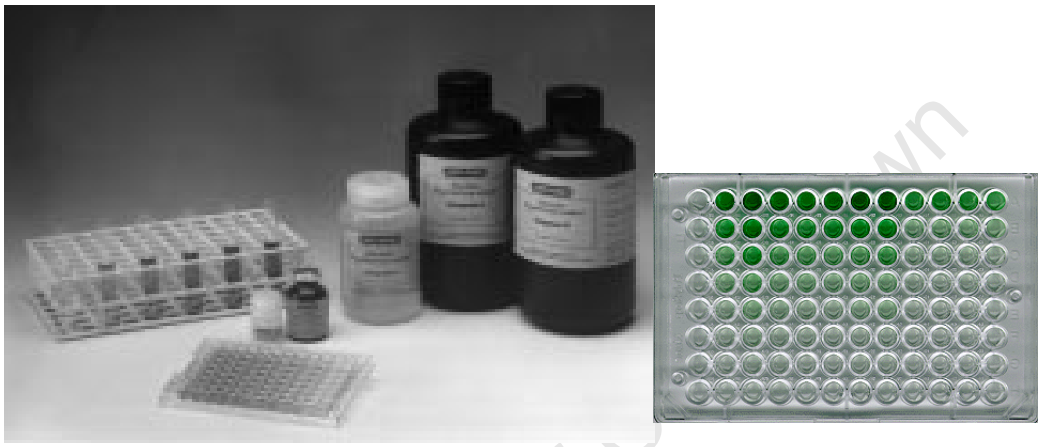

Figure 2.3. Bio-Rad Protein Assay Kit II, 500-0002

The dye reagent of the Bio-Rad protein kit was prepared by diluting the dye reagent concentrate with double distilled water at a ratio 1:4. The prepared dye was filtered through Whatman #1 filter paper to remove any particulates. Six serial dilutions of bovine serum albumin, 1.45mg/ml (the protein standard), were prepared with double distilled water to serve as standards for the ELISA assay. All standards and samples were plated in duplicate.

Ten microlitres of each standard and sample solution was loaded into separate microtiter wells of a Greiner, flat-bottomed plate. 200µl of diluted dye reagent was added to each well in the same sequential order to ensure timing remained constant. The samples and reagents were covered with aluminium foil to protect them from light, and were thoroughly mixed using a microtiter plate shaker at

room temperature for 5-60 minutes. The Bio-Rad protein assay was read at 600nm, single wavelength on an automated ELISA plate reader.

CHAPTER 3

THE DISCORDANCE BETWEEN THE TUBERCULIN SKIN TEST (TST) AND INTERFERON GAMMA RELEASE ASSAY (IGRA)

INTRODUCTION

One third of the world is infected with TB, yet only 10% of these will develop active TB. The immune system may contain the infection to keep the individual symptom free (Kaufmann, 2006). To date the TST, which was introduced in 1890, has been relied upon to diagnose LTBI. The TST contains an extract of cultured filtrate called purified protein derivative (PPD).

The TST is cumbersome to perform, and may be affected by BCG vaccination, recent TST administration (van Zyl-Smit *et al*., 2009), environmental mycobacterial exposure and anergy in individuals who are immunosuppressed (Dheda et al., 2005), thus confounding interpretation of the results. The reading of the TST is prone to reader variability when interpreting the induration width (Huebner *et al*., 1993), and requires a second visit to be read.

More recently, interferon gamma release assays (IGRAs) have been developed using more *M.tb*-specific antigens [early secretory antigenic target-6 (ESAT-6) and culture filtrate protein -6 (CFP-10)] in an attempt to overcome several of these issues. The principle underlying these assays is that T-cells, previously sensitised to tuberculous antigens, will produce IFN-γ upon re-encountering *M.tb*-specific antigens (Pai *et al*., 2007). IGRAs have been found to be advantageous over the TST in terms of their higher specificity in persons previously BCG vaccinated, particularly after birth. The results of the IGRA may be less subjective (Andersen *et al*., 2000).

It has been well documented that the agreement between the TST and IGRA is not 100%. A meta-analysis by Pai *et al.* showed an overall agreement of only 60-80% between the two tests and kappa (k) statistics were highly inconsistent ranging from -0.03 to 0.87 (Pai *et al*., 2004). Similarly, Menzies *et al.* reported a discordance of 29% between the TST and Quantiferon (QFT) assay (Menzies *et al*., 2007).

There are two types of discordance namely TST-positive, IGRA-negative and TST-negative and IGRA-positive. Please see the introduction for more detail. Briefly, a false positive TST can be due to prior BCG vaccination, or previous exposure to NTM, while a false negative result could occur in immunosuppressed patients such as those with HIV/AIDS or taking chemotherapy, diabetics or malnourished individuals. A positive IGRA with negative TST could indicate LTBI, and we hypothesize that a false negative IGRA could result from the immunosuppressive effects of regulatory T cells or cytokines such as IL-4, IL-9 or IL-10.

Despite much literature describing the discordance between the IGRA and TST (Arend *et al*., 2007; Ferrara *et al*., 2006; Lee *et al*., 2006), there are very few studies investigating the underlying mechanisms. I hypothesized that in TST+ve/ IGRA-ve discordant subjects the immunological profile in a skin biopsy is different from that of a TST+ve/ IGRA+ve and TST-ve/ IGRA-ve individual and represents a distinct immunodiagnostic entity which may give insight into the immunopathogenesis of discordance. This study aimed to use quantitative PCR, to investigate the correlation between the immunohistological profiles of TST skin biopsy samples and IGRA status. Of the two subsets of discordance which have been reported, I shall focus on TST-positive, IGRA-negative.

MATERIALS & METHODS

Human subjects and sample processing

A total of 20 individuals were enrolled in the prospective cohort study. 12 individuals were recruited in Cape Town, South Africa, and the remaining 8 were from the Department Infection & Immunity, University College London Hospital (UCLH), UK under the direction of Dr Mahdad Noursadeghi. Please refer to Chapter 2 for details concerning the recruitment and specific methodologies of the TST and T-SPOT®.*TB* assay.

Briefly, a TST was administered to both forearms of all individuals in the study. (At the same time 10ml of peripheral blood was drawn to be processed in the T-SPOT®.*TB* assay.) 2 x 3mm punch skin biopsies were taken from either side of the TST injection site on one forearm. 48 hours later, the TST was read and an additional two punch skin biopsies were taken from forearm 2.

The induration diameter was recorded as positive if it measured > 5mm. Positive and negative categories were then cross-referenced with those of the T-SPOT®.*TB* assay thereby categorizing samples as follows:

- TST positive and T-SPOT®.*TB* positive (+/+); or
- TST positive but T-SPOT®.*TB* negative (+/-); or
- TST negative and T-SPOT®.*TB* negative (-/-); or
- TST negative but T-SPOT®.*TB* positive (-/+).

PCR analysis

qPCR was used to evaluate the changes in levels of specific cytokines and transcription factors over the 48 hour time period. The qScript cDNA Supermix kit (Quanta BioSciences) was used to synthesize first strand cDNA. Quantitative PCR (qPCR) was performed using the Mastercycler Realplex 4 (Eppendorf) with inventoried TaqMan assays as follows:

Table 3.1 TaqMan Gene Expression Inventoried Assays

Gene	Applied Biosystems Assay ID
TNFα	Hs00174128_m1
IL1β	Hs01555410_m1
IL8	Hs99999034_m1
IL12B	Hs99999037_m1
IL2	Hs00174114_m1
IFNγ	Hs00174143_m1
IL4	Hs00174122_m1
IL17A	Hs00174383_m1
Tbet	Hs00894392_m1
GATA3	Hs00231122_m1
RoRc	Hs00172860_m1
FOXP3	Hs01085835_m1
IRF1	Hs00971960_m1
IFIT2	Hs00533665_m1

For these assays, the TaqMan Gen Expression Mastermix (Applied Biosystems) was used according to the manufacturer's instructions. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene to normalise the expression of the relevant cytokines. The 48-hour biopsies had higher levels of GAPDH. Yet this was not due to fluctuation with time, but rather due to the amount of RNA template added. The RNA concentration which was used for cDNA synthesis was not standardised and RNA levels were higher in the 2nd biopsy.

Statistical analyses

Discordance between the IGRAs and the TSTs was compared using appropriate parametric and non-parametric statistical tests with GraphPad Prism version 4.0 (or higher) for Windows. PCR profiling was done using RT² Profiler™ PCR Array system.

RESULTS

Twenty individuals were enrolled in the study, 12 from Cape Town, South Africa and 8 from London, UK. The T-SPOT®.*TB* results of 3 UK individuals were invalid due to a non-reactive positive control well. Thus, these samples were excluded from the study. As the UK cohort was very small, and they were relatively equally distributed across all of the pre-defined sub-groups, all results were pooled for analysis purposes. Three (17.6%) individuals were TST-positive, T-SPOT®.*TB* positive (+/+), 5 (29.4%) were TST-positive, T-SPOT®.*TB* negative (+/-), the majority [8 (47%)] were TST-negative, T-SPOT®.*TB* negative (-/-) while 1 (5.9%) individual was TST-negative, T-SPOT®.*TB* positive (-/+). However, the last-mentioned category could not be considered as it contained only 1 sample. See table 3.1.

Demographics of the patients

There was an equal distribution of TST-positive and TST-negative individuals i.e. 8 in each category. The mean (SD) age of individuals in the +/+ subgroup was 39.3 years (4.5years), the +/- subgroup showed a mean age (SD) of 40.8 years (10.1 years), and the mean (SD) age in the -/- subgroup was 35.9 years (7.3 years). 35% of the overall cohort were male, and all were HIV-negative. Of the 17 individuals included in the study, 3 (17.6%) were black African, 6 (35.3%) of mixed ancestry, 6 (35.3%) of European descent and 1 (5.9%) other. See table 3.1.

Table 3.2 Demographic information of the individuals recruited for the study.

	+/+ n=3	***+/- n=5***	***-/- n=8***	***-/+ n=1***	**p**
Age (SD)	39.3 (4.5)	40.8 (10.1)	35.9 (7.3)	22	
Gender					
Male	0	2 (40%)	3 (37.5%)	1	p= 0.93
Ethnicity					
Black African	1	2(40%)	0	0	
Mixed Ancestry	2 (66.6%)	1	3 (37.5%)	0	
European Ancestry	0	2(40%)	4(50%)	0	
Other	0	0	1	1	
Mantoux					
≤5 mm	0	0	8	1	
6-10mm	0	0	0	0	
11-15mm	0	0	0	0	
16-20mm	0	1	0	0	
21-25mm	1	3 (60%)	0	0	
>25mm	2 (66.6%)	1	0	0	
BCG vaccination					
Yes	3 (100%)	5 (100%)*	7 (87.5%)*	1	*p=0.25
Exposure to NTMs					
	1 (33.3%)	0	1 (12.8%)	0	
HIV Status					
	All subjects were HIV negative				
Previous TB					
Yes	1(33.3%)	0	0	0	

+/+ = TST positive, T-SPOT®.*TB* positive; +/- = TST positive, T-SPOT®.*TB* negative;
-/- = TST negative, T-SPOT®.*TB* negative; -/+ = TST negative, T-SPOT®.*TB* positive

TST results

All TST results were measurable and all individuals returned for TST reading. TST positivity was measured by induration diameter. A diameter >5mm was

categorized as positive. A total of 8 individuals tested TST-positive (47%). When comparing their mean (SD) age of 41.6 (8.45) years to that of the total cohort (37[8]), there was no significant difference (P=0.23).

Of the TST-positive individuals, all had been BCG vaccinated. The median TST diameter of the samples from RSA was 22.5mm (range: 0-52mm) compared to 5mm (range: 0-18mm) of the UK samples. See figure 3.1.

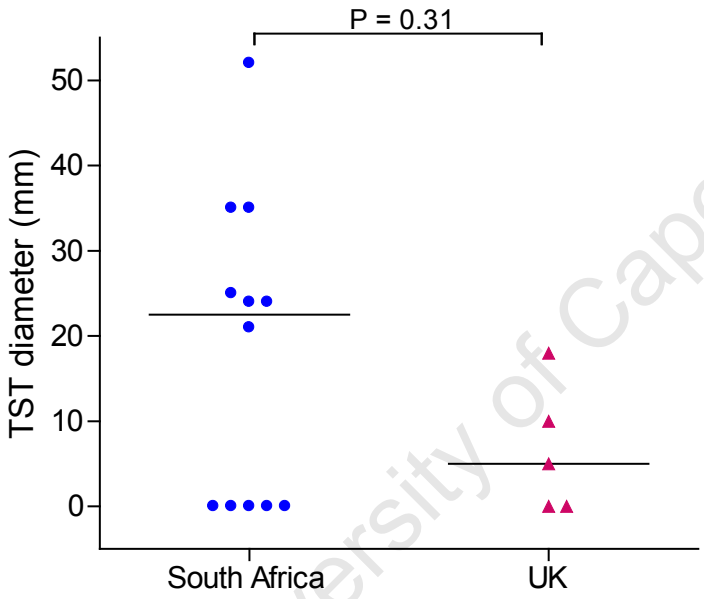

Figure 3.1 Median TST diameter of the South African cohort compared to the median of the cohort of the UK samples. Horizontal lines depict median values.

PCR results

The underlying cytokine changes were assessed over the 48 hour time period between biopsy 1 and biopsy 2. See Figure 3.2.

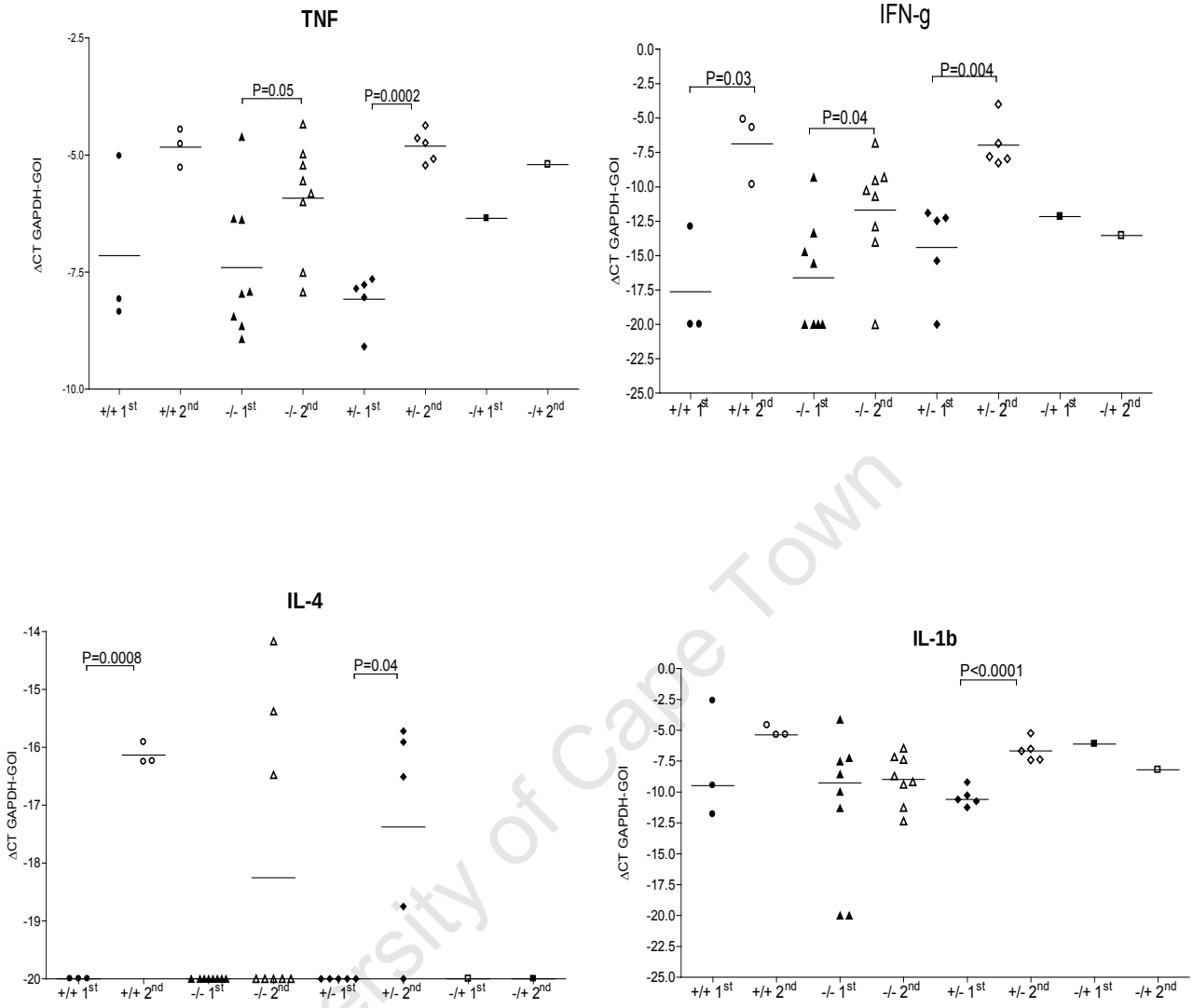

Figure 3.2 The cytokine changes across the 4 pre-defined subgroups compared over 48 hours. The horizontal lines depict median values. Closed symbols represent the first biopsy, while open symbols represent the second biopsy at 48 hours.

+/+1st/2nd = Biopsy 1or 2 in TST positive, T-SPOT®.*TB* positive subgroup;

+/-1st/2nd = Biopsy 1 or 2 in TST positive, T-SPOT®.*TB* negative subgroup;

-/-1st /2nd= Biopsy 1or 2 in TST negative, T-SPOT®.*TB* negative subgroup;

-/+1st/2nd =Biopsy 1 or 2 in TST negative, T-SPOT®.*TB* positive subgroup

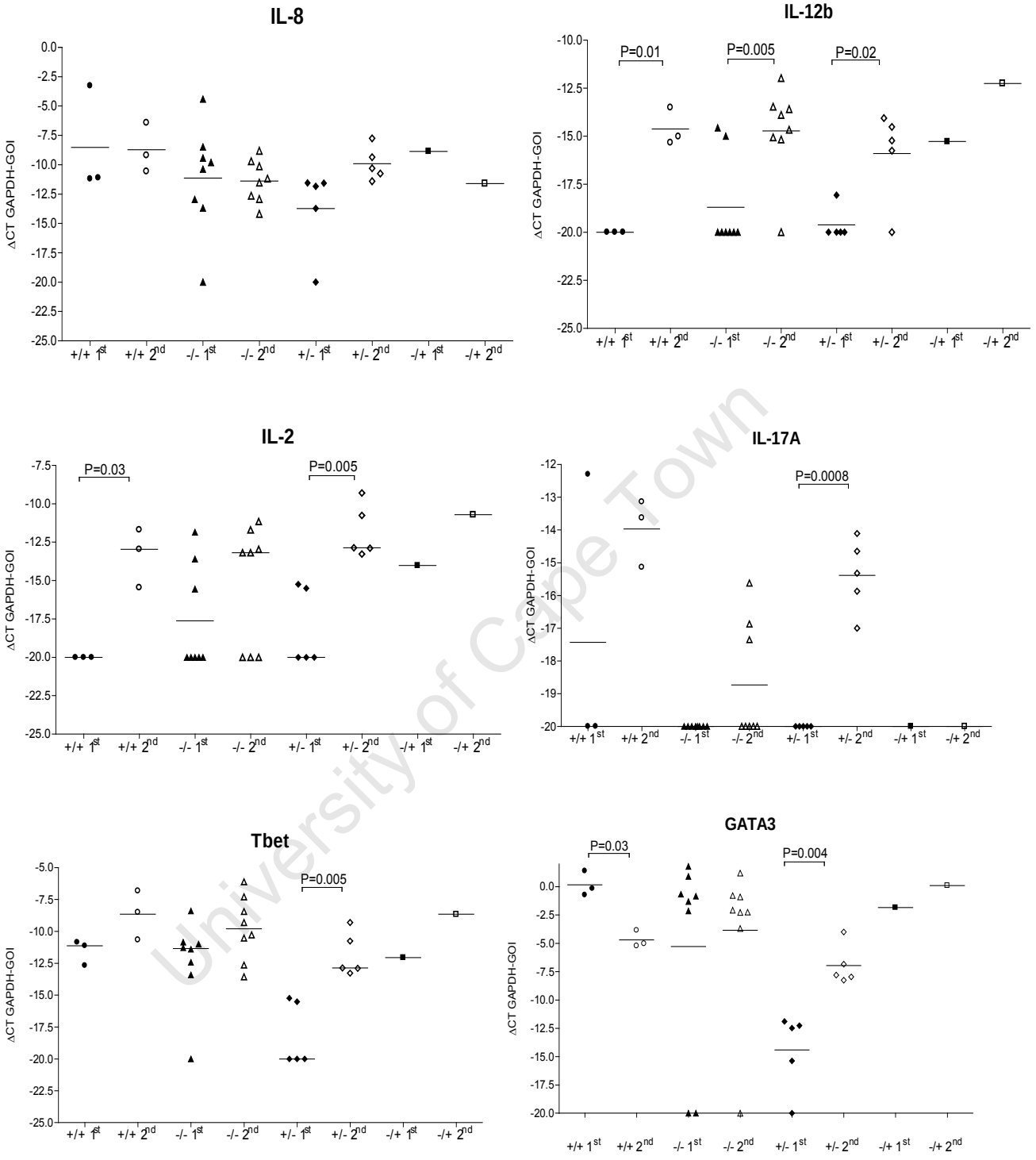

Figure 3.2 The cytokine changes across the 4 pre-defined subgroups compared over 48 hours. The horizontal lines depict median values. Closed symbols represent the first biopsy, while open symbols represent the second biopsy at 48 hours.

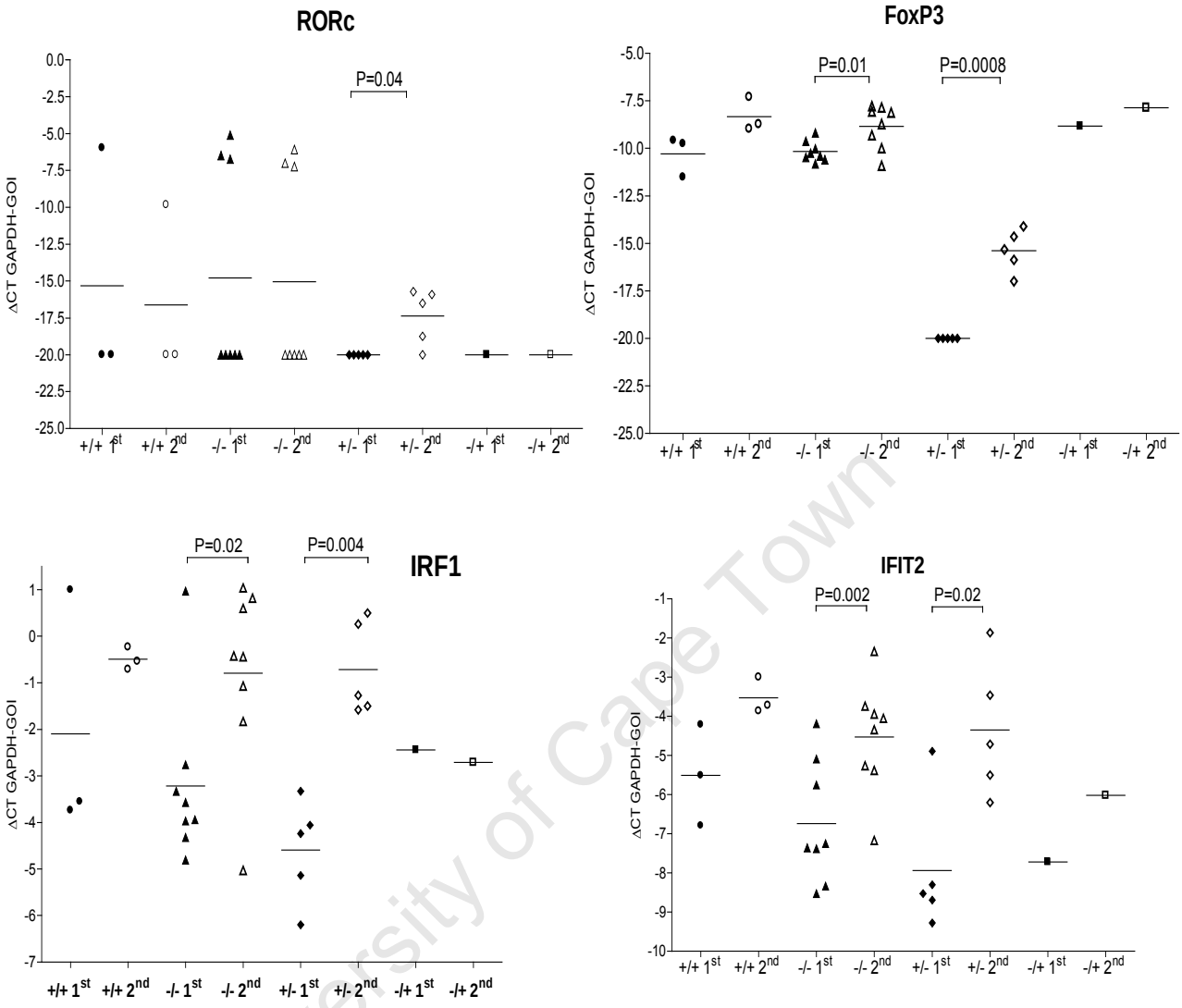

Figure 3.2 The cytokine changes across the 4 pre-defined subgroups compared over 48 hours. The horizontal lines depict median values. Closed symbols represent the first biopsy, while open symbols represent the second biopsy at 48 hours.

+/+1st/2nd = Biopsy 1or 2 in TST positive, T-SPOT®.*TB* positive subgroup;

+/-1st/2nd = Biopsy 1 or 2 in TST positive, T-SPOT®.*TB* negative subgroup;

-/-1st /2nd= Biopsy 1or 2 in TST negative, T-SPOT®.*TB* negative subgroup;

-/+1st/2nd =Biopsy 1 or 2 in TST negative, T-SPOT®.*TB* positive subgroup

All subgroups show an increase in IFN-γ and Tbet indicating a Th1-type immune response. GATA3 (p=0.004) and IL4 (p=0.0008) increase over the 48 hour time period, indicating a simultaneous Th2-type immune response. Thus there was a

mixed Th1/Th2 response. These may dampen the Th1-type immune response accounting for a false-negative IGRA in +/- subgroup. IL-17A and RORc increased significantly (p=0.0008 and p=0.04 respectively). Additionally FoxP3 increased in the +/- subgroup (p=0.0008) while it remained unchanged in the other subgroups. The +/- subgroup showed the greatest change in IL-1β (p<0.0001) and TNF-α (p=0.0002), both cytokines being associated with DC and macrophage activation causing inflammation. IL-8 showed no change in any of the subgroups. When comparing the cytokine changes of the individual subgroups it was evident that the cytokine pattern at baseline biopsy differed between all 3 groups.

Table 3.3 Change in mean cytokine level within each concordant/ discordant subgroup over 48 hours. I.e. Change from the 0 to 48 hour time point.

		+/+	**-/-**	**+/-**	***p* – value comparing 0 and 48 hours**
Th1-type response	IFN-γ	10.75§	4.93#	7.43*	0.004* 0.04# 0.03§
	Tbet	2.89	2.58	6.33*	0.005*
	TNF-α	2.32	1.49	3.27*	0.0002*
	IL-1β	2.86	2.11	3.77*	<0.0001*
Th2-type response	IL-4	3.87§	1.75	2.62*	0.04* 0.0008§
	GATA3	-4.54§	1.44	7.43*	0.004* 0.03§
Th17-type response	IL-17A	3.46	1.27	4.61*	0.0008*
	RORc	-1.93	-0.25	2.62*	0.04*
FoxP3-type response	FoxP3	1.96	1.33#	4.61*	0.0008* 0.01#

Concordant and discordant grouping by TST/ T-SPOT® *TB.* Only 1 individual was TST-ve /T-SPOT® *TB*+ve and thus not included in the analysis. Only significant p-values are indicated. (Note if correction for multiple comparisons is performed, only p values of <0.025 should be considered significant.)
§ = p-value in the +/+ subgroup; # = p-value in the -/- subgroup
* = p-value in the +/- subgroup

Comparison of the 48 hour biopsy cytokine responses across all three subgroups was done using a 1-way ANOVA, Kruskal-Wallis test with Dunn's post- test for multiple comparisons. There was a significant difference in IFN-γ (p=0.003) IL-1β, (p=0.01), IL-17A (p=0.005), GATA-3 (p=0.03) and FOXP3 (p=0.008). When comparing individual groups there was a significant difference between +/+ vs. -/- for IL-1β ($p<0.05$), IL17-A ($p<0.01$), +/+ vs. +/- for FOXP3 ($p<0.05$) and for -/- vs. +/- FOXP3 ($p<0.05$) and GATA-3 ($p<0.05$). The cytokine pattern at the 48 hour time point of each subgroup was unique. See table 3.3.

Table 3.4 Comparison of mean ΔCT GAPDH-GOI values measured between concordant/ discordant subgroups at the 48 hour time point.

		+/+	**-/-**	**+/-**	***p* – value at 48 hours**
Th1-type response	IFN-γ	-6.89	-11.69	-6.97	0.03
	Tbet	-8.66	-9.77	-11.82	0.13
	TNF-α	-4.83	-5.92	-4.81	0.18
	IL-1β	-5.10*	-8.98*	-6.64	0.01
Th2-type response	IL-4	-16.13	-18.25	-17.38	0.43
	GATA3	-4.70	-3.85*	-6.97*	0.03
Th17-type response	IL-17A	-13.97*	-18.73*	-15.39	0.005
	RORc	-16.61	-15.04	-17.38	0.83
FoxP3-type response	FoxP3	-8.33$^{\#}$	-8.84*	-15.39*$^{\#}$	0.008

The comparison was done using the 1-way ANOVA, Kruskal-Wallis test.*, $^{\#}$ indicates significance between groups after correcting for multiple comparisons.

DISCUSSION

The TST has for a long time been the only diagnostic test for LTBI. The newer IGRAs have been shown to be more specific but given the lack of a gold standard, it remains difficult to be certain of the assay's specificity for detecting latent TB infection. In addition, the discordance rates of up to 40% (Pai *et al.*, 2004) remain poorly explained. This study had a comparable discordance rate of 35% (6/17).

When looking at the change in cytokine levels over 48 hours, those in the +/+ subgroup are similar to those in the -/- subgroups, with the exception of IFN-γ and IL-4. The significantly raised IFN-γ response (p=0.03) in the +/+ subgroup would be expected as we believe that these individuals had LTBI. The increased Th2-response could possibly be explained by findings described by Rook *et al.* who showed that individuals living in Africa may have strong Th2-type immune responses to TB with increased levels of IL-4 (Rook, 2007). The -/- subgroup shows a modest, mixed response with IFN-γ and FoxP3 showing a significant increase (p=0.04 and p=0.01 respectively). Yet both TST and IGRA are negative. Perhaps this is because PCR is a more sensitive tool. It has been suggested that our available tools lack the sensitivity to detect LTBI (Dheda *et al*., 2010). The T cells in the skin are possibly more cross-reactive than those in peripheral blood, also making this test more sensitive. Additionally, LTBI may be a spectrum of infection. The level of infection may have been insufficient to be detected by either TST or IGRA but due to the sensitivity of PCR, a response is detected in those thought not to be infected by conventional testing. Further studies are required to explore this hypothesis using blood and tissue samples from different sites.

In the +/- discordant subgroup, however, there were significant changes in all Th1, Th2, Th17 and FoxP3-type responses. As TB drives a Th1-type response, these increases could be expected, yet the IGRA is negative. This could be LTBI with a false negative IGRA due to remote or low burden infection. Alternatively, the host genotype may predict unresponsiveness to ESAT-6 and CFP-10 due to pattern recognition profiles.

TB typically elucidates a Th1-type response, so an increase in IFN-γ, IRF-1, associated with macrophage response to IFN-γ, and Tbet in all subgroups could be expected. However, in the +/- subgroup, a Th2-type immune response is apparent: GATA3 ($p=0.004$) and IL4 ($p=0.0008$) increase over the 48 hour time period. These may suppress the Th1-type cytokines believed to be responsible for a positive IGRA by driving a Th2-type immune response. The increase in IL-17A and RORc and FoxP3 is also unique to the +/- subgroup. IL-17A and RORc favour a T-helper 17 (Th17) response. In addition to it's pro-inflammatory effect, Th17 may be targeted against extracellular bacteria (Tato and O'Shea, 2006). Fox-P3 characterizes regulatory T cells (Tregs) which suppress a variety of immune responses (Fontenot and Rudensky, 2005). Tregs contribute to the persistence of *M.tb in vivo* by suppressing antibacterial immune responses (Guyot-Revol *et al*., 2006; Lundgren *et al*., 2005). It is possible that the immunosuppressive effects of Tregs together with Th17- and Th2-type cytokines may explain the TST+ve/ IGRA-ve discordance. The above-mentioned changes in cytokines and transcription factors could be responsible for an immunosuppressive effect on IGRAs, thus resulting in false negative results. These observations need to be confirmed in antigen-stimulated PBMCs.

The PCR results suggested that the baseline cytokine levels were different in all 3 subgroups. Similarly the cytokine levels at the 48-hour time point were unique for each subgroup. Nemeth *et al.* confirmed such unique cytokine reactivity

patterns in his study of ESAT-6 stimulated cytokines over a 24-hour time period (Nemeth *et al*., 2010). Thus, in the TST+ve/IGRA-ve discordant subset the immunological profile in a skin biopsy differed from that of the TST+ve/IGRA+ve and TST-ve/IGRA-ve subgroups. This represents a distinct immunodiagnostic entity which may give insight into the immunopathogenesis of discordance. PCR-analysis of the +/- discordant subgroup showed cytokine changes confirming activity of Tregs and Th2-like cytokines. These effects could all contribute to a false negative T-SPOT®.*TB*, possibly explaining the discordant +/- subgroup.

An alternative explanation for TST+ve/ IGRA-ve discordance is false TST positivity. The TST measures cell-mediated immunity in the form of delayed-type hypersensitivity response to PPD so is positive whenever the host has previously been exposed to mycobacterial antigens. This could result from prior BCG-vaccination, previous exposure to TB, or exposure to some NTM. The overlap in genetic sequence between various NTM and *M.tb* may result in a false positive result. NTM are generally an important cause of false-positive TST in populations with a very low prevalence of TB infection and high prevalence of NTM sensitisation. However, Farhat *et al.* suggest that only 2% of individuals would have false positive TSTs after exposure to NTM (Farhat *et al*., 2006). If vaccinated at 2 years or older, the BCG vaccine could be responsible for up to 20% of false positive results (Farhat *et al*., 2006). Nevertheless, in developing countries BCG vaccination occurs at birth and may thus explain a false positive TST in this context.

The incidence of TB is far greater in developing, high burden countries. In South Africa 461 000 new cases are reported annually compared to only 9 000 in the UK (World Health Organisation, 2009). Consequently, the South African population will be far more exposed to mycobacteria and have a far greater chance of contracting infection. In this case the TST may be more highly reactive, as confirmed in this study by the median Mantoux of the SA population

being 22.5 (range: 0-52mm) compared to that of the UK population at 5mm (range: 0-18mm) See Figure 3.1. All individuals in this study had been BCG-vaccinated at birth.

Inclusion of an additional antigen in the IGRA which may be associated with disease or latency may prove useful. A novel antigen such as heparin-binding haemagglutinin (HBHA) may be such a possibility. It has been shown to be more sensitive than ESAT-6 and more specific than PPD for the detection of LTBI in small studies. In addition it was not influenced by BCG status of the individual (Hougardy *et al*., 2007).

IGRAs have been designed for the diagnosis of LTBI though their sensitivity and specificity for this purpose, particularly in high burden settings is controversial. Whilst the study of TST/IGRA responses may shed important light on immunopathogenesis, the real clinical challenge in high burden settings is the diagnosis of active TB. This is discussed in the following chapters.

This study served as a pilot study for possible further more detailed work on the discordance between the TST and IGRA. Although the results of this study appear promising and interesting, the sample size was very small. Larger studies are necessary in both high- and low-burden settings to confirm these findings.

CHAPTER 4

FEASIBILITY and DIAGNOSTIC UTILITY of ANTIGEN-SPECIFIC INTERFERON-γ RESPONSES for RAPID IMMUMODIAGNOSIS OF TUBERCULOSIS USING INDUCED SPUTUM

INTRODUCTION

Multiple factors severely hamper efforts to control TB. The sensitivity of routine smear-microscopy is approximately 50% (Cattamanchi *et al*., 2009), culture techniques take several weeks to yield results, and suitable representative biological samples are frequently unobtainable either due to lack of sputum production or poor sample quality. This problem is compounded by the HIV pandemic which increases the incidence of smear-negative and sputum-scarce TB (Moore and Roper, 2007).

Alternative techniques to obtain pulmonary samples are required in these patients such as sputum induction, gastric washings and bronchoscopy (Brown *et al*., 2007; Dheda *et al*., 2009; Schoch *et al*., 2007). However, gastric washings are largely limited to use in children while bronchoscopy is expensive, invasive and not widely available in resource-limited settings. In contrast, sputum induction has been shown to be safe, well tolerated and non-invasive and is more cost-effective than bronchoscopy for pulmonary TB diagnosis (Bhowmik *et al.*, 1998; Fishman *et al*., 1994; Hartung *et al*., 2002; McWilliams *et al*., 2002; Parry *et al*., 1995). Additionally, it has been shown to provide an equal, or higher microbiological yield when compared to bronchoscopy (Breen *et al*., 2007; Conde *et al*., 2000; Robinson *et al*., 1985) and is superior to gastric washings regarding speed to culture positivity and sensitivity (Kawada *et al*., 1996).

It is possible to collect more than one induced sputum sample on the same day resulting in faster diagnosis, treatment and a subsequent shorter hospital stay (Brown *et al*., 2007). Hartung *et al.* showed that induced sputum may diagnose TB in 29% of suspects who were either smear negative or sputum-scarce (Hartung *et al.*, 2002). Smear results from sputum induction and broncho-alveolar lavage (BAL) agreed in 75% of cases (Ganguly *et al*., 2008). Moreover, the predictive value of negative induced sputum culture has been shown to be approximately 96% (Andersen *et al*., 2000). Yet rapid diagnosis of *Mycobacterium tuberculosis* is still frequently impossible given the low yield of smear microscopy, approximately 7-32% in this context (McWilliams *et al*., 2002; Morse *et al*., 2008).

In resource-limited settings, therefore, the use of sputum induction could provide an ideal patient-friendly option. Being less costly, it is a more feasible option for primary healthcare clinics where the first possible contact for diagnosis of TB takes place. Multi-drug resistant (MDR) TB and extensively drug resistant (XDR) TB are becoming progressively more prevalent (Bell *et al*., 2003; Dheda *et al*., 2010). Furthermore, the WHO 2009 TB report indicated that 23% of the estimated 2 million HIV deaths in 2007 were due to TB. Thus early diagnosis is especially important in these two population groups. Sputum induction is particularly useful for smear-negative patients, e.g. HIV-positive patients, and for those who are unable to expectorate.

More recently, the IGRAs have been developed. There are currently two formats, an ELISPOT, and an ELISA. IGRAs have been shown to be highly sensitive and specific blood tests for the diagnosis of latent *M.tb* infection (Dheda *et al*., 2005; Pai *et al*., 2008). These rapid, in vitro immunoassays measure IFN-γ release by antigen-specific effector memory T cells after stimulation by *M.tb*-specific antigens [early secretory antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10)].

The commercially available IGRA (T-SPOT®.*TB*) (Oxford Immunotec, Oxford, UK) uses an ELISPOT format with the standardised TB-specific-antigens to accurately diagnose latent TB infection. Although IGRAs are more commonly used to detect IFN-γ release from peripheral blood T-cells, they have been successfully shown to be an accurate tool for the rapid diagnosis of active TB using cerebrospinal fluid mononuclear cells (Patel *et al*., 2009), and BAL cells (Jafari *et al*., 2009; van Zyl-Smit *et al*., 2010). Thus, it appears that the ELISPOT assay can be accurately used to diagnose active TB using cells from the site of disease. So I hypothesized that mononuclear cells isolated from induced sputum could be used in the ELISPOT assay to diagnose pulmonary TB.

However, there are no data reporting the use of induced sputum using the ELISPOT assay in any population. Therefore, the aim of this study was to determine the feasibility and performance outcome of the RD-1-ELISPOT to diagnose tuberculosis using induced sputum mononuclear cells in a high TB-burden setting.

MATERIALS & METHODS

A prospective cohort study including one hundred and one patients, in whom sputum induction was indicated, was performed. Please refer to chapter 2 for further details about patient recruitment and sample processing.

Briefly, induced sputum was obtained from TB suspects and processed within 2 hours. 28 individuals were included in an optimisation phase to determine the best method of isolating mononuclear cells from induced sputum to be used in an ELISPOT assay. The optimal homogenisation and filtration method, centrifugal speed and number of cells to be plated per ELISPOT well were determined. A validation phase based on these findings followed. The cells were plated in duplicate and incubated overnight with the antigens ESAT-6, and CFP-10. The number of spots seen in each well represents IFN-γ release by peptide-specific T cells and determines the reactivity of the assay. Diagnosis of TB was based on the number of spots/well according to the manufacturer's guidelines for peripheral blood mononuclear cells.

Statistical analyses

Results of the IGRAs were compared with diagnoses made by conventional microbiological sputum staining and bacterial culture. The number and reasons for indeterminate tests were recorded. Categorical variables were compared using the Chi-Squared analysis; continuous variables were compared using Students'-T test when normally distributed and the Mann-Whitney test for non-parametric distribution. A p-value of 0.05 or less was considered significant. GraphPad Prism (version 4.0 or higher) for Windows (Graphpad Software, San Diego California, USA. www.graphpad.com) was used for statistical analyses.

RESULTS

Demographics of the validation phase patients

The validation phase included 73 patient samples. 5 individuals were excluded as patient side-effects necessitated terminating sputum induction prematurely. The mean (SD) age of the definite, probable and non-TB groups was 38 (15) years, 48 (10) years, and 49 (17) years respectively. Approximately half of the cohort was male, with the probable TB group having the highest percentage (75%) of individuals. Similarly, the probable TB group also had the highest number of HIV-positive individuals (75%). Of the 68 samples, 20 were diagnosed with definite of probable TB while 48 had non-TB (Table 4.1).

Table 4.1. Demographic information of patients included in the validation phase excluding 5 patients in whom sputum could not be induced.

	Total	Definite TB	Probable TB	Non-TB	p
Number of subjects	68	16(23%)	4 (6%)	48 (71%)	
Age mean(SD)	46 (17)	38 (15)	48 (10)	49 (17)	p=0.03
Sex					
Male	33(49%)	10(63%)	3(75%)	20(42%)	p=0.14
Racial group					
Black African	29(43%)	9 (56%)	3(75%)	17(35%)	p=0.14
Mixed Ancestry	35(51%)	7 (44%)	1(25%)	27(56%)	p=0.39
European Ancestry	4(6%)	0	0	4(9%)	
Current smoker	20 (29%)	5 (31%)	2(50%)	13(27%)	p=0.75
HIV status					
positive	20 (29%)	7 (44%)	3(75%)	10 (21%)	p=0.09
unknown/ refused testing	10 (15%)	0	0	10 (21%)	
Previous TB (n=54)	16(24%)	5(31%)	2(50%)	9(19%)	p=0.29

Each patient was nebulized with 5% hypertonic saline for 5-20 minutes or until an acceptable sample could be obtained. The median volume of induced sputum was 7ml (range: 4-12ml). Eight patients produced≤ 1ml which was considered inadequate for further processing.

Optimization phase

The optimization phase included 28 TB suspects. It was found that by adding 2 x volume of 0.01% DTT solution to the induced sputum sample, and rolling the suspension for 20 minutes, the mucins were sufficiently digested to dissolve and homogenize the sample. This volume neither destroyed the mononuclear cells nor contributed to background discoloration of the assay. An increased length of mixing time was tested if dissolution and/or homogenization were inadequate. This did not significantly alter the mucus content and viscosity of the sample. In order to select the filtration method which would retain the highest number of viable mononuclear cells with the least particulate and unwanted matter, 2-ply sterile gauze, 100µm cell strainer and density centrifugation gradient were compared. The 2-ply sterile gauze gave a mononuclear cell loss of 18%, compared to 52% with the100µm cell strainer whilst density centrifugation gradient resulted in 87% cell loss. Thus, the gauze was selected as the optimal method (Figure 4.1).

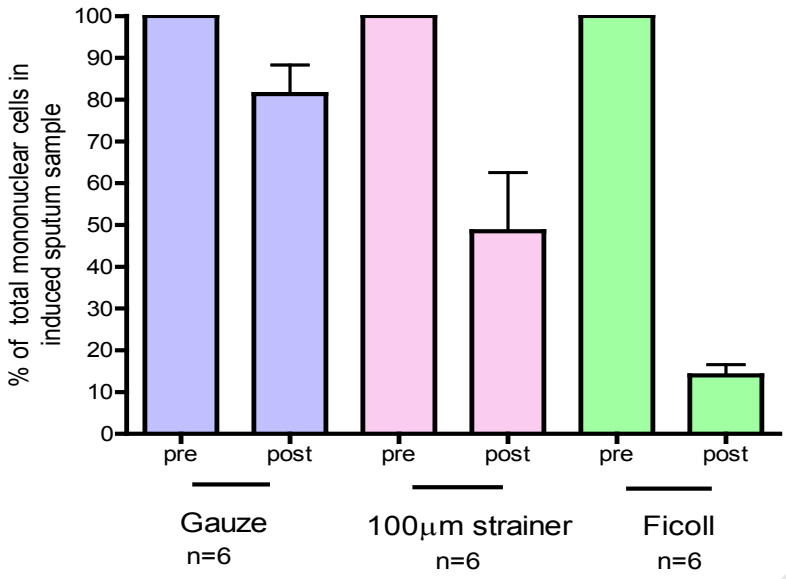

Figure 4.1 Comparison of 3 filtration methods. Three filtration methods were compared to determine the method which allowed the least cell loss, while effectively removing unwanted debris. The T cell number in the induced sputum sample was calculated before and after filtration.

In order to pellet the mononuclear cells to be plated in the ELISPOT wells, the sample was centrifuged at 1200rpm for 10 minutes, at which speed the viability of the induced sputum mononuclear cells was preserved. The optimal number of cells per well was determined as that which produced the highest number of ESAT-6-specific spot-forming cells (SFCs) per well with the same volume of antigen. The highest number of spots was seen when the induced sputum mononuclear cells were plated at 250 000 cells/well (Figure 4.2).

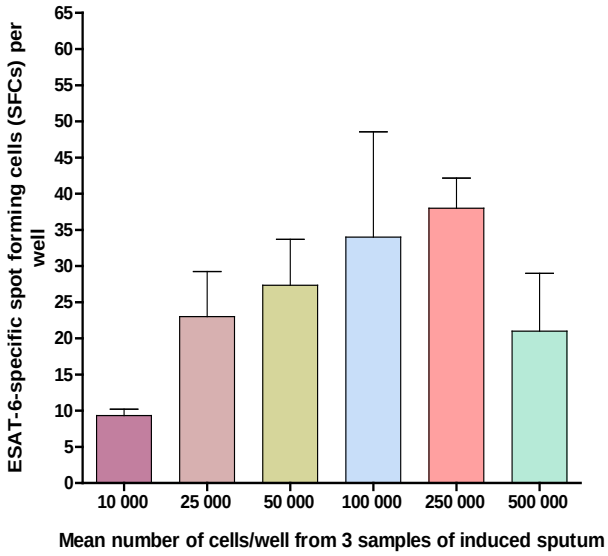

Figure 4.2A

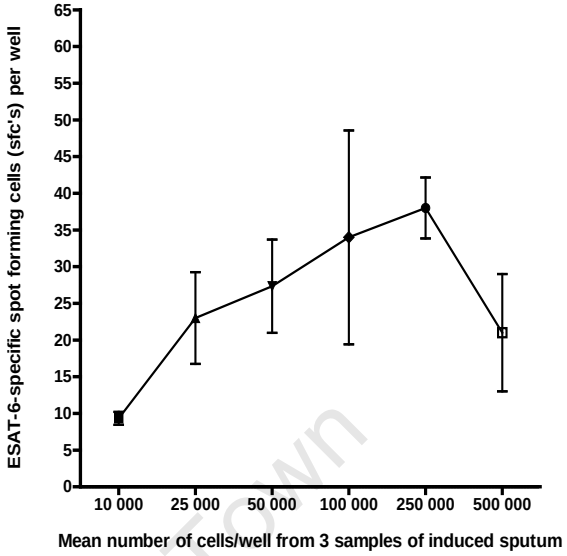

Figure 4.2B

Figure 4.2 Determination of optimal number of cells/well. ESAT-6-specific spot-forming cells from 3 samples of induced sputum were plated in duplicate at 5 increasing concentrations. The mean number of SFCs is shown (4.2A) and then reflected as a summated trendline of all 3 patients (4.2B).

Validation phase

The validation phase was carried out using the methods established in the optimisation phase. Seventy three of these subjects were included. 5/73 (7%) of individuals could not expectorate and sputum induction was prematurely terminated. Two patients experienced nausea and/or vomiting, while 3 suffered chest pain. Thus 68 TB suspects were enrolled in the validation phase of the study. 59 of the 68 (86.8%) assays failed (Table 4.3).

In the 8 samples where the induced sputum volume (less salivary content) was insufficient for further processing, the nebulization time or sample volume appeared adequate in the nebulization booth. There was no correlation between

induced sputum volumes, cell concentrations or age, and HIV status. A further 7 samples could not be used as adequate filtration was prevented due to large mucus plugs disallowing homogenization. After successful homogenization, the samples were filtered and washed twice with phosphate buffered saline (PBS). The median (range) sputum cell concentration (cells/millilitre sputum) was 0.1 x 10^6 cells/ml (0-2.4 x 10^6).

The average mononuclear cell viability which was checked prior to plating was greater than 90%. Calculation of the total sputum cell numbers yielded highly variable values. In 24 individuals there were insufficient cells to perform the T-SPOT®.*TB* assay which was the most common cause of assay failure. In 19 cases the samples' total cell yields were insufficient to plate the optimal 250 000 cells/well. Consequently, these assays could be performed using concentrations above 100 000 cells/well. Differential cell counts were performed using the Wright stain on slides prepared after a cytospin. The differential cell counts showed a median (range) lymphocyte count of 21% (0-75%) and neutrophil count of 79% (10-95%).

Twenty eight samples could be plated in the T-SPOT®.*TB* assay. However, of these 14 assays the positive or negative control well did not fulfil the manufacturer's requirements i.e. clear positive well, or SFCs visible in the negative control well. In 6 samples, the background debris in the wells was too excessive to permit interpretation of the assay. (Figure 4.3)

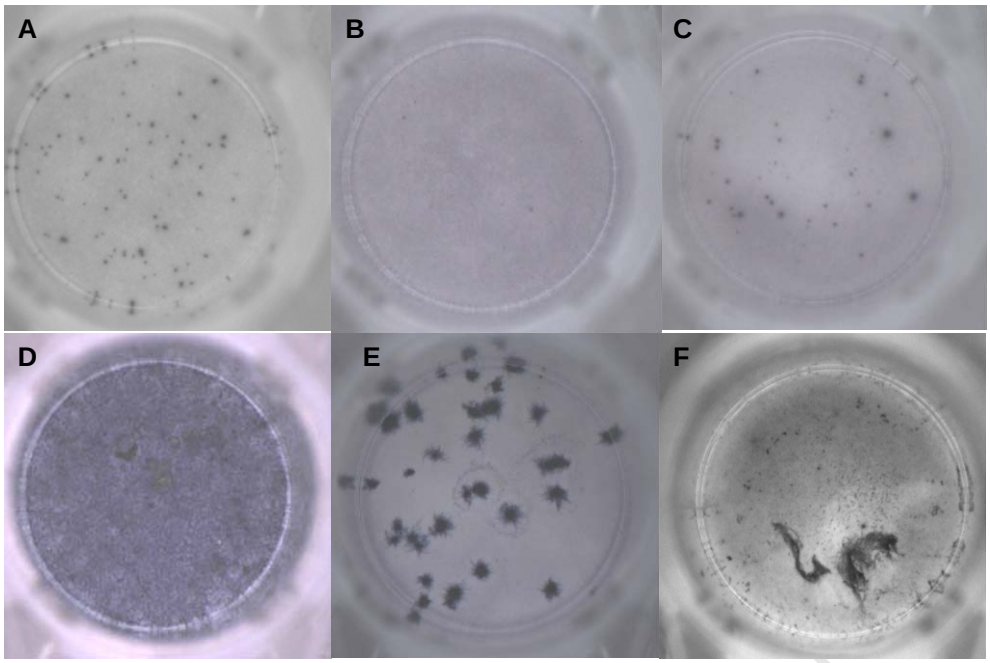

Figure 4.3 Photographs showing ELISPOT results using mononuclear cells from induced sputum. PHA-driven IFN-γ-specific spot-forming cells using mononuclear cells from induced sputum are shown in Panel (A). A negative control well is shown in (B). (C) shows ESAT-6-specific spot-forming cells in a patient with TB. (D) shows an inconclusive result due to high background discoloration. (E) and (F) show artifacts due to non-specific debris and mucus.

Therefore, despite optimisation of the assay to process the induced sputum only 8 (11.8%) of the 68 assays included in the validation phase produced an evaluable result (Table 4.2). Seven assays were positive with a median (IQR) of 96 (21,164) SFUs/10^6cells for the ESAT-6 and 64 (0,250) CFP-10 wells, respectively. All 7 positive samples were from TB patients and the single negative sample was from a non-TB patient. In 3 of the assays, wells with CFP-10 antigen could not be plated due to an insufficient number of mononuclear cells isolated from the induced sputum sample. The reasons for failure of sputum ELISPOT results by final diagnosis are summarized in table 4.3.

Table 4.2 Characteristics of the 8 evaluable assays using induced sputum in the T-SPOT®.*TB* assay.

T-SPOT®.*TB* result	Final diagnosis	Spot forming units/10^6cells				SI smear/culture	
		ESAT-6	CFP-10	Positive control*	Negative control*	AFB smear	MGIT culture
positive	Definite TB	120	not done[#]	100	40	positive	positive
positive	Definite TB	176	not done[#]	64	40	positive	positive
positive	Definite TB	160	not done[#]	80	20	positive	positive
positive	Definite TB	28	>250	>250	0	negative	positive
positive	Definite TB[+]	0	>250	>250	0	negative	negative
positive	Definite TB[+]	>250	0	60	0	negative	negative
positive	Probable TB	72	64	>250	12	negative	negative
negative	Non TB	0	0	40	0	not done[$]	not done[$]
Median		96	64	90	0		
IQR		21/164	0/250	63/250	0/25		

*As per the manufacturer's guidelines, an assay was considered valid if the number of SFU's/10^6cells in the negative control well was twice that of the positive control well. A value of >250SFU's/10^6cells was selected as the cut-off positive value.

[#]Insufficient cells to plate both wells.

[+] Patients were found to be culture positive on other biological samples and therefore classified as definite TB.

[$] Asymptomatic control patient with COPD and normal chest x-ray (sputum smear and culture was not indicated)

Table 4.3 T-SPOT®.*TB* assay outcomes and reasons for inconclusive test results in the validation phase.

Validation phase (n=68)[+] **Test outcomes/reasons for test failures**	**Definite and Probable TB** (n=20)[+]	**Non TB*** (n=48)
Sputum induction-related factors (n=39)		
1. Inadequate volume of sputum	1	7
2. Failure to homogenise sputum	1	6
3. Insufficient cells	6	18
T-SPOT®.*TB* related factors (n=20)		
1. Excessive debris (high background)	1	5
2. Positive control well failed	1	10
3. Negative control well failed	2	1
Valid T-SPOT®.*TB* result (n=8)		
1. Positive	7	0
2. Negative	0	1

*The Non-TB group included 1) 29 TB suspects classified as non-TB after follow-up and 2) non-TB control patients with alternative respiratory diseases e.g. interstitial lung disease.

[+]1 sample was rejected due to laboratory error

DISCUSSION

Patients who are either smear negative and sputum-scarce pose a diagnostic problem as diagnosis of TB is often made by identification of AFB in sputum. A disadvantage of this test is the number of false negative results obtained due to the need for a minimum of 5 000-10 000 bacilli/ml of sputum (Teixeira *et al*., 2007). However, recent studies show that the yield of sputum induction compares well to bronchoscopy (Anderson *et al*., 1995; McWilliams *et al*., 2002). Sputum induction is far less invasive, requires less medical expertise, and is less costly making it a feasible option for primary clinics where the first possible contact for diagnosis of TB takes place.

In this study, sputum induction was performed using a method based on that first described by Pin *et al.* to examine inflammatory cells in the airways (Pin *et al*., 1992). A single specimen of induced sputum can be used for immunological (IGRA) and bacterial or histological (stain/culture) diagnosis of *M.tb*. While the volume of induced sputum produced in this study ranged between 4-12ml, a study by Brown *et al.* in 2007 showed that positive culture results are not dependent upon sputum volume (Brown *et al*., 2007). Similarly, the number of mononuclear cells isolated from the induced sputum samples in our study did not correlate with volume. Therefore, neither obtaining a larger sample volume, nor exposing the patient to sputum induction for longer, was likely to overcome the problem of insufficient cells which occurred in approximately 36% of our assays. Cell viability was greater than 90% in all samples analysed which compared favourably with 83% (SD=15.6%) reported by Pizzichini *et al.* (Pizzichini *et al*., 1996). Consequently this factor is unlikely to have contributed to the poor test outcome.

Insufficient cell yield was the most common reason for unevaluable assays [34/68(50%)]. This is not surprising as sputum induction samples the large airways rather than the alveolar spaces where the majority of mononuclear cells reside. The low cellular yield is exacerbated by the variable though low proportion of lymphocytes in pulmonary samples. Nevertheless, increasing the numbers of cells per well is unlikely to have increased the number of evaluable assays as demonstrated by the cell-cytokine response curves.

Contamination with particulate debris and salivary squamous epithelial cells poses a problem when processing sputum, and has been approached using two alternative techniques. The entire expectorate, comprising sputum and variable amounts of saliva, can be processed as described by Fahy *et al.* (Fahy *et al*., 1993). This method has subsequently been modified whereby saliva and sputum are collected separately (Gershman *et al*., 1996). Alternatively, the expectorate can be poured into a Petri dish and the more opaque, viscid sputum selected using an inverted microscope (Pavord *et al*., 1997). The former technique was selected in this study as it is far more feasible, quicker, and doesn't require an inverted microscope which is often unavailable in resource-poor settings. However, there remains conflicting data as to whether or not the differential cell counts differ between the two techniques (Efthimiadis *et al*., 2002). This would be an important factor in determining assay reproducibility.

Filtration further minimizes contamination of the samples. While 48μm nylon mesh was used by Pizzichini *et al.,* this study showed 2-ply sterile gauze to be optimal (Pizzichini *et al*., 1996). In fact, the suspension of induced sputum homogenized with 0.1% DTT would not flow through the 48μm nylon mesh. Any remaining, contaminating squamous cells would only reduce the total and absolute cell counts of the induced sputum sample, but should not significantly alter the differential cell count. (The latter only becomes a consideration in immunological studies). Although fluid phase markers of inflammation such as

ECP and IL-8 do seem to be adversely affected by squamous contamination, to date this effect on IFN-γ remains unconfirmed (Simpson *et al*., 2004).

Of the 20 T-SPOT®.*TB* assays which were plated but unevaluable, 3 of the negative control wells failed i.e. had more than 6 SFCs. A study by (Martinez-Maza *et al*., 1984; Quiding *et al*., 1993; Robinson *et al*., 1985) suggests this may be unavoidable. They showed that blood mononuclear cells may release a high degree of spontaneous IFN-γ in the absence of any exogenous stimuli. Although the positive control well failed in 11 of the 20 assays, the optimisation findings demonstrated that 250 000 cells/well was optimal and thus the concentrations of cells was not altered during the validation experiments.

In contrast to these findings, Breen *et al.* demonstrated that induced sputum could successfully be used for immunological diagnosis of TB in 9 patients using PPD-driven cells in a flow cytometric or ELISPOT assay (Breen *et al*., 2007). However, the use of PPD to stimulate induced sputum mononuclear cells results in non-specific responses even in suspects with latent TB infection (LTBI). By contrast, RD-1 specific responses in the lung, as used in this study, are highly specific even in high burden countries where the prevalence of LTBI is over 50% (Dheda *et al*., 2009; Jafari *et al*., 2009). In addition, the ELISPOT assay requires far less training than flow cytometry, and is less costly making it more suitable for use in resource-poor settings. Breen *et al.* reported a total cell yield almost 6 times greater than those found in our study. However, we used unselected and consecutively recruited patients, a different population group, and different sputum processing methodology. Several other factors including disease extent, the small number of patients evaluated by ELISPOT in the Breen study, the duration of sputum induction and volume of hypertonic saline used, and malnutrition and hence attenuated immune responses, may explain the discordant results.

Eight of the RD-1 ELISPOT assays in this study were evaluable (in seven the diagnosis was TB). The immunological and clinical diagnoses of the positive and probable TB patients were concordant. Of the 20 positive TB patients in the validation phase, 7 (35%) were positively diagnosed by the T-SPOT®.*TB* assay. This low sensitivity would prevent this IGRA using induced sputum mononuclear cells from being clinically useful, if used in isolation, to diagnose TB.

In order to improve the ELISPOT utility, specific variables such as different media, culture of induced sputum mononuclear cells before plating in the wells, or variations in the concentration of capture-antibody in the wells, may be altered. As this small study was conducted in a high burden, resource poor setting, the findings require confirmation in different geographical settings with larger cohorts of patients.

In conclusion, despite the low frequency of valid results, it was confirmed in this study that rapid immunodiagnosis of pulmonary TB by antigen-specific IFN-γ ELISPOT responses, using mononuclear cells from induced sputum, is indeed possible (Cashmore et al.). However, due to several technical factors the proportion of inconclusive results is too high to warrant current clinically utility. Pizzichini *et al.* showed that the induced sputum non-cellular fraction, after isolation of the cellular components, could be used to measure molecular markers indicative of airway inflammation such as eosinophil activation and mast cell activation (Pizzichini *et al*., 1996). Recent studies have shown that an antigen-detection ELISA assay using the non-cellular fraction of biological fluids can be successful for the diagnosis of TB (Boehme et al., 2005; Dheda et al., 2009; Patel et al., 2009). Consequently, I proceeded to study the possibility of detecting LAM in the non-cellular fraction of induced sputum as a diagnostic marker of *M.tuberculosis.*

CHAPTER 5

FEASIBILITY OF A LAM ANTIGEN-CAPTURE ELISA ASSAY USING INDUCED SPUTUM TO DIAGNOSE TUBERCULOSIS

INTRODUCTION

Current diagnostic tools for tuberculosis are inadequate resulting in increased healthcare costs, higher patient mortality or morbidity, and greater long-term disability. TB diagnosis is particularly difficult in HIV-positive patients where smear microscopy is negative in 24-61% of pulmonary TB cases (Getahun *et al*., 2007) and radiological and clinical features are often atypical (Burman and Jones, 2003; Getahun *et al*. 2010). Mycobacterial culture takes several weeks for positive results (Pai *et al*., 2006). Newer T-cell assays cannot be used as reliable rule-in tests for active TB diagnosis (Dheda *et al*., 2009), and molecular assays are not widely available in resource-poor, high-burden settings (Pai *et al*., 2006). Thus, reliable, affordable, rapid point of care (POC) diagnostic tests using non-invasive, easily obtainable clinical specimens which are not influenced by the host's level of immunosuppression are urgently needed (Pai *et al*., 2009; Urdea *et al*., 2006).

More recently assays detecting secreted or circulating mycobacterial antigens, such as LAM, have been developed. LAM is an antigenic marker of the genus *Mycobacterium* and features amongst the candidate antigens for diagnostic TB serology. In order to measure the amount of LAM representative of *M.tuberculosis* infection, a LAM ELISA-based assay has been developed. This technique was first explored in sera (Sada *et al*., 1990). Many studies investigating the diagnostic performance of LAM in urine have been done. The LAM ELISA has also shown promise with the use of cerebrospinal fluid (Patel *et al*., 2009) and pleural fluid (Dheda *et al*., 2009). Pereira Arias-Bouda *et al.*

developed an in-house capture ELISA to detect sputum LAM (Pereira Arias-Bouda *et al*., 2000) and Dheda *et al.* tested the feasibility of using expectorated sputum in a LAM ELISA commercial assay (Dheda *et al*., 2010; van Zyl-Smit *et al*., 2009). The assay's poor performance was attributed to cross-reactivity between the mycobacterial-LAM and that of normal mouth flora and other mouth commensals.

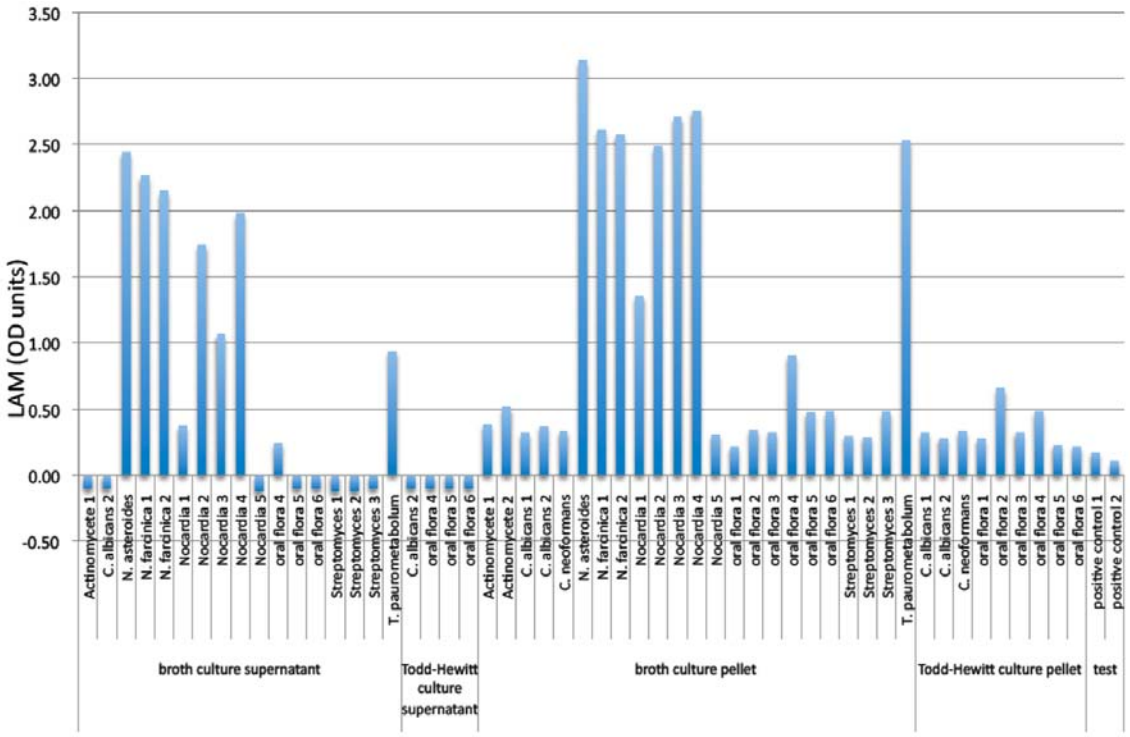

Figure 5.1 LAM positivity (>zero OD units=positive for LAM after subtraction of the negative control ie. Cutpoint is zero) in cultures of oral mouth flora and in organism-specific cultures (various Actinobacteria, including different strains of Nocardia and Streptomyces, and C.albicans, T.paurometabolum, and C.neoformans inoculated into normal broth culture [containing yeast extract] and Todd-Hewitt culture media [without yeast extract]. Normal oral flora from 6 different healthy control subjects was also cultured) (Dheda *et al*., 2010)

LAM-like molecules are not specific to mycobacteria as they are also found in actinomycetes (including the genera Rhodococcus, Corynebacterium or Gordonia), bacteria which are typical of mouth flora. Therefore, I hypothesized that an induced sputum sample would yield far better results as the mouth flora would be minimized or eliminated.

Due to the potential advantages of using induced sputum as a biological sample for rapid and user-friendly diagnosis, this study aimed to evaluate the feasibility of using induced sputum in a LAM ELISA-based assay to diagnose *M.tuberculosis* infection.

MATERIALS & METHODS

Human subjects & sample processing

A substudy involving 79 of the 101 TB suspects recruited for the ELISPOT assay was undertaken. Please refer to Chapter 2 for further details regarding collection and processing of induced sputum samples.

Briefly, the induced sputum sample was homogenized, filtered, then centrifuged to isolate mononuclear cells. This non-cellular fraction was drawn off then heated and centrifuged. The suspension was evaluated for LAM content using the Clearview® TB ELISA assay. The samples were evaluated as per the manufacturer's instructions.

Assays were normalised for volume, total number of cells, and protein content as the initial volume of sputum varied. Each normalisation strategy was compared.

Statistical Analysis

Performance of the LAM assay to correctly diagnose tuberculosis was compared to the reference standard (culture positivity). The appropriate parametric and non parametric tests were performed with GraphPad Prism version 4.00 (or higher) for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Additional tests were performed using OpenEpi, Version 2, open source calculator.

RESULTS

Patient demographics

Seventy nine TB suspects were included in the study. When stratified into the 4 pre-defined subgroups, there were 12 individuals with definite TB, 14 with probable TB and 43 with non-TB. 10 individuals remained indeterminate and were thus excluded from the study. The mean (SD) age was 47 years (17 years). 30% (21/69) of the cohort were HIV-positive (Table 5.1). In the definite TB group 33% (4/12) were HIV positive compared to 16% (7/43) in the non TB group (p=0.23).

Table 5.1 Demographic information of the TB suspects recruited for the study

	Total	**Definite TB**	**Probable TB**	**Non-TB**	***P***
Number of subjects	69*	12	14	43	
Age mean (SD)	47 (17)	41 (16)[+]	44 (14)	50 (17)[+]	P=0.09
Sex					
Male	32 (46%)	8 (67%)[+]	6 (43%)	18 (42%)[+]	P=0.15
Race					
Black African	27 (39%)	6 (50%)[+]	8 (57%)	13 (30%)[+]	P=0.23
Mixed Ancestry	40 (58%)	6 (50%)	6 (43%)	28 (65%)	
European Ancestry	2 (3%)	0	0	2 (5%)	
HIV status					
Positive	21 (30%)	4 (33%)[+]	10 (71%)	7 (16%)[+]	P=0.23
Unknown	11 (16%)	0	2 (14%)	9 (21%)	
Previous TB (n=54)					
Yes	19/54 (35%)	4/8 (50%)[+]	8/11 (72%)	7/35 (20%)[+]	P=0.12

* 10 suspects could not be categorized, so were excluded from the analysis

The Clearview® ELISA assay was used to calculate the LAM concentration (ng/ml) in the induced sputum samples. There was no statistically significant difference in the LAM concentrations (median, IQR) between the definite TB (0.66ng/ml, 0.12-3.21ng/ml) and the non-TB groups (0.13ng/ml, 0.0-1.14ng/ml) P=0.14. When combining the definite and probable TB groups no difference in LAM concentration was detected (P=0.59) (Figure 5.2)

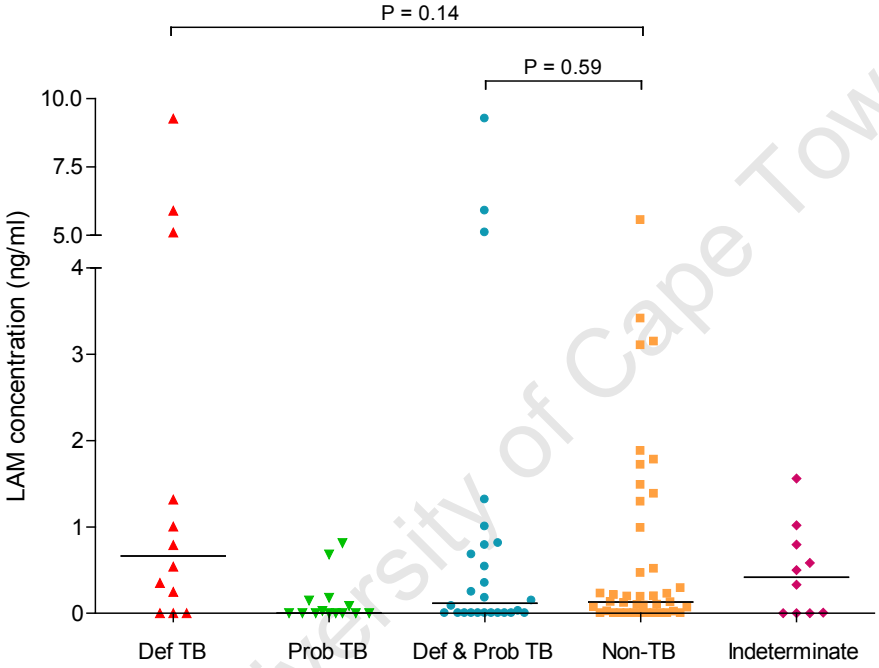

Figure 5.2 Induced sputum LAM concentrations in the 4 pre-defined diagnostic subgroups. LAM concentration in ng/ml and horizontal line depicts median values. Def TB = Definite TB, Prob TB = Probable TB

Normalising LAM concentrations for volume, protein concentration and cell count.

As the LAM-containing supernatants were isolated from various volumes of induced sputum (range: 4-12ml), the LAM concentrations were normalised for original volume of sputum obtained. There was no significant difference between the total LAM (median; IQR) content of the definite TB group (1.80ng; 0.0–

8.32ng) compared to the non-TB group (0.90ng; 0.0-4.0ng) (P=0.62). There was also no difference when combining the definite & probable TB groups (P= 0.31). (Figure 5.3)

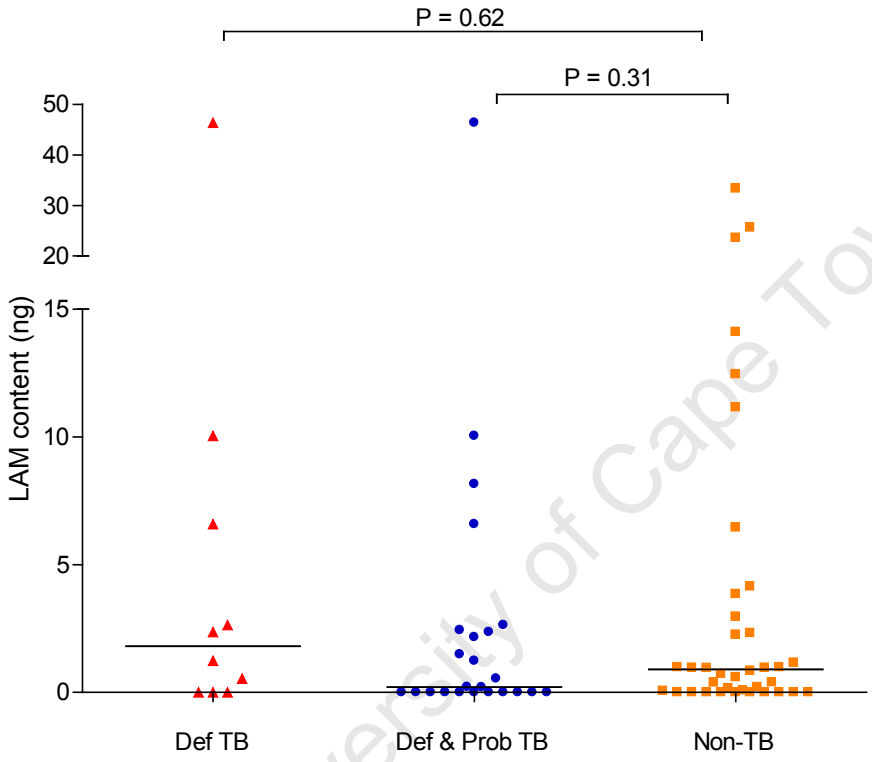

Figure 5.3 Induced sputum LAM concentrations (ng/ml) normalised for original sputum volume (ml) LAM content in ng, horizontal lines depict median values. Def TB = Definite TB, Prob TB = Probable TB

Similarly, the LAM concentration was normalised for protein content of the induced sputum. There was no significant difference when comparing the non-TB group with neither the definite TB group (P=0.19) nor with the combined definite & probable TB group (P=0.68), despite the median concentration of the definite TB group [0.78ng/mg (IQR: 0.17-4.33ng/mg)] being more than double that of the non-TB group [0.23ng/ml (IQR: 0-2.04ng/mg)]. (Figure 5.4)

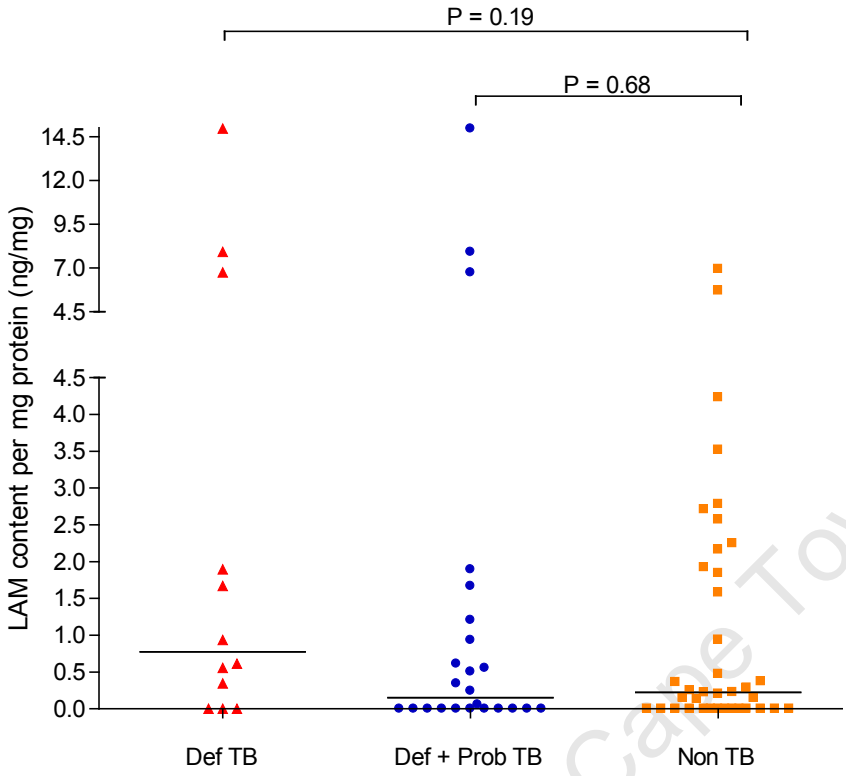

Figure 5.4 Induced sputum LAM concentrations (ng/ml) normalised for protein concentration (mg/ml) LAM content in ng/mg protein, horizontal lines depict median values. Def TB = Definite TB, Prob TB = Probable TB,

Similarly no significant difference in LAM content between the pre-defined subgroups could be found when the LAM concentration was normalised for total number of cells. (Figure 5.5). There are 7 individuals in the Definite TB subgroup with zero LAM content, and 10 individuals in the Non-TB subgroup with a positive LAM content. This could be due to the indirect relationship between the cell-free fraction in which the LAM was measured and the number of induced sputum mononuclear cells which were isolated from the sample.

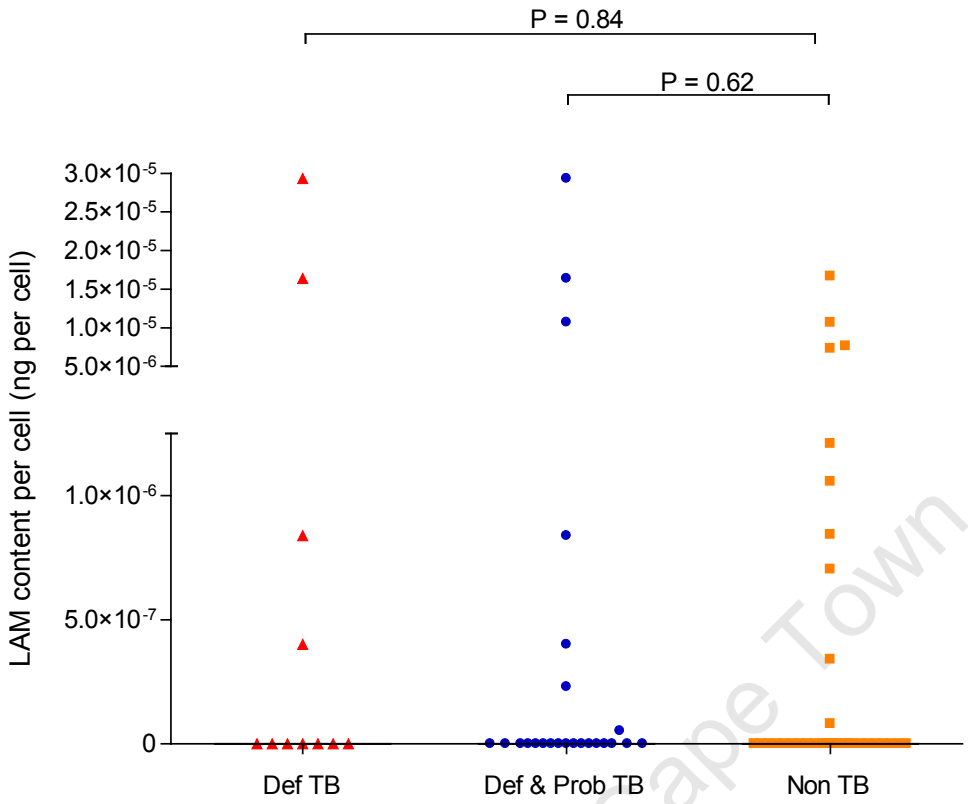

Figure 5.5 Induced sputum LAM concentrations (ng/ml) normalised for total number of cells. LAM content in ng per cell. Def TB = Definite TB, Prob TB = Probable TB,

Diagnostic performance of the induced sputum LAM

Using the manufacturer's pre-defined cut-point for LAM positivity (specifically defined for urine samples) in the definite TB group, the sensitivity of the LAM assay was 66.67% (95%CI 39.06-86.19%) and specificity was 53.49% (95%CI 38.92-67.49%). Combining the definite and probable TB groups, the sensitivity decreased to 46.15% (95%CI 28.76-64.54%), while the specificity remained unchanged (Table 5.2). The effect of using a ROC-defined cut-point is discussed later.

The assay performed worse in the HIV-positive compared to the HIV-negative subgroup. However, specificity in the HIV-positive subgroup was also poor at

57.14% (95%CI 25.05-84.18%) compared to the HIV-negative category [44.44% (95%CI 27.59-62.69%)] (Table 5.2).

Characteristics of the test results are depicted in Figure 5.6.

Table 5.2 The performance characteristics of LAM as a diagnostic test in the overall study population and subdivided into HIV positive and HIV negative subgroups using the manufacturer's defined cut-point.

	Sensitivity *(95%CI)*	*Specificity* *(95%CI)*	*PPV* *(95%CI)*	*NPV* *(95%CI)*
Def TB	66.67% (39.06-86.19%)	53.49% (38.92-67.49%)	28.57% (15.25-47.06%)	85.19% (67.52-94.08%)
Def & Prob TB	46.15% (28.76-64.54%)	53.49% (38.92-67.49%)	37.5% (22.93-54.75%)	62.16% (46.1-75.94%)
HIV-positive, Def TB	25% (4.55-69.94%)	57.14% (25.05-84.18%)	25% (4.55-69.94%)	57.14% (25.05-84.18%)
HIV-positive, Def & Prob TB	21.43% (7.571-47.59%)	57.14% (25.05-84.18%)	50% (18.76-81.24%)	26.67% (10.9-51.95%)
HIV-negative, Def TB	87.5% (52.91-97.76%)	44.44% (27.59-62.69%)	31.82% (16.36-52.68%)	92.31% (66.69-98.63%)
HIV-negative, Def & Prob TB	80% (49.02-94.33%)	44.44% (27.59-62.69%)	34.78% (18.81-55.11%)	85.71% (60.06-95.99%)

LAM interpretation	Definite & probable TB	Non-TB	Total
positive	12	20	**32**
negative	14	23	**37**
Total	**26**	**43**	**69***

Total cohort

LAM interpretation	Definite & probable TB	Non-TB	Total
positive	3	3	**6**
negative	11	4	**15**
Total	**14**	**7**	**21***

HIV-positive individuals

LAM interpretation	Definite & probable TB	Non-TB	Total
positive	8	15	**23**
negative	2	12	**14**
Total	**10**	**27**	**37***

HIV-negative individuals

Figure 5.6 LAM interpretation of the overall study cohort.

*HIV status of 11 individuals was unknown.

Determining a ROC-derived LAM cut point

LAM results were analysed using a receiver operating characteristic (ROC) curve to assess the global performance. This suggested that manipulation of the assay positive/ negative cut point would not improve the diagnostic performance. See Figure 5.7.

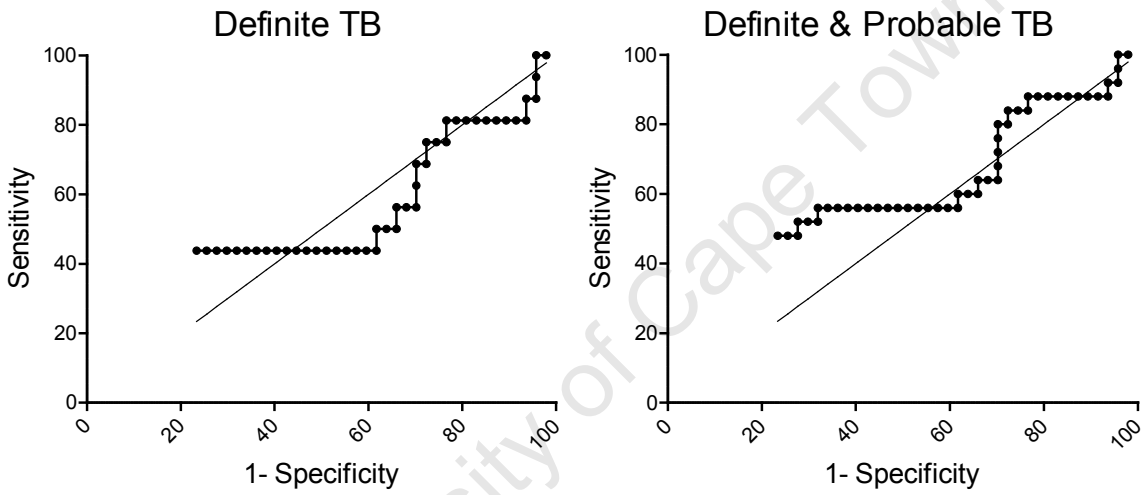

Figure 5.7 ROC curves showing definite TB and combined definite & probable TB groups.

Table 5.3 ROC curve table for Definite TB subgroup

Definite TB					
Cutoff	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
< 0.009047	43.75	19.75% to 70.12%	76.60	61.97% to 87.70%	1.87
< 0.01955	43.75	19.75% to 70.12%	74.47	59.65% to 86.06%	1.71
< 0.0430	43.75	19.75% to 70.12%	72.34	57.36% to 84.38%	1.58
< 0.0675	43.75	19.75% to 70.12%	70.21	55.11% to 82.66%	1.47
< 0.0800	43.75	19.75% to 70.12%	68.09	52.88% to 80.91%	1.37
< 0.0935	43.75	19.75% to 70.12%	65.96	50.69% to 79.14%	1.29
< 0.0980	43.75	19.75% to 70.12%	63.83	48.52% to 77.33%	1.21
< 0.1085	43.75	19.75% to 70.12%	61.70	46.38% to 75.49%	1.14
< 0.1235	43.75	19.75% to 70.12%	59.57	44.27% to 73.63%	1.08
< 0.1300	43.75	19.75% to 70.12%	57.45	42.18% to 71.74%	1.03
< 0.1401	43.75	19.75% to 70.12%	55.32	40.12% to 69.83%	0.98
< 0.1648	43.75	19.75% to 70.12%	53.19	38.08% to 67.89%	0.93
< 0.1852	43.75	19.75% to 70.12%	51.06	36.06% to 65.92%	0.89
< 0.1915	43.75	19.75% to 70.12%	48.94	34.08% to 63.94%	0.86
< 0.1945	43.75	19.75% to 70.12%	46.81	32.11% to 61.92%	0.82
< 0.2035	43.75	19.75% to 70.12%	44.68	30.17% to 59.88%	0.79
< 0.2185	43.75	19.75% to 70.12%	42.55	28.26% to 57.82%	0.76
< 0.2275	43.75	19.75% to 70.12%	40.43	26.37% to 55.73%	0.73
< 0.2386	43.75	19.75% to 70.12%	38.30	24.51% to 53.62%	0.71
< 0.2701	50.00	24.65% to 75.35%	38.30	24.51% to 53.62%	0.81
< 0.3115	50.00	24.65% to 75.35%	36.17	22.67% to 51.48%	0.78
< 0.3412	50.00	24.65% to 75.35%	34.04	20.86% to 49.31%	0.76
< 0.4083	56.25	29.88% to 80.25%	34.04	20.86% to 49.31%	0.85
< 0.4895	56.25	29.88% to 80.25%	31.91	19.09% to 47.12%	0.83
< 0.5271	56.25	29.88% to 80.25%	29.79	17.34% to 44.89%	0.80
< 0.6645	62.50	35.43% to 84.80%	29.79	17.34% to 44.89%	0.89
< 0.8879	68.75	41.34% to 88.98%	29.79	17.34% to 44.89%	0.98
< 0.9958	68.75	41.34% to 88.98%	27.66	15.62% to 42.64%	0.95
< 1.012	75.00	47.62% to 92.73%	27.66	15.62% to 42.64%	1.04
< 1.155	75.00	47.62% to 92.73%	25.53	13.94% to 40.35%	1.01
< 1.304	75.00	47.62% to 92.73%	23.40	12.30% to 38.03%	0.98
< 1.350	81.25	54.35% to 95.95%	23.40	12.30% to 38.03%	1.06
< 1.436	81.25	54.35% to 95.95%	21.28	10.70% to 35.66%	1.03
< 1.604	81.25	54.35% to 95.95%	19.15	9.149% to 33.26%	1.00
< 1.750	81.25	54.35% to 95.95%	17.02	7.647% to 30.81%	0.98
< 1.830	81.25	54.35% to 95.95%	14.89	6.204% to 28.31%	0.95
< 2.492	81.25	54.35% to 95.95%	12.77	4.832% to 25.74%	0.93
< 3.125	81.25	54.35% to 95.95%	10.64	3.546% to 23.10%	0.91
< 3.281	81.25	54.35% to 95.95%	8.511	2.368% to 20.38%	0.89
< 4.259	81.25	54.35% to 95.95%	6.383	1.336% to 17.54%	0.87
< 5.329	87.50	61.65% to 98.45%	6.383	1.336% to 17.54%	0.93
< 5.726	87.50	61.65% to 98.45%	4.255	0.5196% to 14.54%	0.91
< 7.584	93.75	69.77% to 99.84%	4.255	0.5196% to 14.54%	0.98
< 10.01	100.0	79.41% to 100.0%	4.255	0.5196% to 14.54%	1.04
< 11.26	100.0	79.41% to 100.0%	2.128	0.05385% to 11.29%	1.02

Table 5.4 ROC curve table for Definite & Probable TB subgroup.

Definite & Probable TB					
Cutoff	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
< 0.009047	48.00	27.80% to 68.69%	76.60	61.97% to 87.70%	2.05
< 0.01955	48.00	27.80% to 68.69%	74.47	59.65% to 86.06%	1.88
< 0.02402	48.00	27.80% to 68.69%	72.34	57.36% to 84.38%	1.74
< 0.04602	52.00	31.31% to 72.20%	72.34	57.36% to 84.38%	1.88
< 0.0675	52.00	31.31% to 72.20%	70.21	55.11% to 82.66%	1.75
< 0.07765	52.00	31.31% to 72.20%	68.09	52.88% to 80.91%	1.63
< 0.08765	56.00	34.93% to 75.60%	68.09	52.88% to 80.91%	1.75
< 0.0935	56.00	34.93% to 75.60%	65.96	50.69% to 79.14%	1.65
< 0.0980	56.00	34.93% to 75.60%	63.83	48.52% to 77.33%	1.55
< 0.1085	56.00	34.93% to 75.60%	61.70	46.38% to 75.49%	1.46
< 0.1235	56.00	34.93% to 75.60%	59.57	44.27% to 73.63%	1.39
< 0.1300	56.00	34.93% to 75.60%	57.45	42.18% to 71.74%	1.32
< 0.1401	56.00	34.93% to 75.60%	55.32	40.12% to 69.83%	1.25
< 0.1648	56.00	34.93% to 75.60%	53.19	38.08% to 67.89%	1.20
< 0.1852	56.00	34.93% to 75.60%	51.06	36.06% to 65.92%	1.14
< 0.1915	56.00	34.93% to 75.60%	48.94	34.08% to 63.94%	1.10
< 0.1945	56.00	34.93% to 75.60%	46.81	32.11% to 61.92%	1.05
< 0.2035	56.00	34.93% to 75.60%	44.68	30.17% to 59.88%	1.01
< 0.2185	56.00	34.93% to 75.60%	42.55	28.26% to 57.82%	0.97
< 0.2275	56.00	34.93% to 75.60%	40.43	26.37% to 55.73%	0.94
< 0.2386	56.00	34.93% to 75.60%	38.30	24.51% to 53.62%	0.91
< 0.2701	60.00	38.67% to 78.87%	38.30	24.51% to 53.62%	0.97
< 0.3115	60.00	38.67% to 78.87%	36.17	22.67% to 51.48%	0.94
< 0.3412	60.00	38.67% to 78.87%	34.04	20.86% to 49.31%	0.91
< 0.4083	64.00	42.52% to 82.03%	34.04	20.86% to 49.31%	0.97
< 0.4895	64.00	42.52% to 82.03%	31.91	19.09% to 47.12%	0.94
< 0.5271	64.00	42.52% to 82.03%	29.79	17.34% to 44.89%	0.91
< 0.6100	68.00	46.50% to 85.05%	29.79	17.34% to 44.89%	0.97
< 0.7343	72.00	50.61% to 87.93%	29.79	17.34% to 44.89%	1.03
< 0.8015	76.00	54.87% to 90.64%	29.79	17.34% to 44.89%	1.08
< 0.9006	80.00	59.30% to 93.17%	29.79	17.34% to 44.89%	1.14
< 0.9958	80.00	59.30% to 93.17%	27.66	15.62% to 42.64%	1.11
< 1.012	84.00	63.92% to 95.46%	27.66	15.62% to 42.64%	1.16
< 1.155	84.00	63.92% to 95.46%	25.53	13.94% to 40.35%	1.13
< 1.304	84.00	63.92% to 95.46%	23.40	12.30% to 38.03%	1.10
< 1.350	88.00	68.78% to 97.45%	23.40	12.30% to 38.03%	1.15
< 1.436	88.00	68.78% to 97.45%	21.28	10.70% to 35.66%	1.12
< 1.604	88.00	68.78% to 97.45%	19.15	9.149% to 33.26%	1.09
< 1.750	88.00	68.78% to 97.45%	17.02	7.647% to 30.81%	1.06
< 1.830	88.00	68.78% to 97.45%	14.89	6.204% to 28.31%	1.03
< 2.492	88.00	68.78% to 97.45%	12.77	4.832% to 25.74%	1.01
< 3.125	88.00	68.78% to 97.45%	10.64	3.546% to 23.10%	0.98
< 3.281	88.00	68.78% to 97.45%	8.511	2.368% to 20.38%	0.96
< 4.259	88.00	68.78% to 97.45%	6.383	1.336% to 17.54%	0.94
< 5.329	92.00	73.97% to 99.02%	6.383	1.336% to 17.54%	0.98
< 5.726	92.00	73.97% to 99.02%	4.255	0.5196% to 14.54%	0.96
< 7.584	96.00	79.65% to 99.90%	4.255	0.5196% to 14.54%	1.00
< 10.01	100.0	86.28% to 100.0%	4.255	0.5196% to 14.54%	1.04
< 11.26	100.0	86.28% to 100.0%	2.128	0.05385% to 11.29%	1.02

DISCUSSION

A simple, rapid, low cost POC diagnostic test is urgently needed, especially in smear-negative patients and in TB-high burden countries where the prevalence of HIV-co-infection can be as high as 80% (Corbett *et al*., 2003). Induced sputum appears to be ideal as it is non-invasive, relatively easy to obtain, offers a yield equal to that of bronchoscopy and is patient-friendly. Similarly, antigen-detection ELISA assays may be advantageous as they are able to directly detect bacterial replication, potentially enabling them to differentiate active infection from latent TB and to reflect disease stage and/or treatment progression. In addition, these assays do not rely on optimal host immunological functioning.

The performance of LAM in induced sputum using the Clearview® ELISA assay was disappointing. Despite correcting for several factors, the diagnostic performance could not be improved upon. In the overall cohort, the sensitivity was 66.67% (95%CI 39.06-86.19%) with a specificity of 53.49% (95%CI 38.92-67.49%). In the combined definite and probable TB group, the sensitivity decreased to 46.15% (95%CI 28.76-64.54%), while the specificity remained unchanged (Table 5.2). The reason for these results could be attributed to the sputum quality as sensitivity and specificity are affected by this. Sputum induction samples the larger airways, so samples may be susceptible to contamination by environmental lung contaminants such as air pollution and cigarette smoke. Furthermore, mucins contain charged, glycosylated proteins which could cross-react with the glycosylated proteins making up mycobacterial LAM (Mall *et al*., 1999). This may explain the high LAM levels in the non-TB patients. As these glycosylated proteins could adhere to each other, the filtration step may remove LAM caught in the mucus globules, thereby decreasing assay sensitivity. However, more likely the polyclonal antibodies fail to recognise the LAM molecules in sputum because LAM is already complexed with human anti-LAM

antibodies. We performed a heating step to try and dissociate LAM from antibody in such complexes. The efficacy of this heating step is unclear.

Interestingly, the test is marketed for use in HIV-positive patients (as per the manufacturer's instructions), so one could expect its performance to improve in the HIV-positive subgroup. However, HIV is a paucibacillary disease which could explain the low sensitivity of 25% (95%CI 4.55-69.94%) in the TB-HIV-co-infected subgroup versus the improved sensitivity of 87.5% (95%CI 52.91-97.76%) in the HIV-negative individuals. The sputa of HIV-positive patients have a low burden of *M.tb* compared to the tissues. A question exists whether patient TB-burden may impact on LAM concentration. In disseminated HIV-disease with advanced immunosuppression, sputum LAM concentration may be relatively low despite a high-TB burden elsewhere in the body e.g. high urine LAM as it is filtered through the kidneys. This hypothesis is confirmed by Mutetwa *et al.* who reported that test sensitivity in urine was higher in HIV-positive (52%) patients than in HIV-negative (21%) individuals (Mutetwa *et al*., 2009). Dheda *et al.* showed similar findings where LAM in urine was associated with HIV-positivity and test sensitivity was significantly higher in the HIV-positive population compared to uninfected individuals (21% versus 6%; p< 0.001) (Dheda et al.,2010). A later study of LAM in urine reported a sensitivity of 50.5% and specificity of 78.3% (Tessema *et al*., 2002). It was hypothesized that their assay's poor performance could have been attributed to patient HIV-status, latent TB, intravascular LAM-anti-LAM complex formation, or environmental mycobacterial infections, such as *M.leprae,* or *M.avium.*

Dheda *et al.'s* most recent study examined expectorated sputum and reported sensitivity and specificity to be 86% and 15% respectively. Dheda attributes the low specificity found in expectorated sputum to cross-contamination of mycobacterial LAM antigen in the cell walls of mouth flora and mouth commensals (Dheda *et al*., 2010). Their results compared favourably with those

reported by LM Pereira Arias-Bouda *et al.* who reported 91% sensitivity using expectorated sputum in an in-house LAM capture ELISA assay (Pereira Arias-Bouda *et al*., 2000). Induced sputum, as used in my study, showed poor specificity yet better than the studies using expectorated sputum. This may be due to less contamination with mouth flora as the technique of sputum induction samples secretions from the lower airways. Nevertheless, one must acknowledge that some contamination during expectoration is unavoidable.

The sensitivity measured in induced sputum in this study has been shown to be less than in expectorated sputum (Dheda *et al*., 2010). This may partly be due to laboratory processing methods. The homogenization and digestion of mucins and debris could lead to the destruction of target antigens. The induced sputum was filtered through 2-ply sterile gauze and centrifuged before the non-cellular fraction was drawn off and heated as part of the defined protocol described in the previous chapter. In comparison, Dheda *et al.* placed the spontaneous sputum directly into the heated waterbath (Dheda *et al*., 2010). It is uncertain how this may have affected the final LAM concentrations. As already mentioned, LAM and immunoglobulins may form LAM-anti-LAM complexes thereby decreasing the available LAM to bind to the assay antibodies (Tessema *et al*., 2002).

These poor results may, in part, be due to the use of an alternative body, fluid i.e. induced sputum, as the Clearview® ELISA assay was commercially designed for use with urine. Consideration would need to be made of differing sputum characteristics, compared with urine, such as viscosity and pH. A further practical difficulty in this project was the blurring of clinical and radiographic features of pulmonary TB (PTB) by host immunosuppression, especially in HIV-positive patients. This made it especially difficult to categorize patients hence we used culture positivity to categorize definite TB patients.

This study is the first to evaluate the use of induced sputum in the Clearview® LAM ELISA kit. The sensitivity, specificity, and positive and negative predictive values are too low to warrant clinical utility. It was conducted in a TB-high burden, resource-poor population therefore a larger study in a different geographical setting is needed to verify the results.

In future, adjustments to the existing Clearview® LAM ELISA assay for specific use with induced sputum may perhaps improve its efficacy. Reagents designed specifically for use with sputum and/or induced sputum may improve performance. This could necessitate the initial design of in-house assay, such as that of Chan *et al*. (2000). Additionally, use of ethanol precipitation, which has been used to concentrate antigens from bacterial cultures, may result in LAM being more detectable. Further investigation is required to find alternative and better antigens for TB diagnosis.

CHAPTER 6

CONCLUSION & FUTURE PROSPECTS

Tuberculosis has become a public health catastrophe in the developing world. It is endemic in South Africa and is especially prevalent in the Western Cape, where the disease incidence in certain areas is above 1 500/100 000 per year (Wood *et al*., 2007). Current tools are suboptimal thus there is an urgent need for user friendly and accurate, rapid TB diagnostic tests.

Most of the studies concerning interferon gamma release assays (IGRAs) done in high incidence areas appear to contradict the findings of those studies performed in low incidence settings. A meta-analysis performed by Dheda *et al*. involving studies done in high incidence settings, such as Uganda, The Gambia and India showed that IGRA sensitivity might be lower than in low incidence settings (Dheda *et al*., 2009). Although the TST is less specific in BCG-vaccinated individuals, it is as sensitive as the IGRAs in active disease and concordance with the TST is modest to good. Any discordance is predominantly TST-positive, IGRA-negative as is the case in my study. (Dheda *et al*., 2009). This may suggest variable T cell activity across populations depending on such immuno-modulatory factors as tuberculosis prevalence, malnutrition, HIV/AIDS, BCG vaccination, exposure to NTMs, leprosy, helminth and other such tropical infections.

The tuberculin skin test (TST) has historically been the only diagnostic test for latent TB infection. There is a large amount of prospective data to validate the positive predictive value of the TST (Comstock, 1975). However, its efficacy may be compromised by many factors including patient immune status, reader bias and cross-reactivity with non-tuberculous mycobacteria (NTM) and prior Bacillus

Calmette-Guérin (BCG) vaccination. In addition, it is subject to the "booster" phenomenon so patients cannot be tested more than once within a short period of time. The IGRAs have been developed as a potential replacement or adjunctive, diagnostic test for LTBI. They show arguably better sensitivity and specificity than the TST yet there is a 10-40% discordance rate between the two tests (Pai *et al*., 2004). The reasons for this are unclear.

In this thesis, TST-positive, IGRA-negative individuals showed an increased expression of biomarkers associated with regulatory T cells (Tregs) and immunosuppressive cytokines such as IL-4 in skin biopsy samples. Guyot-Revol *et al.* showed that e*x vivo* Tregs have an attenuating effect on peripheral blood IFN-γ-producing CD4-cells responsible for the Th1-immune response associated with active TB (Guyot-Revol *et al*., 2006). Such suppression of IFN-γ production by effector cells, may compromise the IGRA resulting in "false" negative tests. Thus, it could be beneficial to directly explore the effect of Tregs in the T-SPOT®.*TB* assay by using peripheral blood mononuclear cells (PBMCs) with and without Tregs and then determining whether they affect the number of spot-forming cells (SFCs) obtained per well. Future work is required to address this question.

While the TST is useful in detecting LTBI, the more recently developed IGRAs may be useful for the immunodiagnosis of active TB. If there is a high frequency of *M.tb*-specific T cells within a specific disease compartment, it may be possible to diagnose active TB. For example, T cell assays have been both diagnostically and clinically useful in diagnosing PTB when using BAL fluid (Dheda *et al*., 2009). Similarly, I have shown that IGRAs can be successfully performed to diagnose TB using cells from induced sputum, although this assay is not clinically viable in it's current format (Cashmore *et al*., 2010). The T-SPOT®.*TB* was tested in this thesis using an alternative biological fluid, namely induced sputum. Sputum induction is far less costly and invasive than bronchoscopy, but yet has a

comparable yield. In addition, a single induced sputum specimen can be used for immunological (IGRA), and microbiological diagnosis of *M.tb*. Sputum induction doesn't require much medical expertise and is ideal for patients who are sputum-scarce e.g. HIV, or unable to self-expectorate; therefore, I reasoned that it could potentially be an ideal technique for resource-poor areas. This study evaluated TB antigen-specific (ESAT-6 and CFP-10) enzyme-linked immunospot assay (ELISPOT) responses using mononuclear cells isolated from induced sputum samples. The results showed that very few (<12%) assays could be evaluated as there were insufficient mononuclear cells obtained from the sample to plate the required 250 000 lymphocytes/well for the T-SPOT®.*TB* kit. The efficiency of the assay could possibly be improved by changing assay-specific variables such as i) proliferation of induced sputum mononuclear cells before plating, ii) depletion of immunosuppressive cells, e.g. Tregs, before the assay, iii) use of stimulatory antigens other than or in addition to ESAT-6 and CFP-10, iv) use of staphylococcal enterotoxin B (SEB), rather than PHA, as a positive control, or v) increase of the cut-off number of SFCs for negativity (It has been shown that airway pathogens activate BAL alveolar T cells possibly contributing to an increased spot count in the negative control (Dheda *et al*., 2009). Future work is required to address these issues.

For the IGRA assay to diagnose active TB in sputum it may be possible to simultaneously investigate the response of a combination of cytokines, such as IL-2 and IFN-γ, in the same test to better understand the underlying cellular activities. It would also be especially helpful in highly TB endemic areas to identify and validate novel antigens or biomarkers, capable of discriminating LTBI from active TB or helpful in following effectiveness of treatment (Sargentini *et al*., 2009). Demmissie *et al.* have reported that the 16-kDa Rv2031c antigen is largely restricted to latently infected individuals (Demissie *et al*., 2006). New biomarkers, other than IFN-γ, are already under investigation e.g. IFN-γ-induced

protein-10 (IP-10) and monocyte chemotactic protein-2 (MCP-2) (Dheda *et al*., 2009; Jafari and Lange, 2008; Ruhwald *et al*., 2007; Wilkinson *et al*., 2005)

An alternative to cellular assays using induced sputum is the lipoarabinomannan (LAM), enzyme-linked immunosorbent assay (ELISA). However, the Clearview® TB LAM ELISA performed disappointingly as an indicator of *M.tb* infection with a sensitivity of only 66.67% and specificity of 53.49%. Mycobacterial LAM may be cross-reacting with LAM-like microbial surface molecules in the cell walls of mouth-residing mouth flora. Similarly, there may be cross-reactivity between mycobacterial LAM and mucins in induced sputum as both are made up of glycosylated proteins (Mall *et al*., 1999). Mucins can be extracted then purified by caesium chloride density gradient ultracentrifigation. LAM could possibly then be isolated by gel filtration, SDS-PAGE and Western blotting of these isolated mucins (Mall *et al*., 1999). As the Clearview® TB ELISA assay was designed for use in urine, its usefulness using induced sputum could potentially be improved by alternative sputum-specific reagents.

In conclusion, a rapid cost effective and ideally point of care test is urgently needed for the diagnosis of active TB. Unfortunately induced sputum ELISPOT and LAM testing do not appear to be promising candidates. In addition an accurate, cheap and reliable test for latent TB is also required. Although the IGRA assays hold great promise, understanding the reasons for TST-IGRA discordance may help drive the development of an improved test platform. The lack of a gold standard for LTBI however remains a major stumbling block in the development of any novel LTBI diagnostic assay. Finally it remains imperative to test any novel TB assay in both TB endemic areas and low burden settings as assay performance, cost and acceptability is not always comparable between high and low income settings.

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