Tropical Biomedicine 34(3): 556–569 (2017)

Serological biomarker screening and host factor analysis elucidating immune response heterogeneity in active pulmonary tuberculosis

Mohd Hanafiah, K.[1,2,3*], Liu, J.J.[1], Lieschke, K.[1], Barnes, N.C.[1], Garcia, M.L.[1] and Anderson, D.A.[1]
[1]Macfarlane Burnet Institute, Life Sciences, 85 Commercial Road, Melbourne, Victoria, Australia 3004
[2]Universiti Sains Malaysia, School of Biological Sciences, 11600, Pulau Pinang, Malaysia
[3]Monash University, Faculty of Medicine, Nursing and Health Sciences, Department of Immunology, Clayton, Victoria, Australia 3800
[*]Corresponding author e-mail: kye@usm.my.
Received 6 December 2016; received in revised form 8 April 2017; accepted 10 April 2017

Abstract. While mortality and morbidity from pulmonary tuberculosis (PTB) have improved, diagnosis of this infectious disease remains suboptimal without a point-of-care test. Antibody/antigen-based serodiagnostics is the most amenable for point-of-care translation but hampered by a lack of validated biomarkers and a heterogeneous patient antibody response. Using a case-control design, we assessed serodiagnostic potential of immunoglobulins G, A, and dimeric IgA responses against 18 antigenic preparations, followed by antibody-subclass responses against antigen 60 (A60), and four markers of host innate immunity by enzyme-linked immunoassay using sera samples (n=110) collected from April to October 2007 in VietNam from human immunodeficiency-negative patients with provisional diagnosis of PTB. We further analyzed host variables to investigate factors driving biomarker heterogeneity observed in patients. Among active pulmonary tuberculosis patients, low correlation was observed between anti-A60 antibody-classes, and between anti-A60 immunoglobulin G subclasses, but anti-A60 immunoglobulin A subclasses were significantly correlated. The best diagnostic combination of anti-A60 immunoglobulin G/A and a C-reactive protein "rule-out" remains insufficient at 82%/92% sensitivity/specificity (95%CI: 72-92%/82-98%). Heterogeneity of anti-A60 immunoglobulins G2, G3, M, as well as C-reactive protein and serum amyloid A levels observed in this study population appeared to be significantly associated with history of previous tuberculosis, hemoptysis, age, vaccination, night sweats, smoking, chest pain, fever, alcohol, and solid culture count. Further research on tuberculosis serological biomarkers may require consideration of host factors and new approaches using multiple biomarkers.

INTRODUCTION

Since Robert Koch's isolation of *Mycobacterium tuberculosis* (MTB) as the causative agent of tuberculosis (TB) in 1882, diagnosis of active pulmonary TB (PTB) has relied on mycobacterial detection in sputum; either through smear microscopy or culture or recently using molecular tests (Pai, 2015; Wallis *et al.*, 2010). However, microscopy lacks sensitivity, culture takes weeks to produce results and even newer diagnostics such as the rapid automated GeneXpert MTB/RIF (Cepheid; Sunnyvale, CA) (Pai, 2015) or blood-based interferon-gamma release assays (IGRA) are all confined to laboratories. Hence, while mortality and cure rates of this highly infectious disease have improved ("Global tuberculosis control: WHO report 2015," 2015), gaps remain between case detection rates and estimated incidence, which may be attributed to under-diagnosis in people with limited access to laboratory infrastructure ("Global

tuberculosis control: WHO report 2015," 2015). A biomarker-based non-sputum assay has been highlighted as a high-priority target product profile (TPP) for TB diagnostic research (Denkinger *et al.*, 2015). As serodiagnostic tests are more amenable to point-of-care (POC) use (Mohd Hanafiah *et al.*, 2013), detection of MTB antigens, MTB-specific antibodies or other blood-based markers remains an attractive approach despite lackluster progress (Flores *et al.*, 2011; Steingart *et al.*, 2009; K. Steingart *et al.*, 2007).

Heterogeneity of host antibody response has been consistently observed across studies (Ireton *et al.*, 2010; Kunnath-Velayudhan & Gennaro, 2011; Lyashchenko *et al.*, 1998; Morrison *et al.*, 2008). Factors such as immunogenetic background of host (Bothamley *et al.*, 1989), mycobacterial burden (Achkar *et al.*, 2010), stage and spectrum of disease (Kunnath-Velayudhan *et al.*, 2012; Lu *et al.*, 2016) and variation due to different strains of MTB (Carmona, 2013) have been hypothesized to influence the pattern of immune response in infected individuals (Lyashchenko *et al.*, 1998). Since all forms of TB begins at respiratory sites (Manabe *et al.*, 2008), local immunity and the host-pathogen interaction at these sites could provide further insight. Hence, dimeric/ polymeric immunoglobulin (Ig) A (dIgA) as the main antibody produced locally at mucosal sites (Woof & Russell, 2011), may be an additional target for TB serology. However, despite its role in early responses of host immunity, antigen-specific dIgA has been ignored due to lack of reagents and protocols to screen for this proportion of IgA in serum (Mohd Hanafiah, 2015).

Despite several serological studies published covering single, fusion, native and recombinant candidate MTB antigens (Abebe *et al.*, 2007; Ashraf *et al.*, 2014; Flores *et al.*, 2011; Senoputra *et al.*, 2015; Steingart *et al.*, 2009), only a few have analyzed different isotypes (She & Litwin, 2015; Steingart, *et al.*, 2007; Tiwari *et al.*, 2014) or combination with markers of host innate immunity. In particular, markers of host innate immunity such as C-reactive protein, serum amyloid A and apoptosis-associated markers have been posited to possess diagnostic potential (Drain *et al.*, 2014; Sandhu *et al.*, 2012; Shu *et al.*, 2013; Wilson *et al.*, 2011). Using well-characterized panels of patient sera from Foundation for Innovative New Diagnostics (FIND), this study screened antibody responses (IgG, IgA, dIgA) against 18 antigen preparations, C-reactive protein (CRP), serum amyloid A (SAA) and prostaglandin E (PGE) by enzyme-linked immunoassay (ELISA), then analyzed the factors driving heterogeneity by describing reactivity of antibodies (IgG, IgA, IgM, dIgA) and antibody subclasses (IgG1, IgG2, IgG3, IgA1, IgA2) against A60 (as one of the best performing antigens evaluated here) in relation to host variables of the study population to inform TB serodiagnostic research.

METHODS AND MATERIALS

Study population

Serum samples (n=110) from human immunodeficiency (HIV)-negative patients with provisional PTB diagnosis collected from April to October 2007 in VietNam then confirmed using concentrated Ziehl-Neelsen microscopy post-NALC-NaOH and solid or liquid TB culture (genotype information unavailable), were acquired from the FIND TB repository ("FIND's standard specimen banking," 2015). Sputum sample from active PTB patients (active TB) (n=60) have detectable bacilli on smear microscopy and colony-forming units (CFU) on culture. Non-TB patients (n=50) tested negative on smear and culture at enrolment and at 2-months follow up to exclude PTB, but latent and extrapulmonary TB (LTB, ETB) could not be excluded. This sample size is estimated to be able to detect a difference of 0.10, at AUC 0.90 with 95% confidence and 80% power (Hajian-Tilaki, 2014). All samples were collected pre-treatment and stored frozen at -20°C prior
to use.

Enzyme-linked immunosorbent assays

In-house ELISAs: Medisorp Nunc microtiter plates (Thermo Scientific; Waltham, MA) were coated with antigens obtained from the

M. tuberculosis collection at BEI Resources, NIAID, NIH: Culture Filtrate Proteins (CFP, NR-14825) SDS-Soluble Cell Wall Proteins (SCWP, NR-14840), Cell Wall Fraction (CWF, NR-14830), and Cytosol Fraction (CYT, NR-14836); Trehalose Dimycolate (TDM, NR-14844), Sulfolipid-1 (SL1, NR-14845), Phosphatidylinositol Mannosides (PIM) 1 & 2 (NR-14846), PIM 6 (NR-14847), Mycolyl arabinogalactan peptidoglycan complex (mAGP, NR-14851), Lipomannan (LM, NR-14850), Lipoarabinomannan (LAM, NR-14848), PstS1 (NR-14859), Ag85 Complex (NR-14855), MPT32/Apa (NR-14862), GroES (NR-14861); and recombinant CFP-10 (NR-14869) and ESAT-6 (NR-14862). For proteins, 100µL/well of antigens diluted to 1 µg/mL in bicarbonate buffer pH 9 were coated on microtiter plates overnight at 4°C. For glycolipids, 50µL/well of antigens were diluted to 2 µg/mL in heptane (Julian *et al.*, 2001), while LAM was diluted to 1 µg/mL in ethanol (Brown *et al.*, 2003). Microtiter plates coated with glycolipids were air-dried in a fume hood at RT overnight. Microtiter plates pre-coated with 7µg/mL of antigen 60 (A60) were commercially acquired (PBC Maes; Strasbourg, France). Buffer-coated wells without antigen were included to estimate non-zero (background) binding. Plates were washed thrice by dispensing and aspirating 350µL/well wash buffer (PBS 0.05% Tween-20; sans Tween-20 for glycolipid antigens (Julian *et al.*, 2001), 0.5% NaCL for A60) and blotted dry between steps. Plates were blocked with 300 µL/well PBS 1% BSA for an hour at 37°C before sample addition. 100µL/well sera samples diluted 1:400 for IgG, 1:100 for IgA and dIgA (pre-incubated 1:5 in supernatant containing chimeric secretory component expressed in-house (Mohd Hanafiah, 2015)) in PBS 1% BSA, 0.05% Tween-20 were added in duplicate to antigen coated and non-zero binding wells, and incubated for an hour at 37°C (overnight at 4°C for dIgA). For dIgA (Mohd Hanafiah, 2015), 100µL/well monoclonal anti-secretory component (Abcam; Cambridge, UK) diluted 1µg/mL in diluent were added and incubated for one hour at 37°C prior to addition of 100 µL/well of HRP-labelled goat anti-mouse (Dako; Glostrup, Denmark) diluted 1:2,000 in diluent. For IgG and IgA, 100µL/well of either HRP-labelled rabbit anti-human IgG (Dako; Glostrup, Denmark), or HRP-labelled goat anti-human IgA (Abcam; Cambridge, UK) diluted 1:5,000 in ELISA diluent were added. Plates with conjugate were incubated at 37°C for 30 minutes. 100µL/well of tetramethylbenzidine (KPL; Gaithersburg, MD) was added and incubated at RT for five minutes before 100µL/well of 0.5M H_2S0_4 was added to stop assay development. Absorbance was read at 450/620nm.

Anti-A60 IgM and IgG/IgA subclass responses were detected by adding 100µL/well samples diluted 1:100, then 100µl/well of either mouse anti-human IgG1, IgG2, IgG3, or IgG4 (Lifespan Bio; Seattle, WA) or mouse anti-human IgA1 or IgA2 (Nordic-MUbio; Susteren, Netherlands) diluted 1:8,000 (for anti-IgG1, IgG2), 1:4,000 (for anti-IgA1) or 1:1,000 (anti-IgG3, IgG4, IgA2), and finally 100 µL/well of HRP-labelled goat anti-mouse (Dako; Glostrup, Denmark) diluted 1:2,000 or goat anti-human IgM (Millipore; Temecula, CA) diluted 1:5,000 – using diluents, washing, incubation and assay development methods described above.

Markers of host innate immunity: Markers reportedly elevated during systemic inflammation such as C-reactive protein (CRP) (Drain *et al.*, 2014) and serum amyloid A (SAA) (Sandhu *et al.*, 2012), and apoptosis-associated markers such as prostaglandin E2 (PGE2) and human decoy receptor 3 (dcR3) (Shu *et al.*, 2013) were measured using human CRP ELISA kit (eBioscience; San Diego, CA), human SAA ELISA kit (Hycult Biotech; Uden, Netherlands), Parameter Prostaglandin E2 and DcR3 ELISA kits (R&D Systems; Abingdon, UK) according to manufacturers' protocols.

Statistical analysis

Average optical density (OD) and variance of duplicates were calculated in Microsoft Excel 2011 (Microsoft; Redmond, WA). Samples with less than 10% variance were included in analysis. Concentration of CRP, SAA, PGE2 and dcR3 for samples were interpolated by generating a four-parameter logistic curve-fit; in-house assays comparing antibody reactivity of active TB and non-TB

patients were plotted graphically; and area-under receiver operating characteristic (ROC) curves (AUC) of assays were calculated in GraphPad Prism-7 (GraphPad Software; La Jolla, CA). For calculating sensitivity (sn) and specificity (sp) of assays, cut-offs were determined using the mean plus one or two standard deviations (SD) of reactivity of non-TB patients (considered to be the upper limit of normal (ULN)) or from ROC analysis. The following statistical tests were conducted in Stata-11 (StataCorp LP; College Station, TX): 1) T-tests with unequal variances and Pearson's chi-square tests for pairwise comparisons of numerical and categorical variables between active TB and non-TB patients, respectively; 2) Pearson's test for correlations between anti-A60 antibody class/subclasses; 3) Global multiple regression model including all variables to eliminate non-significant predictors of immune response followed by multiple regression models using predictors of interest (controlled for TB diagnosis) to calculate beta coefficients (β) or odds ratio (OR); 4) comparison of diagnostic model AUCs. P <0.05 was considered significant. Due to potential issues of multiplicity and increased type I error in multiple comparisons in biomarker studies, p values were adjusted according to number of comparisons made using Bonferroni adjustment.

RESULTS

Demographic and clinical description of study population

Table 1 summarizes demographic and clinical characteristics of the study population, and pairwise comparisons of these variables between active TB and non-TB patients. Multiple logistic regression analysis suggests active TB patients had higher odds of BCG vaccination (OR: 3.0; 95% CI: 1.2 – 7.4; p=0.015), viscous sputum (OR: 2.7; 95% CI: 1.6 – 4.7; p< 0.001) and longer night sweats duration (OR: 3.6; 95% CI: 1.5 – 8.8; p=0.005) compared to non-TB patients. Other variables analyzed were comparable between active and non-TB patients.

Serodiagnostic performance

Table 2 summarizes the diagnostic potential of each antigen assessed. As the sn/sp using ULN and ROC cut-offs were comparable for the majority of antigen-specific antibody classes evaluated, diagnostic values are presented using the ROC-determined cut-offs (Table 2). With the exception of LAM, antigens with higher sensitivity across IgG, IgA and dIgA responses appear to be complex antigens such as SCWP and A60. The highest AUC values were obtained on anti-MTB IgG assays, but these assays had high reactivity from non-TB patients, resulting in very high cut-offs, which limits diagnostic application.

Active TB patients had significantly higher levels of CRP (p: 0.004), SAA (p <0.001), and PGE2 (p: 0.023) compared to non-TB patients. Unlike previously reported (Shu *et al.*, 2013), dcR3 (p: 0.826) levels were not higher in non-TB patients. Among markers of host innate immunity, SAA had the highest diagnostic value (AUC: 0.778), followed by CRP (AUC: 0.711), PGE2 (AUC: 0.615), and dcR3 (AUC: 0.583) (Fig. 1).

Anti-SCWP (p < 0.001), anti-CFP (p: 0.017), and anti-A60 (p: 0.011) dIgA was higher in active TB compared to non-TB patients, but not anti-LAM (p: 0.063), or anti-CYT (p: 0.334) dIgA. While sensitivity and specificity may reflect cut-off values, anti-MTB dIgA also had negligible background reactivity from non-TB patients. However, only a small proportion of the active TB samples had detectable anti-MTB dIgA, rendering overall diagnostic sensitivity of the assay very low (Fig. 1).

The heterogeneity of antibody response that was previously reported among TB patients (Ireton *et al.*, 2010; Kunnath-Velayudhan *et al.*, 2010; Lyashchenko *et al.*, 1998) was also observed among non-TB patients (Fig. 2). Four active TB patients (6.7%) did not have reactivity against any antigen, while 26 (52%) non-TB patients had antibody reactivity against at least one antigen. The heat map shows that while active TB patient IgG are reactive against most MTB antigens, dIgA and IgA appear to have higher reactivity on select antigens, due to the lower background reactivity from non-TB patients

Table 1. Active TB and non-TB patient characteristics

Variable Frequency (%)	Active TB (C+ SS+) (n=60)	Non-TB (C- SS-) (n=50)	p value
Age group: Number (%)			0.190
18–24 years	15 (25)	11 (22)	
25–35 years	17 (28)	12 (24)	
36–45 years	17 (28)	9 (18)	
46 years and above	11 (18)	18 (36)	
Age in years: Mean (SD)	37 (14)	40 (16)	0.248
Male	43 (72)	35 (70)	0.852
Cigarette smoker: Number (%)	29 (48)	23 (46)	0.807
Total cigarettes per day: Mean (SD)	6.4 (9)	5.9 (9)	0.791
Alcohol consumption: Number (%)			0.527
Does not consume	34 (57)	28 (56)	
Consumes infrequently	19 (32)	19 (38)	
Consumes weekly or more often	7 (11)	3 (6)	
Cough duration (weeks)			0.050
0–9	34 (59)	40 (80)	
10–19	20 (34)	9 (18)	
20 and above	4 (7)	1 (2)	
Total cough duration in weeks: Mean (SD)	7.4 (9)	4.8 (3.5)	0.039
Haemoptysis	9 (15)	11 (22)	0.343
Night sweats	27 (45)	3 (6)	<0.001*
Total night sweats (weeks): Mean (SD)	2.1 (3.2)	0.06 (0.24)	<0.001*
Weight loss	25 (42)	13 (26)	0.085
Fever above 38°C	34 (59)	26 (52)	0.625
Chest pain	20 (33)	17 (34)	0.941
Contact with active TB case	5 (8)	7 (14)	0.342
BCG vaccination status			0.014
Yes/Scar	38 (63)	21 (42)	
No	12 (20)	20 (40)	
Unknown	10 (17)	9 (18)	
Previous TB	4 (7)	5 (10)	0.525
General appearance			0.055
Mildly ill	17 (28)	23 (46)	
Moderately/gravely ill	43 (72)	27 (54)	
Sputum viscosity			
Not viscous/watery	6 (10)	21 (42)	<0.001*
Viscous	25 (42)	17 (34)	
Very viscous	29 (48)	12 (24)	

Note: Pairwise comparisons were conducted using t-test with unequal variances for numerical variables and Pearson chi-square tests for categorical variables, two-tailed. P values (Bonferroni adjusted) <0.002 are significant.

Table 2. Sensitivity of assays using ROC method with specificity at 96% (95% CI: 86-100)*

Antibody	IgG		IgA		dIgA	
	Sn (95% CI)	Cut-off	Sn (95% CI)	Cut-off	Sn (95% CI)	Cut-off
*Glycolipid antigens***						
LAM	37% (25-51)	>2.6	15% (7.2-27)	>1.1	18% (9.5-30)	>0.22
LM						
mAGP	24% (14-37)	>1.4	28% (17-41)	>0.51	6.9% (1.9-17)	>0.042
PIM 1,2						
PIM 6	13% (5.3-24)	>0.23	14% (6.1-25)	>0.3	8.6% (2.9-19)	>0.06
SL-1			10% (3.9-21)	>0.37		
TDM	17% (8.6-29)	>0.16	19% (9.9-31)	>0.31	5% (1.1-14)	>0.065
*Protein antigens***						
PstS1	10% (3.8-21%)	>3.2	28% (17-41)	>0.94	20% (11-32)	>0.12
Ag85	42% (29-56)	>1.5	7.1% (2-17)	>1.5		
MPT32	26% (16-40)	>2.3	5.4% (1.1-15)	>1.9		
GroES	25% (24-38)	>1.4	16% (7.6-28)	>0.64		
CFP10	21% (11-34)	>0.3	28% (17-42)	>0.4		
ESAT-6	18% (8.7-30)	>0.047				
Crude antigens						
CFP	17% (8.3-29)	>2.9	32% (20-45)	>0.91	23% (13-36)	>0.12
CWF	8.3% (2.8-18)	>2.5	32% (21-46)	>0.27	20% (11-33)	>0.072
CYT	15% (7.1-27)	>2.6	28% (17-41)	>0.35	32% (20-45)	>0.025
SCWP	42% (29-56)	>2.1	44% (31-58)	>0.6	21% (11-34)	>0.19
A60	42% (28-57)	>1.1	47% (34-60)	>0.72	27% (16-40)	>0.16

*With the exception of anti-PstS1 IgG and anti-A60 IgG, determined at specificity of 98% (95% CI: 89-100). Sensitivity and cut-offs rounded to two significant digits. **Where values missing, ROC analysis was not performed due to non-TB having similar or higher values than active TB patients.

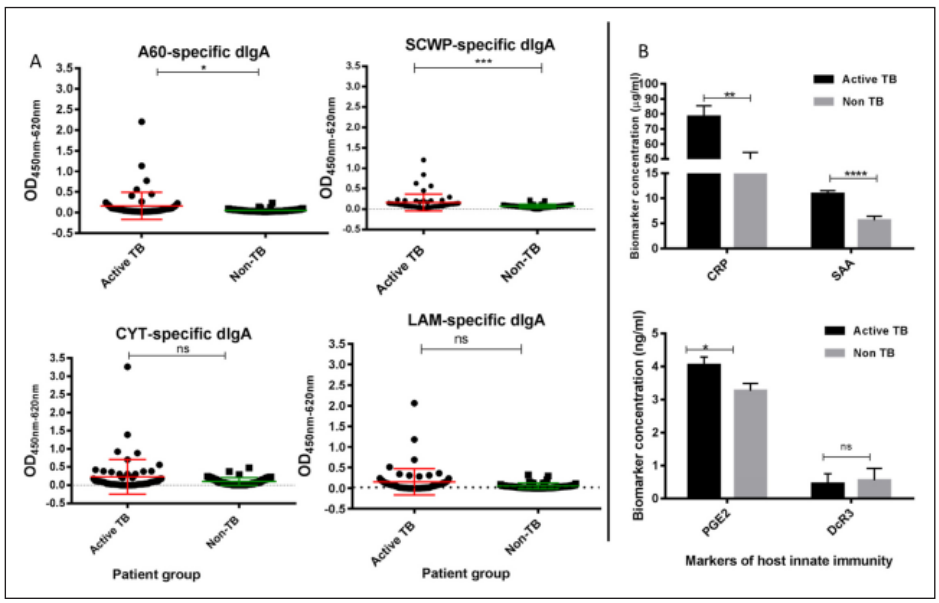

Figure 1. Antigen-specific dIgA and markers of host innate immunity. A) Scatterplots of A60, SCWP, CYT, LAM-specific dIgA assays demonstrate their low sensitivity for detecting active TB, but also very high specificity (almost negligible background in non-TB). B) Pairwise comparisons reveal elevated levels of CRP, SAA, PGE2, and DcR3 in active TB compared to non-TB patients; although not sufficiently diagnostic. Error bars represent SD. Asterisks indicate statistical significance of p value <0.05 (*), <0.01 (**), <0.001 (***).

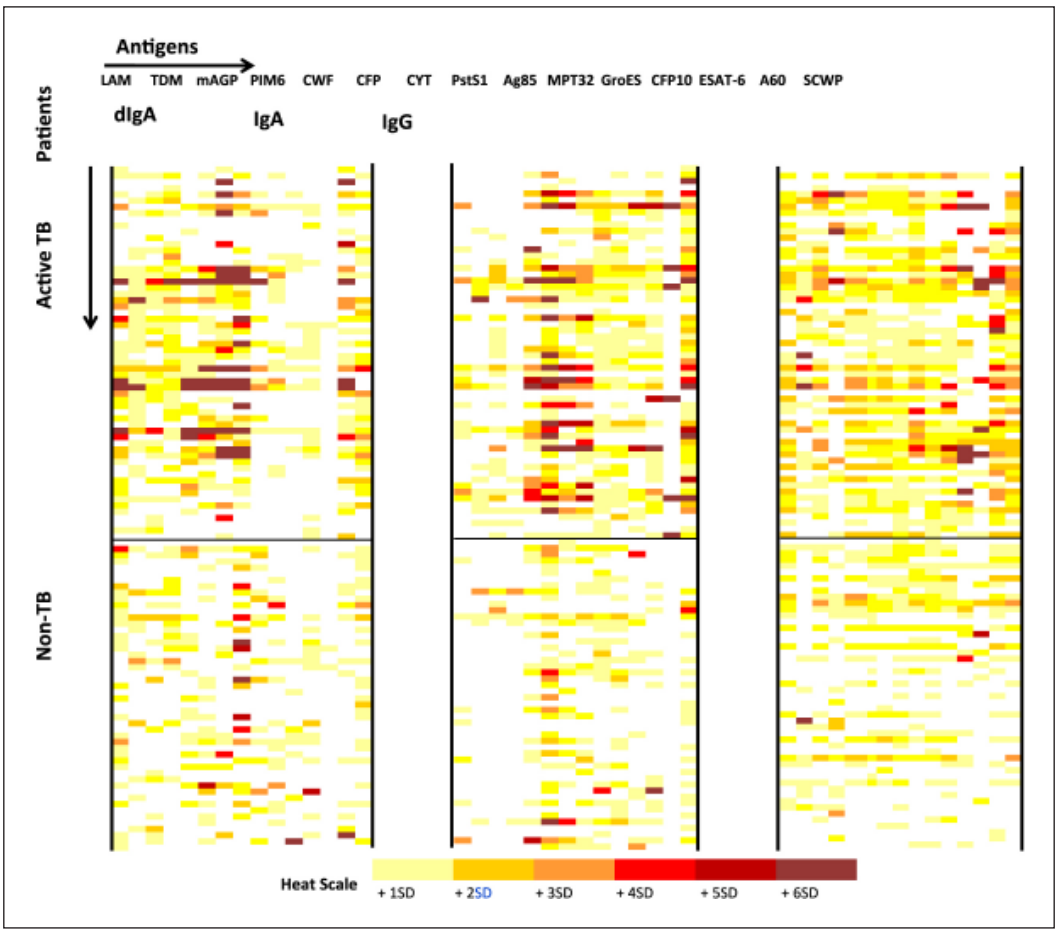

Figure 2. Heterogeneity of MTB-specific antibody reactivity. Each column of the heat map represents an antigen-specific antibody response (dIgA, IgA, IgG separated), while each row represents an individual patient (active TB and non-TB patients separated). Intensity of cell color corresponds to the strength of reactivity (OD) determined at one, two, three, four, five, or six SDs from mean of non-TB reactivity.

which lowers the positive cut-off value for dIgA and IgA.

Anti-A60 antibody class and subclass correlation

As A60 had the best sn/sp values in initial screening, A60 antibody-class/subclass reactivity and correlation were further analyzed. Compared to non-TB, active TB patients had higher mean reactivity of anti-A60: IgG1 ($p < 0.001$), IgG2 ($p=0.003$), IgG3 ($p=0.010$), and IgA1 ($p < 0.001$), but not IgA2 ($p= 0.158$). No anti-A60 IgG4 reactivity was detected in either group.

Low correlation was observed for anti-A60 antibody classes (Fig. 2). For active TB patients, anti-A60 IgG was most strongly correlated with anti-A60 IgG1 (r: 0.645; $p<0.001$) as expected due to its predominance in total IgG, followed by anti-A60 IgG3 (r: 0.508; $p=0.002$), and least strongly though significantly correlated with anti-A60 IgG2 (r: 0.460; $p=0.010$). Anti-A60 IgA was more strongly correlated with anti-A60 IgA1 (r: 0.819; $p<0.001$) compared to anti-A60 IgA2 (r: 0.650; $p<0.001$), again as expected from the predominance of total IgA1. Anti-A60 IgG1, IgG2 and IgG3 reactivity were not correlated, while anti-A60 IgA1 and IgA2

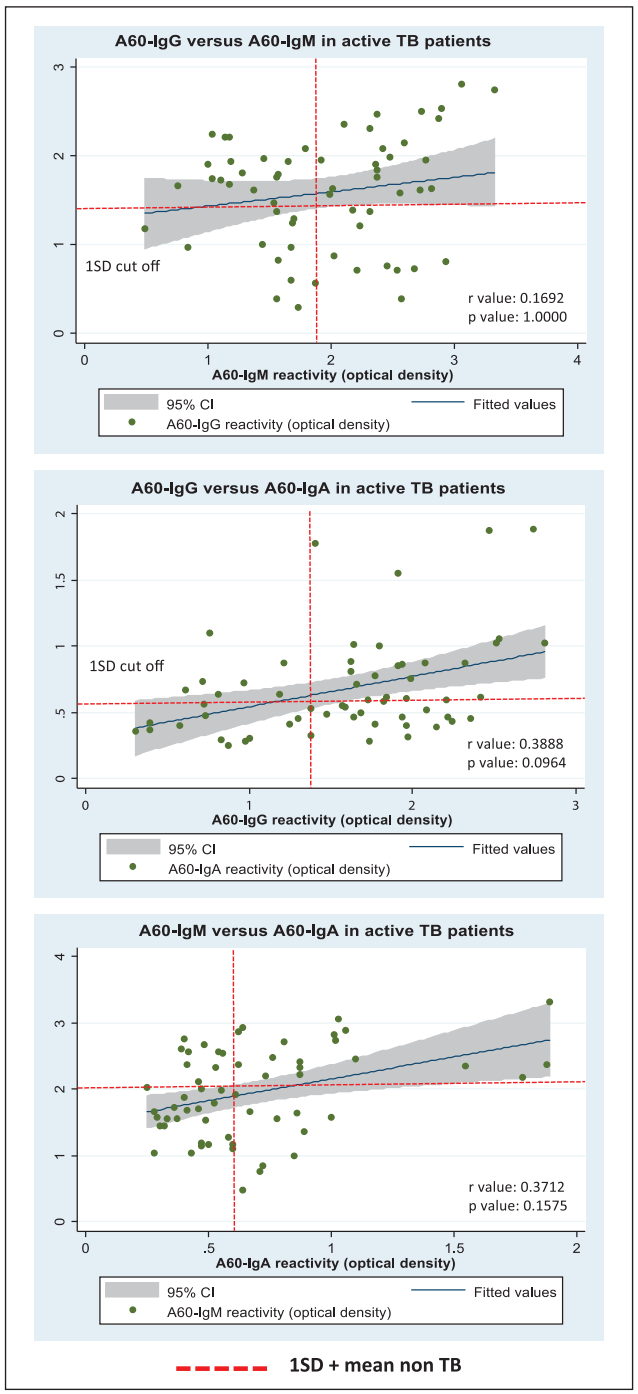

Figure 3. Anti-A60 antibody class correlation. Scatterplots of anti-A60 IgG versus IgM, anti-A60 IgA versus IgG and anti-A60 IgM versus IgA among active TB patients (n=60) demonstrates random distribution of reactivity, with Pearson correlation coefficient (r) values and p values indicating low correlation between all antibody classes in active TB (similarly seen in non-TB) patients. 95% CI flanking fitted line (grey shade). Clear outliers seen on plots with 1SD cut-off, particularly between IgG and IgA in active TB patients, suggest the benefit of combining these antibody classes.

correlated significantly. Among non-TB patients, correlation between anti-A60 IgG and subclasses IgG1 (r: 0.698; p<0.001), IgG2 (r: 0.667; p<0.001 and IgG3 (r: 0.686; p<0.001) were comparable. Anti-A60 IgA correlated with anti-A60 IgA1 (r: 0.727; p <0.001) but not with anti-A60 IgA2 (r: 0.230; p=1.000). Anti-A60 IgG1 and IgG2 were correlated, but not anti-A60 IgG1 and IgG3 or anti-A60 IgA1 and IgA2.

Markers of host innate immunity were not correlated with anti-A60 antibody response in active TB and non-TB patients. However, CRP and SAA significantly correlated in active TB (r: 0.543; p<0.001) and non-TB patients (r: 0.867; p<0.001), while PGE2 correlated with SAA (r: 0.591; p <0.001) and CRP (r: 0.556; p <0.001) in non-TB patients. Due to the heterogeneity and low correlation between antibody classes, combined antibody responses resulted in higher sensitivity but lowered specificity. Specificity is regained when an innate immunity marker is used as a "rule out" test. For example, a combination of anti-A60 IgA and anti-A60 IgG (OD >1SD cut off) with "rule out" of samples with less than 6 µg/mL CRP resulted in sn/sp of 82%/92% (95%CI: 72-92%/82-98%).

Predictors of antibody response against A60

Multiple regression analysis of host factors and variables identified history of previous TB, hemoptysis, age, BCG, night sweats, smoking, chest pain, fever, alcohol, and solid culture count as significant host predictors of antibody response, CRP and SAA in this study population. The nature of these associations are detailed in Table 3.

Table 3. Predictors of antibody response

Outcome (unit)	Predictor	β	95% CI (LB, UB)	p value
Total study population (controlled for TB diagnosis)				
IgG2 (OD)	Previous TB	0.30	0.04, 0.56	0.025
IgG3 (OD)	Haemoptysis	-0.29	-0.54, -0.03	0.030
IgM (OD)	Age	-0.02	-0.03, -0.08	0.001
	BCG	-0.41	-0.71, -0.12	0.007
	Night sweats	-0.32	-0.61, -0.03	0.032
CRP (µg/ml)	BCG	-38.55	-67.70, -9.41	0.010
SAA (µg/ml)	Night sweats	-3.05	-5.75, -0.35	0.028
Active TB patients				
IgG1 (OD)	Smoking	-0.014	-0.03, -0.004	0.009
IgG3 (OD)	BCG	0.54	0.10, 0.98	0.018
	Previous TB	0.96	0.12, 1.80	0.026
	Haemoptysis	-0.48	-0.94, -0.02	0.043
	Chest pain	0.57	0.12, 1.01	0.015
IgM (OD)	BCG	-0.66	-1.13, -0.18	0.008
	Previous TB	1.26	0.36, 2.16	0.008
	Night sweats	-0.60	-1.01, -0.20	0.005
CRP (µg/ml)	BCG	-57.17	-104.89, -9.45	0.020
	Solid culture count	-6.80	-13.13, -0.46	0.036
	Fever above 38°C	35.31	1.66, 68.96	0.040
SAA (µg/ml)	Alcohol	-2.24	-4.04, -0.44	0.017

Note: Predictors significant with p value <0.05, two-tailed. β represents the coefficient of association; i.e. change in unit of outcome (antibody (OD) or marker concentration) for every unit/level change in predictor of interest. Smoking (no. cigarettes smoked per day). Previous TB (0=No, 1=Yes); BCG (0=No, 1=Yes); Severity (0=Mildly ill 1=Moderately/gravely ill); Age (years); Haemoptysis (0=No, 1=Yes); Chest pain (0=No, 1=Yes); Solid culture count (CFU); Night sweats (0=No, 1=Yes); Fever above 38°C (0=No, 1=Yes); Alcohol consumption (1=Does not consume, 2=Consumes infrequently, 3=Consumes weekly or more often).

DISCUSSION

Studies evaluating antibody response against purified antigens often concluded that combining antigens resulted in increased sensitivity but decreased specificity (Abebe *et al.*, 2007; Araujo *et al.*, 2010; Steingart *et al.*, 2009; Wu *et al.*, 2010). Expectedly, crude antigens such as A60 and SCWP, being native cocktails of immunogenic antigens (Cocito *et al.*, 1987; Wolfe *et al.*, 2010) elicited higher reactivity from patient antibodies compared to purified antigens, with the exception of LAM (which constitutes 40% of MTB cell wall (Brennan, 2003)). While this may be the first assessment of antibodies against SCWP, the A60, which comprises immunodominant antigens such as groEL2 and hspX, have well documented immunogenicity (Cocito *et al.*, 1987). Unlike previous reports (Drain *et al.*, 2014; Sandhu *et al.*, 2012; Shu *et al.*, 2013; Wilson *et al.*, 2011), markers of host innate immunity such as CRP, SAA, PGE2 and dcR3 had low diagnostic potential and did not outperform anti-MTB antibodies, which may reflect the fact that studies investigating non-specific markers typically used healthy volunteers or LTB controls, while non-TB patients in this study may be infected with other agents that stimulate production of these markers.

TB manifests over an extended period, resulting in a wide spectrum of disease states in infected populations (Sridhar *et al.*, 2011). This study, with the first demonstration of detectable antigen-specific dIgA in serum of active TB patients, suggests that dIgA has limited utility for diagnosing the majority of active TB patients in high prevalence settings who may have delayed clinical presentation (Li *et al.*, 2013). However, the heat map illustrating heterogeneous antibody responses (Kunnath-Velayudhan *et al.*, 2010; Lyashchenko *et al.*, 1998) indicates that anti-MTB dIgA may detect unique true positives. Furthermore, correlation analyses indicate that 1) antibody-classes are independent biomarkers providing unique information on host immune response against MTB antigens; 2) measuring only one antibody-class response towards antigens provides an incomplete understanding of their utility; and 3) predominance of IgA1, IgG1 and IgG3 reactivity against A60 suggest predominance of protein components in the complex, since IgA2 and IgG2 responses are produced against polysaccharides and lipids (Sousa *et al.*, 1998) – although poor adsorption of glycolipids to polystyrene may play a role. This underscores the limitation of screening antigens on only one antibody-class, and contributes to increasing evidence that a combination of different biomarkers (Baumann *et al.*, 2014) contributes to higher sensitivity. Typically, a sensitive general marker of host innate immunity such as CRP can be useful as a "rule out" as opposed to a "rule in" test (which requires higher specificity) (Lee, 1999). In practical terms, a non-TB patient with MTB-specific antibodies from a previous TB exposure can be eliminated from contributing to a "false positive" result if the CRP is not elevated beyond a minimum value. However, despite CRP "rule out" buffering some loss of specificity in combining MTB-specific biomarkers, the best diagnostic combination/algorithm derived after broad biomarker screening in this study population remains insufficient at only 82%/92% sn/sp, short of TPPs of 98%/98% sn/sp (Denkinger *et al.*, 2015).

Using a regression model including all host variables to separately predict anti-A60 antibodies and markers of host innate immunity, we did not detect a significant association between antibody response and increased bacterial burden (proxied by solid culture CFU) as postulated previously (Achkar *et al.*, 2010). However, anti-A60 IgG3, which likely increases progressively throughout disease duration (Beyazova *et al.*, 1995), was significantly higher in "Moderately/Gravely ill" compared to "Mildly ill" patients and patients with chest pain, while CRP, which is an acute phase protein produced early in infection (Yeh & Willerson, 2003), was significantly higher in patients with fever but lower in patients with longer duration of cough and higher solid culture CFU. Smoking is an important TB risk factor associated with suppression of immune

response (van Zyl-Smit *et al.*, 2013), and a reduction in anti-A60 IgG1 per increase in cigarettes smoked per day was observed in active TB patients. Additionally, BCG vaccination, age, history of previous TB, alcohol consumption, night sweats, and hemoptysis also appear to be important predictors of anti-A60 IgG3, IgM, CRP and SAA. Notably, history of BCG vaccination is associated with reduced levels of anti-A60 IgM and CRP, but higher level of anti-A60 IgG3 among active TB patients. Increased IgG against MTB surface antigens among BCG-immunized patients has been reported previously (Perley *et al.*, 2014), and as similarly observed with anti-A60 IgG2 in patients with history of TB, may correspond with contribution from persistence of IgG previously raised against the vaccine/MTB antigens (Beyazova *et al.*, 1995). The apparent decrease in SAA with increasing alcohol consumption may relate to the fact that SAA is predominantly produced in the liver (Eklund *et al.*, 2012). Conversely, although the inverse relationship between age and IgM has been reported (Stoica *et al.*, 1978), it is unclear why IgM is decreased in BCG-immunized active TB patients. And while IgM and SAA both possess proinflammatory properties (Achkar & Casadevall, 2013; Eklund *et al.*, 2012), their inverse relationship with night sweats also appears tenuous.

The observations presented should be interpreted with the following caveats: 1) relatively small sample size and cross-sectional nature of sampling; 2) LTB, ETB and other mycobacterial infections, such as with *M. avium* (Senoputra *et al.*, 2015), cannot be excluded in the non-TB group – but these also reflect the real-world scenario of presumptive TB diagnosis; 3) use of frozen serum which may be more reflective of biomarkers with long-term stability in storage such as CRP (Yeh & Willerson, 2003). Further research on host factors and their association with biomarker response may benefit from studying samples collected in longitudinal studies of TB contacts.

Despite a broad evaluation of different antigen preparations and antibody-classes, this study has not found biomarkers able to meet TPP requirements for further consideration and development. Being the most amenable for translation into an instrument-free test, serodiagnostics remains an important field that requires continued research. Nevertheless, the results here indicate that host factors including but not limited to age, history of TB, and BCG, should be considered when evaluating serological biomarkers. Finally, a shift in serodiagnostic methodology – including innovative methods of biomarker investigation, manipulation and improvement of existing antigens, as well as an incorporation of multiple biomarkers – may be necessary in the quest to discover useful serological biomarkers that indicate active PTB regardless of host characteristics.

Abbreviations:
Antigen 60 (A60); Area-under receiver operating characteristic curves (AUC); C-reactive protein (CRP); Colony forming unit (CFU); Confidence interval (CI); Culture filtrate protein (CFP); Enzyme-linked immunosorbent assays (ELISA); Heat-shock proteins (HSP); Human immunodeficiency (HIV); Immunoglobulin (Ig); Interferon-gamma Release Assays (IGRA); Lipoarabinomannan (LAM); Odds ratio (OR); Optical density (OD); Point-of-care (POC); Prostaglandin E (PGE); SDS-Soluble Cell Wall Proteins (SCWP); Sensitivity (Sn); Serum amyloid A (SAA); Specificity (Sp) Standard deviation (SD); Upper limit of normal (ULN).

Acknowledgements. This work was supported by the Bill and Melinda Gates Foundation, Seattle, WA (grant number: OPP1039637) and by the Victorian Operational Infrastructure Support Program. We are grateful to the Foundation for Innovative New Diagnostics (Eloise Valli and Nora Champouillon), for provision of serum panels, BEI Resources (NIAID, NIH) for MTB antigen preparations and to Rosemary Ffrench for helpful discussions.

REFERENCES

Abebe, F., Holm-Hansen, C., Wiker, H.G. & Bjune, G. (2007). Progress in serodiagnosis of Mycobacterium tuberculosis infection. *Scandinavian Journal of Immunology*, **66**: 176-191.

Achkar, J.M. & Casadevall, A. (2013). Antibody-mediated immunity against tuberculosis: implications for vaccine development. *Cell Host & Microbe*, **13**: 250-262.

Achkar, J.M., Jenny-Avital, E., Yu, X., Burger, S., Leibert, E. & Bilder, P.W., *et al.* (2010). Antibodies against immunodominant antigens of Mycobacterium tuberculosis in subjects with suspected tuberculosis in the United States compared by HIV status. *Clinical and Vaccine Immunology : CVI*, **17**: 384-392.

Araujo, L.S., Maciel, R.M., Trajman, A. & Saad, M.H. (2010). Assessment of the IgA immunoassay diagnostic potential of the Mycobacterium tuberculosis MT10.3-MPT64 fusion protein in tuberculous pleural fluid. *Clinical and Vaccine Immunology*, **17**: 1963-1969.

Ashraf, S., Saqib, M.A., Sharif, M.Z., Khatak, A.A., Khan, S.N. & Malik, S.A., *et al.* (2014). Evaluation of diagnostic potential of RV3803c and RV2626c recombinant antigens in TB endemic country Pakistan. *Journal of Immunoassay & Immunochemistry*, **35**: 120-129.

Baumann, R., Kaempfer, S., Chegou, N.N., Oehlmann, W., Loxton, A.G. & Kaufmann, S.H., *et al.* (2014). Serologic diagnosis of tuberculosis by combining Ig classes against selected mycobacterial targets. *The Journal of Infection*, **69**: 581-589.

Beyazova, U., Rota, S., Cevheroglu, C. & Karsligil, T. (1995). Humoral immune response in infants after BCG vaccination. *The International Journal of Tuberculosis and Lung Disease*, **76**: 248-253.

Bothamley, G.H., Beck, J.S., Schreuder, G.M., D'Amaro, J., de Vries, R.R. & Kardjito, T., *et al.* (1989). Association of tuberculosis and M. tuberculosis-specific antibody levels with HLA. *Journal of Infectious Diseases*, **159**: 549-555.

Brennan, P.J. (2003). Structure, function, and biogenesis of the cell wall of Mycobacterium tuberculosis. *Tuberculosis (Edinb)*, **83**: 91-97.

Brown, R.M., Cruz, O., Brennan, M., Gennaro, M.L., Schlesinger, L. & Skeiky, Y.A., *et al.* (2003). Lipoarabinomannan-reactive human secretory immunoglobulin A responses induced by mucosal bacille Calmette-Guerin vaccination. *Journal of Infectious Diseases*, **187**: 513-517.

Carmona, J., Cruz, A., Moreira-Teixeira, L., Sousa, C., Sousa, J., Osorio, N.S., Saraiva, A.L., Svenson, S., Kallenius, G., Pedrosa, J., Rodrigues, F., Castro, A.G. & Saraiva, M. (2013). Mycobacterium tuberculosis strains are differentially recognized by TLRs with an impact on the immune response. *PloS One*, **8**(6): e67277.

Cocito, C., Baelden, M.C. & Benoit, C. (1987). Immunological properties of antigen 60 of BCG. Induction of humoral and cellular immune reactions. *Scandinavian Journal of Immunology*, **25**: 579-585.

Denkinger, C.M., Kik, S.V., Cirillo, D.M., Casenghi, M., Shinnick, T. & Weyer, K., *et al.* (2015). Defining the needs for next generation assays for tuberculosis. *Journal of Infectious Diseases*, **211**: S29-38.

Drain, P.K., Mayeza, L., Bartman, P., Hurtado, R., Moodley, P. & Varghese, S., *et al.* (2014). Diagnostic accuracy and clinical role of rapid C-reactive protein testing in HIV-infected individuals with presumed tuberculosis in South Africa. *The International Journal of Tuberculosis and Lung Disease*, **18**(1): 20-26.

Eklund, K.K., Niemi, K. & Kovanen, P.T. (2012). Immune functions of serum amyloid A. *Critical Reviews in Immunology*, **32**(4): 335-348.

FIND's standard specimen banking. (2015). from http://www.finddx.org/specimen-banks/

Flores, L.L., Steingart, K.R., Dendukuri, N., Schiller, I., Minion, J. & Pai, M., *et al.* (2011). Systematic review and meta-analysis of antigen detection tests for the diagnosis of tuberculosis. *Clinical and Vaccine Immunology* **18**(10): 1616-1627.

Global tuberculosis control: WHO report 2015. (2015) (Vol. 20, pp. 192). France: World Health Organization.

Hajian-Tilaki, K. (2014). Sample size estimation in diagnostic test studies of biomedical informatics. *Journal of Biomedical Informatics*, **48**: 193-204.

Ireton, G.C., Greenwald, R., Liang, H., Esfandiari, J., Lyashchenko, K.P. & Reed, S.G. (2010). Identification of Mycobacterium tuberculosis antigens of high serodiagnostic value. *Clinical and Vaccine Immunology*, **17**(10): 1539-1547.

Julian, E., Cama, M., Martinez, P. & Luquin, M. (2001). An ELISA for five glycolipids from the cell wall of Mycobacterium tuberculosis: Tween 20 interference in the assay. *Journal of Immunological Methods*, **251**(1-2): 21-30.

Kunnath-Velayudhan, S., Davidow, A.L., Wang, H.Y., Molina, D.M., Huynh, V.T. & Salamon, H., *et al.* (2012). Proteome-scale antibody responses and outcome of Mycobacterium tuberculosis infection in nonhuman primates and in tuberculosis patients. *The Journal of Infectious Diseases*, **206**(5): 697-705.

Kunnath-Velayudhan, S. & Gennaro, M.L. (2011). Immunodiagnosis of tuberculosis: a dynamic view of biomarker discovery. *Clinical Microbiology Reviews*, **24**(4): 792-805.

Kunnath-Velayudhan, S., Salamon, H., Wang, H.Y., Davidow, A.L., Molina, D.M. & Huynh, V.T., *et al.* (2010). Dynamic antibody responses to the Mycobacterium tuberculosis proteome. *Proceedings of the National Academy of Sciences United States of America*, **107**(33): 14703-14708.

Lee, W.C. (1999). Selecting diagnostic tests for ruling out or ruling in disease: the use of the Kullback-Leiber distance. *International Journal of Epidemiology*, **28**(3): 521-525.

Li, Y., Ehiri, J., Tang, S., Li, D., Bian, Y. & Lin, H., *et al.* (2013). Factors associated with patient, and diagnostic delays in Chinese TB patients: a systematic review and meta-analysis. *BMC Medicine*, **11**: 156.

Lu, L.L., Chung, A.W., Rosebrock, T.R., Ghebremichael, M., Yu, W.H. & Grace, P.S., *et al.* (2016). A Functional Role for Antibodies in Tuberculosis. *Cell*, **167**(2): 433-443 e414.

Lyashchenko, K., Colangeli, R., Houde, M., Al Jahdali, H., Menzies, D. & Gennaro, M.L. (1998). Heterogeneous antibody responses in tuberculosis. *Infection and Immunity*, **66**(8): 3936-3940.

Manabe, Y.C., Kesavan, A.K., Lopez-Molina, J., Hatem, C.L., Brooks, M. & Fujiwara, R., *et al.* (2008). The aerosol rabbit model of TB latency, reactivation and immune reconstitution inflammatory syndrome. *Tuberculosis*, **88**(3): 187-196.

Mohd Hanafiah, K. (2015). *Dimeric immunoglobulin A and serodiagnosis of mucosal infections*. (PhD Thesis), Monash University, Clayton, Victoria, Australia.

Mohd Hanafiah, K., Garcia, M. & Anderson, D. (2013). Point-of-care testing and the control of infectious diseases. *Biomarkers in Medicine*, **7**(3): 333-347.

Morrison, J., Pai, M. & Hopewell, P.C. (2008). Tuberculosis and latent tuberculosis infection in close contacts of people with pulmonary tuberculosis in low-income and middle-income countries: a systematic review and meta-analysis. *Lancet Infectious Diseases*, **8**(6): 359-368.

Pai, M. (2015). Innovations in Tuberculosis Diagnostics: Progress and Translational Challenges. *EBioMedicine*, **2**(3): 182-183.

Perley, C.C., Frahm, M., Click, E.M., Dobos, K.M., Ferrari, G. & Stout, J.E., *et al.* (2014). The human antibody response to the surface of Mycobacterium tuberculosis. *PloS One*, **9**(2): e98938.

Sandhu, G., Battaglia, F., Ely, B.K., Athanasakis, D., Montoya, R. & Valencia, T., *et al.* (2012). Discriminating active from latent tuberculosis in patients presenting to community clinics. *PloS One*, **7**(5): e38080.

Senoputra, M.A., Shiratori, B., Hasibuan, F.M., Koesoemadinata, R.C., Apriani, L. & Ashino, Y., *et al.* (2015). Diagnostic value of antibody responses to multiple antigens from Mycobacterium tuberculosis in active and latent tuberculosis. *Diagnostic Microbiology and Infectious Disease*, **83**(3): 278-285.

She, R.C. & Litwin, C.M. (2015). Performance of a Tuberculosis Serologic Assay in Various Patient Populations. *American Journal of Clinical Pathology*, **144**(2): 240-246.

Shu, C.C., Wu, M.F., Hsu, C.L., Huang, C.T., Wang, J.Y. & Hsieh, S.L., *et al.* (2013). Apoptosis-associated biomarkers in tuberculosis: promising for diagnosis and prognosis prediction. *BMC Infectious Diseases*, **13**: 45.

Sousa, A.O., Henry, S., Maroja, F.M., Lee, F.K., Brum, L. & Singh, M., *et al.* (1998). IgG subclass distribution of antibody responses to protein and polysaccharide mycobacterial antigens in leprosy and tuberculosis patients. *Clinical and Experimental Immunology*, **111**(1): 48-55.

Sridhar, S., Pollock, K. & Lalvani, A. (2011). Redefining latent tuberculosis. *Future Microbiology*, **6**(9): 1021-1035.

Steingart, K.R., Dendukuri, N., Henry, M., Schiller, I., Nahid, P. & Hopewell, P.C., *et al.* (2009). Performance of purified antigens for serodiagnosis of pulmonary tuberculosis: a meta-analysis. *Clinical and Vaccine Immunology*, **16**(2): 260-276.

Steingart, K.R., Henry, M., Laal, S., Hopewell, P.C., Ramsay, A., Menzies, D., Cunningham, J., Weldingh, K. & Pai, M. (2007). Commercial serological antibody detection tests for the diagnosis of pulmonary tuberculosis: a systematic review. *PloS Medicine*, **4**(6): 1041-1060.

Stoica, G.H., Samborschi, C. & Michiu, V. (1978). Influence of sex and age on serum immunoglobulin concentrations in healthy subjects. *Medecine Interne*, **16**(1): 23-31.

Tiwari, D., Tiwari, R.P., Chandra, R., Bisen, P.S. & Haque, S. (2014). Efficient ELISA for Diagnosis of Active Tuberculosis Employing a Cocktail of Secretory Proteins of Mycobacterium tuberculosis. *Folia Biologica*, **60**(1): 10-20.

van Zyl-Smit, R.N., Binder, A., Meldau, R., Semple, P.L., Evans, A. & Smith, P., *et al.* (2013). Cigarette smoke impairs cytokine responses and BCG containment in alveolar macrophages. *Thorax*, **69**(4): 363-70.

Wallis, R.S., Pai, M., Menzies, D., Doherty, T. M., Walzl, G. & Perkins, M.D., *et al.* (2010). Biomarkers and diagnostics for tuberculosis: progress, needs, and translation into practice. *Lancet*, **375**(9729): 1920-1937.

Wilson, D., Badri, M. & Maartens, G. (2011). Performance of serum C-reactive protein as a screening test for smear-negative tuberculosis in an ambulatory high HIV prevalence population. *PloS One*, **6**(1): e15248.

Wolfe, L.M., Mahaffey, S.B., Kruh, N.A. & Dobos, K.M. (2010). Proteomic definition of the cell wall of Mycobacterium tuberculosis. *Journal of Proteome Research*, **9**(11): 5816-5826.

Woof, J.M. & Russell, M.W. (2011). Structure and function relationships in IgA. *Mucosal Immunology*, **4**(6): 590-597.

Wu, X., Yang, Y., Zhang, J., Li, B., Liang, Y. & Zhang, C., *et al.* (2010). Comparison of antibody responses to seventeen antigens from Mycobacterium tuberculosis. *Clinica Chimica Acta*, **411**(19-20): 1520-1528.

Yeh, E.T. & Willerson, J.T. (2003). Coming of age of C-reactive protein: using inflammation markers in cardiology. *Circulation*, **107**(3): 370-371.