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Adjunctive biomarkers for improving diagnosis of tuberculosis and monitoring therapeutic effects

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Treatment

Summary *Objectives:* To identify host biomarkers associated with latent tuberculosis infection (LTBI), active tuberculosis (TB), and nontuberculous mycobacteria (NTM) diseases to improve diagnosis and effective anti-TB treatment.
Methods: Active TB and NTM patients at diagnosis, recent TB contacts, and normal healthy subjects were recruited. Tuberculin skin tests, QuantiFERON-TB Gold In-Tube tests, and multiplex bead arrays with 17 analytes were performed. TB patients were re-evaluated after 2 and 6 months of treatment.
Results: Mycobacterium tuberculosis (*M. tb*) antigen-specific IFN-γ, IL-2, and CXCL10 responses were significantly higher in active TB and LTBI compared with controls ($P < 0.01$). Only serum VEGF levels varied between the active TB and LTBI groups (AUC $= 0.7576$, $P < 0.001$). Active TB and NTM diseases were differentiated by serum IL-2, IL-9, IL-13, IL-17, TNF-α and sCD40L levels ($P < 0.05$). Increased sCD40L and decreased *M. tb* antigen-specific IFN-γ levels correlated with sputum clearance of *M. tb* after 2 months of treatment ($P < 0.001$).
Conclusions: Serum IL-2, IL-9, IL-13, IL-17, TNF-α, sCD40L and VEGF-A levels may be adjunctive biomarkers for differential diagnosis of active TB, LTBI, and NTM disease. Assessment of serum

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sCD40L and *M. tb* antigen-specific IFN-γ, TNF-α, and IL-2 levels could help predict successful anti-TB treatment in conjunction with *M. tb* clearance.

Introduction

Tuberculosis (TB) remains a major global health problem with an estimated 8.6 million new cases of TB worldwide in 2012.[1] Incidence of TB and its mortality rate have been falling since 1990, but the global burden remains substantial due to the slow rate of decline in TB incidence (2% per year).[1] For effective control of TB, rapid and accurate laboratory diagnosis is of utmost importance. Sputum smear microscopy of acid-fast bacilli (AFB) and culture of *M. tb* have been widely used for diagnosis of active TB.[2] However, AFB smear microscopy has limited sensitivity (50—60%) and is inappropriate for monitoring therapeutic effects, because it cannot distinguish live from dead bacilli.[2] A favourable outcome of anti-TB treatment is conventionally predicted by sputum culture conversion within the first two months of treatment,[3] whereas definitive identification of *M. tb* by culture takes several weeks.[2] The AFB smear test is not specific to pulmonary TB, because patients with nontuberculous mycobacteria (NTM) lung disease may show positive results by the AFB smear test.[4] Thus, there is a need for early clinical identification of NTM lung disease among AFB smear-positive patients as the therapeutic regimens for pulmonary TB and NTM lung diseases differ. A recently developed molecular diagnostics such as the Xpert® MTB/RIF and line probe assay contributed to rapid diagnosis of pulmonary TB and differentiation between *M. tb* and NTM in AFB smear-positive specimen.[5,6] However, the need of infrastructure and its high cost compared to smear microscopy are the major issue for implementation of the technology in low- and middle-income countries.[5]

Individuals with latent tuberculosis infection (LTBI) have a lifetime risk of 10% for progression to active disease. Thus, control of LTBI with early diagnosis may help effective TB control accompanied by appropriate treatment of active cases. A tuberculin skin test (TST) is a traditional method for detecting LTBI. However, the TST frequently provides false positive responses in individuals with recent BCG vaccination or exposure to NTM.[7] An IFN-γ release assay (IGRA) can rapidly detect LTBI by measuring *in vitro* release of IFN-γ in response to *M. tb*-specific peptide antigens, including early secreted antigen target, 6 kDa (ESAT-6), culture filtrate protein 10 kDa (CFP-10), and TB 7.7.[8] The IGRA overcomes the drawbacks of the TST, such as cross-reactivity with antigens derived from *M. bovis* BCG and most NTM species.[6] However, the IGRA does not discriminate LTBI from active TB upon diagnosis.[9] Discordant performance of IGRA in NTM patients has been reported; the IGRA holds potential to differentiate between NTM and *M. tb* infection in a TB low-incidence setting[10] whereas false positive IGRA in NTM patients was observed due to high prevalence of LTBI in a population with a TB high-incidence.[11,12] These reports indicate that IFN-γ assessment, by itself, is not sufficient for differential diagnosis of active TB, LTBI, or NTM diseases, and therefore putative biomarkers for improving diagnosis and monitoring therapeutic effects need to be identified for effective TB control.

In this study, we examined a panel of cytokines in patients with active TB or NTM diseases, TB contacts, and normal healthy controls to determine cytokine signatures according to disease, infection, or treatment state. We hypothesized that individuals with active TB would have different cytokine signatures compared with those with NTM disease or LTBI. In addition, measurement of multiple cytokines may help identify potential biomarkers not only for differentiating active TB from LTBI or NTM disease, but also for predicting host responses during anti-TB treatment. We aimed to characterize biosignatures as putative biomarkers, which may be useful at the early phase of diagnosis and for monitoring therapeutic effects even before confirmation of *M. tb* growth or clearance in culture. Because changes in circulating cytokine or chemokine levels are associated with human diseases, we performed multiplex bead arrays measuring 17 analytes including cytokines, chemokines, and a growth factor in serum, as well as plasma samples that were derived from QuantiFERON-TB Gold In-Tube (QFT-IT) tests.

Materials and methods

Enrolment of study participants and anti-TB treatment

From November 2010 to December 2013, 86 TB patients (mean age of 32 ranged from 20 to 76, 44 males and 42 females) at diagnosis, and 51 individuals who were recently exposed to TB patients but had no active disease (mean age of 44 ranged from 18 to 82, 13 males and 38 females) were enrolled (Fig. 1). A total of 133 normal healthy individuals (mean age of 31 ranged from 20 to 61, 63 males and 70 females) recruited had no history of contact with TB patients and no symptoms of TB with normal observation on chest X-ray (Fig. 1). Forty-two NTM patients aged 43—84 years at diagnosis (10 males and 32 females) were also enrolled and NTM isolates were confirmed from the 42 patients (Fig. 1). Active pulmonary TB at diagnosis was confirmed by smear/culture of *M. tb* from sputa or radiological examination. Individuals who had immunosuppressants, or any form of cancer or diabetes, were excluded. Those who had HIV or renal disease were also excluded. LTBI and normal control groups were defined based on TSTs and QFT-IT tests: 26 of the 51 TB contacts showed positive IFN-γ responses by the QFT-IT tests and were considered most likely to have LTBI compared with the 25 TB contacts with negative IFN-γ responses. The control group consisted of 55 of the 133 normal healthy individuals with negative IFN-γ responses by the QFT-IT tests and with $<$10 mm of TST induration size. Therefore 58 TB patients, 26 TB

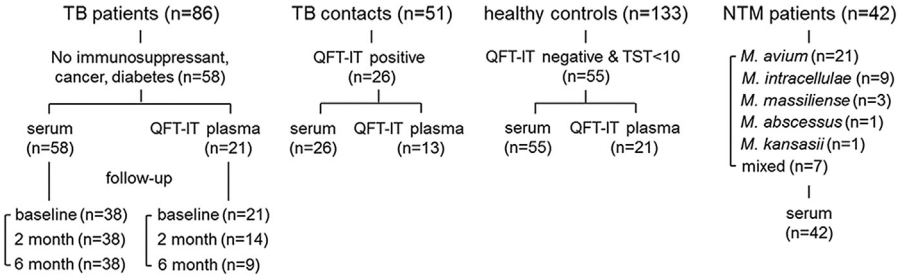

Figure 1 Enrolment of subjects and collection of samples. Active TB patients at diagnosis ($n = 86$), TB contacts ($n = 51$), normal healthy controls ($n = 133$), and NTM patients at diagnosis ($n = 42$) were recruited. TB patients with cancer, diabetes, or who had taken any immunosuppressant ($n = 28$) were excluded. QFT-IT negative TB contacts ($n = 25$) were excluded. Among the 133 healthy controls recruited, only the subjects who showed negative responses in both TSTs (<10 mm) and QFT-IT tests were included ($n = 55$). TB patients were re-evaluated after 2 and 6 months of anti-TB treatment. Sera and QFT-IT plasma samples were collected from each group.

contacts and 55 normal healthy controls were included in the analysis of this study (Table 1).

Anti-TB treatment for TB patients included rifampicin, isoniazid, ethambutol, and pyrazinamide for at least 6 months based on the Korean Guidelines for Tuberculosis 2011.[13] The standard treatment regimen includes the 4 drugs for the first two months after which the continuation phase consists of four months of rifampicin, ethambutol and isoniazid. In the case of patients with drug resistance, known patterns of resistance, drug susceptibility testing data and drug intolerance were considered for the anti-TB therapy. TB patients were re-evaluated with blood collection after 2 months of anti-TB treatment and post treatment (6 months), and 38 of the TB patients recruited were included in the analysis of the 2 and 6 month re-evaluations during anti-TB treatment (Table 1). However, much less patients were included for the analysis with QFT-IT plasma samples as many of the QFT-IT plasma samples were not available; 21 TB patients at pre-treatment, 14 after 2 months of treatment, and nine after 6 months of treatment (Fig. 1). The immune responses of 21 TB patients were compared with those of 13 individuals with LTBI and 21 controls (Fig. 1).

All patients were prospectively recruited at Severance Hospital in Seoul, South Korea, and the study was explained to the study participants, and informed written consent was obtained for interviews and all tests, including TST, clinical examination (e.g. chest X-ray), and blood sampling for immunological testing such as QFT-IT tests. Ethical permission for this study was granted by the Severance Hospital Ethics Review Committee: approval number 4-2010-0213 for active pulmonary TB patients, TB contacts, normal healthy controls, and approval number 4-2011-0241 for NTM patients.

Table 1 Characteristics of subjects involved in the analysis at baseline. Pulmonary TB was diagnosed in all of the 58 TB patients except for one patient with extrapulmonary TB. Among the 38 TB patients who were included in follow-ups at 2 and 6 months post-treatment, 31 patients were *M. tb* culture positive at baseline while 30 out of the patients showed culture conversion at 2 months post-treatment. Eight of the 58 patients (13.8%) and 3 of the 38 patients (7.9%) were AFB smear-positive. Based on the results of chest radiograph, TST and QFT-IT test, 26 TB contacts and 55 normal healthy controls were defined as a group of LTBI and control.

	TB ($n = 58$)	TB fir follow-up ($n = 58$)	LTBI ($n = 26$)	Control ($n = 55$)
Mean age (range)	32 (22–76)	32 (22–69)	47 (22–69)	30 (22–57)
Male, *n*[a] (%)	32 (55.2)	18 (47.4)	9 (34.6)	24 (43.6)
Body mass index, median (IQR[b])	20.3 (18.9–21.9)	19.9 (18.7–21.9)	22.6 (21.3–24.0)	20.3 (20.3–23.7)
Presence of BCG scar, *n* (%)	33 (56.9)	23 (60.5)	22 (84.6)	35 (63.6)
Prior TB treatment, *n* (%)	5 (8.6)	2 (5.3)	2 (7.7)	0 (0)
Drug resistant TB, *n* (%)	8 (16.3)	2 (6.5)		
Extrapulmonary TB, *n* (%)	1 (1.7)	1 (2.6)		
Pulmonary TB diagnosis, n (%)				
M. tb. culture, positive	50 (86.2)	31 (81.6)		
M. tb. culture, negative	7 (12.1)	6 (15.8)		
Extent of lesion in pulmonary TB, n (%)				
One-third of lung field	44 (75.9)	29 (76.3)		
Two-thirds of lung field	9 (15.5)	7 (18.4)		
More than two-thirds of lung field	5 (8.6)	2 (5.3)		
Culture conversion at 2 months, *n* (%)	45/50 (90.0)	30/31 (96.8)		

[a] Number.
[b] Interquartile range.

TST

TSTs were administered by intradermal injection of 0.1 mL of tuberculin purified protein derivative (RT-23, Statens Serum Institute, Copenhagen, Denmark) for TB patients, TB contacts and normal healthy controls. The reaction was read at 48 and 72 h later and the induration size of 10 mm was considered as a cut-off point for a positive reaction.

QFT-IT tests

Serum samples were obtained from 4 mL of blood (VACUETTE® serum tube, Greiner Bio-One GmbH, Frickenhausen, Germany) and 3 mL of blood was collected directly into each of three QFT-IT tubes (Nil, *M. tb* Ag tube; ESAT-6, CFP-10, and TB 7.7 peptide antigens, and mitogen tube; PHA, Cellestis, Valencia, CA, USA). The QFT-IT tubes were incubated upright at 37 °C for 24 h, and plasma was harvested. Plasma samples were divided into aliquots for IFN-γ ELISAs and multiplex bead arrays. The IFN-γ ELISAs were performed according to the manufacturer's protocol (QuantiFERON-TB Gold, Cellestis), and the data were analysed using QFT-IT Analysis Software (Cellestis).

Measurement of cytokine concentrations

Multiplex bead arrays with 17 different analytes, including cytokines, chemokines, and a growth factor, were performed using sera and QFT-IT plasma samples using BD FACSVerse™ (BD Biosciences, San Jose, CA, USA). The analytes included IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17A, IL-22, IFN-γ, TNF-α, IFN-α, sCD40L, CXCL10 (IP-10), and vascular endothelial growth factor A (VEGF-A). The manufacturer's protocol (eBioscience, San Diego, CA, USA) was followed for the multiplex bead arrays. The concentration of each analyte was calculated using FlowCytomix Pro software (eBioscience), and values out of standard curve ranges were adjusted by setting minimum and maximum values. Values of 17 analytes in QFT-IT plasma were corrected for background levels by subtracting negative control values (nil tubes). In order to abate false positive responses, responders were defined as those who showed higher values than twice the limits of detection in standard curves: 5.5 pg/mL for IL-9, 27 pg/mL for IL-17A, 34.5 pg/mL for CXCL10, 55 pg/mL for IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IFN-γ, TNF-α, IFN-α, VEGF-A, 110 pg/mL for sCD40L, and 220 pg/mL for IL-22.

Statistical analysis

Concentration differences of the 17 analytes from sera and QFT-IT plasma samples from active TB patients, TB contacts with LTBI, and normal healthy controls were analysed by Kruskal–Wallis tests and Dunn's multiple comparison tests. Mann Whitney tests were used to analyse concentration differences of 17 analytes between active TB and NTM diseases. Concentrations of the 17 analytes between pre- and post-treatment in TB patients were analysed by Wilcoxon signed rank tests. *P* values were adjusted using Bonferroni correction to account for multiple comparisons. Diagnostic values of 17 analytes in sera and QFT-IT plasma were examined by analysis of the area under the receiver operating characteristic (ROC) curves (AUC).

Results

Biosignature of 17 analytes in sera from active TB and NTM patients, TB contacts with LTBI, and normal healthy controls

Median concentrations of serum IL-22, CXCL10, and VEGF-A were significantly higher in 58 TB patients than in 55 controls ($P < 0.05$) while only VEGF-A concentration differed between active TB and LTBI groups ($P < 0.01$) (Fig. 2A). Analysis of the AUC indicated that serum VEGF-A could be a good biomarker for discriminating active TB from LTBI (AUC = 0.7576, $P < 0.001$; Supplementary Fig. 1).

Concentrations of the 17 analytes in the sera from 38 TB patients (Table 1), before treatment, were compared with those from 42 NTM patients at diagnosis. TB patients had significantly higher concentrations of Th1 and Th2 cytokines, as well as IL-17, than did the NTM patients. Five out of the 17 analytes (IL-2, IL-9, IL-13, IL-17 and TNF-α) were detected at statistically significant higher levels in TB patients than in NTM patients (Fig. 2B). On the other hand, TB patients showed significantly lower concentrations of sCD40L ($P < 0.01$) than did the NTM patients. IFN-γ, CXCL10 and VEGF-A did not differ between the two groups (Fig. 2B).

Biosignature of 17 analytes in QFT-IT plasma from TB patients, TB contacts with LTBI, and controls

In response to *M. tb* antigen stimulation, QFT-IT plasma IFN-γ, IL-2, and CXCL10 responses were significantly higher in active TB and LTBI groups than in the control group ($P < 0.01$, Fig. 3A). TB patients also presented higher levels of IL-13 than did the control group although the differences were not significant ($P > 0.05$). QFT-IT plasma VEGF-A did not differentiate between active TB and LTBI groups unlike serum VEGF-A, and none of the 17 analytes differed between the two groups in response to *M. tb* antigens (Fig. 3A). All cytokines were highly produced in response to mitogen (PHA) without any significant difference between the groups ($P > 0.05$), suggesting that there were no non-specific immunosuppression effects on the cytokine responses to *M. tb* antigens in the QFT-IT plasma samples (Fig. 3B).

Longitudinal analysis of immune responses in sera from active TB patients during anti-TB treatment

The effect of anti-TB treatment on immune responses was monitored 2 and 6 months after the initiation of anti-TB treatment. In the sera from TB patients, the sCD40L concentration significantly increased along with *M. tb* clearance in culture at the 2-month evaluation ($P < 0.001$, Fig. 4). Increased serum sCD40L concentrations were present in 79% (30 out of 38) of TB patients after 2 and 6 months of treatment. One out of 38 patients at pre-treatment and 6 months post treatment did not have

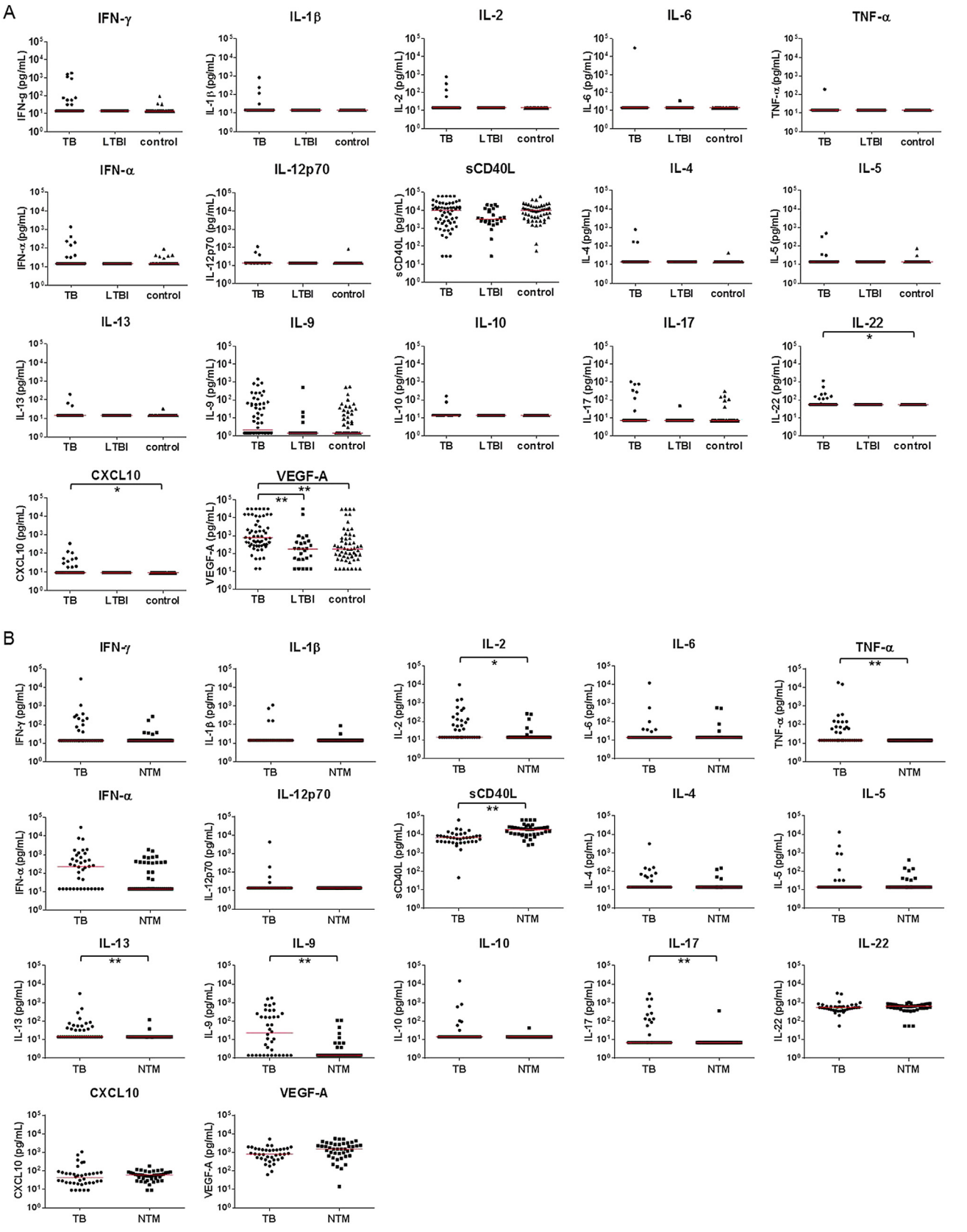

Figure 2 Comparison of serum cytokine concentrations in groups of TB, LTBI, control and NTM. A. Concentrations of 17 cytokines in TB patients ($n = 58$), TB contacts with LTBI ($n = 26$), and controls ($n = 55$). TB patients presented significantly higher IL-22, CXCL10, and VEGF-A serum levels compared with controls. Only VEGF-A level differed between TB patients and individuals with LTBI ($P < 0.01$). B. Serum cytokine concentrations in TB ($n = 38$) versus NTM ($n = 42$) patients. Five of the 17 analysed cytokines

positive sCD40L concentration while all of the 38 patients showed positive sCD40L concentrations (>110 pg/mL) after 2 months of anti-TB treatment (Supplementary Fig. 2). The proportion of the responders who showed <7000 pg/mL of serum sCD40L at baseline (59.5%; 22 out of 37) was reduced to 18.4% (7 out of 38) and 18.9% (7 out of 37) after 2 and 6 months of treatment, respectively (Supplementary Fig. 2). Meanwhile, the number of TB patients showing >7000 pg/mL of sCD40L increased from 16 (43.2%) to 32 (86.5%) following anti-TB treatment (Supplementary Fig. 2). Serum VEGF-A concentrations were reduced in more than half of TB patients (55.3%; 21 out of 38) after 6 months of treatment, whereas the change in median concentrations between pre- and post-treatment was not statistically significant ($P > 0.05$). Sera concentrations of the other analytes, including IFN-γ, did not change during anti-TB treatment in 38 TB patients (Fig. 4).

Longitudinal analysis of immune responses in QFT-IT plasma from active TB patients during anti-TB treatment

In the QFT-IT plasma obtained from active TB patients, the IFN-γ responses were dramatically decreased in 85.7% (12 out of 14) of the TB patients after 2 months of treatment. Eight out of the 12 patients showed confirmed *M. tb* in culture at diagnosis while *M. tb* clearance was observed along with the reduced IFN-γ responses at 2 months post treatment. Additionally, all patients showed reduced IFN-γ responses post-treatment ($P < 0.001$, Fig. 5). Eight out of 14 TB patients showed positive TNF-α responses at baseline and the TNF-α responses decreased in all of the responders after 2 months of treatment ($P < 0.05$, Fig. 5). Furthermore, 69.2% (9 out of 13) and 58.3% (7 out of 12) of the responders after 2 months of treatment showed reduced IL-2 and CXCL10 responses, respectively, though the magnitude of the immune responses was not significant compared to pre-treatment levels. QFT-IT plasma TNF-α and CXCL10 responses were decreased in only 33% of the TB patient post-treatment, indicating that *M. tb* antigen-specific TNF-α and CXCL10 may act as regulating cytokines during the early phase of treatment. The percentage of responders showing relatively low IFN-γ production (<500 pg/mL) gradually increased to 50% after 2 months of treatment and 78% post-treatment (6 months). Meanwhile, the percentage of the responders with high IFN-γ production (>1000 pg/mL) was significantly reduced to 11.1% from 47.6% following 6 months of treatment (Supplementary Fig. 2). A similar pattern was found with TNF-α and IL-2 responders throughout treatment (Supplementary Fig. 2).

Discussion

Current diagnostic tests for TB mainly depend on detection of clinical isolates by AFB smear microscopy and culture, both of which have limited accuracy and speed.[2,3] Recently, the IGRA was developed to quickly determine M. tb infection with higher specificity compared with TST, whereas the IFN-γ levels alone are not sufficient to differentiate between LTBI and active TB disease.[8,9] Based on the need for biomarkers to improve diagnosis of active TB, LTBI, and NTM disease and for monitoring therapeutic effects, we examined the biosignatures of 17 analytes in serum and *M. tb* antigen-stimulated plasma samples (QFT-IT plasma) that were obtained from active TB and NTM patients, TB contacts with LTBI, and normal healthy controls. Our results suggest that serum VEGF-A concentrations may help to differentiate between active TB and LTBI in addition to the diagnosis of TB by culture-confirmed *M. tb*. Measurement of serum IL-2, IL-9, IL-13, IL-17, TNF-α and sCD40L concentrations may also improve diagnosis discriminating between TB and NTM. Increased concentrations of serum sCD40L and decreased *M. tb*-specific IFN-γ, TNF-α, and IL-2 responses were associated with *M. tb* conversion in culture after 2 months of treatment, indicating the usefulness of the cytokines as indicators for monitoring therapeutic effects in active TB patients.

Increased VEGF levels have been reported in granulomatous diseases, such as pulmonary TB,[14] Crohn's disease,[15] and sarcoidosis.[16] Higher levels of serum VEGF were found in patients with active TB[11] and *mycobacterium avium* complex (MAC) infection[17] compared with normal controls, and circulating VEGF concentrations correlated with disease severity in active TB.[18] In this study, the median concentration of serum VEGF-A was significantly higher in TB patients than in the LTBI and control groups. Higher levels of VEGF have been also reported in saliva or plasma of TB patients compared with healthy controls.[19,20] However, in response to M. tb antigen stimulation, we did not observe any differences in VEGF responses between active TB and LTBI although VEGF responses in QFT-IT supernatant differed between the groups in other studies.[9,21] Levels of IFN-γ, IL-2, and CXCL10 in QFT-IT supernatant were significantly higher in TB patients than in normal controls whereas none of the 3 analytes clearly differentiated between TB and LTBI as previously reported.[9,22,23] These data indicate that assessment of a combination of IL-2 and CXCL10 may enhance the sensitivity of IGRA that measures only IFN-γ levels for diagnosis of M. tb infection. In addition, serum VEGF-A concentrations may serve as a biomarker to discriminate TB from LTBI. The relatively low specificity of serum VEGF-A concentrations may be improved by the combined measurement of IFN-γ, IL-2 and CXCL10 in response to M. tb antigens. Molecular tests have high specificity and sensitivity for rapid diagnosis and differentiation between pulmonary TB and NTM diseases,[5,6] but our data also provide a panel of serum cytokines (IL-2, IL-9, IL-13, IL-17, TNF-α and sCD40L) for differential diagnosis of active TB and NTM ($P < 0.01$). This panel may aid in early diagnosis prior to identification of clinical isolates by culture.

such as IL-2, TNF-α, IL-13, IL-9 and IL-17 were detected at higher levels in TB patients than in NTM patients. In contrast, sCD40L concentration was significantly higher in NTM patients than in TB patients ($P < 0.01$). Median concentrations are indicated with horizontal bars. (*$P < 0.05$, **$P < 0.01$).

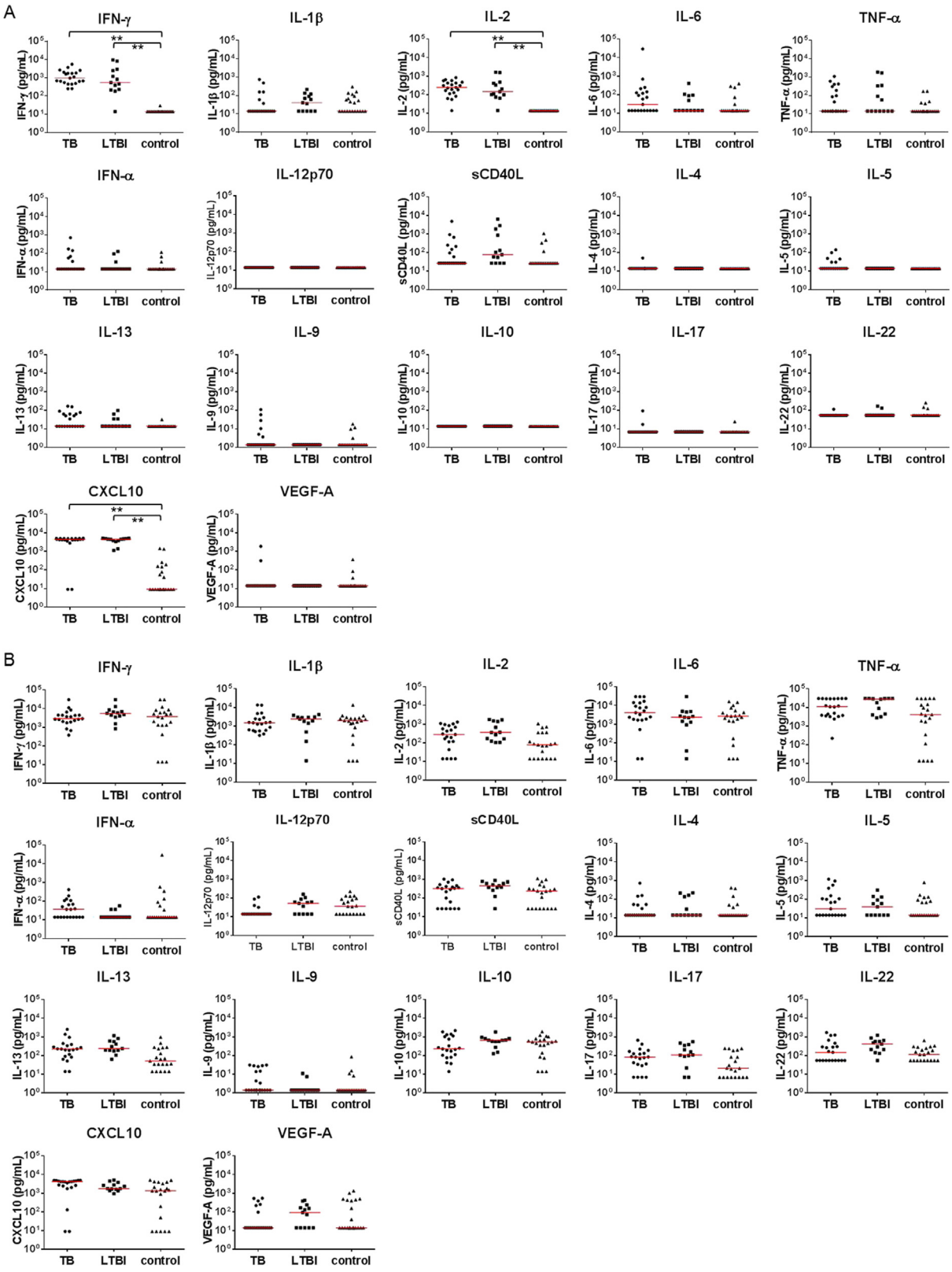

Figure 3 Cytokine responses to *M. tb* ESAT-6, CFP, and TB 7.7 in QFT-IT plasma of TB (n = 21), LTBI (*n* = 13), and control (*n* = 21) groups. A. In response to *M. tb*-specific antigens, IFN-γ, IL-2 and CXCL10 responses were significantly higher in active TB and LTBI than in controls. None of the analytes differed between TB and LTBI groups. B. In response to PHA, all cytokines were greatly expressed in all three groups. Median responses are indicated with horizontal bars. (**$P < 0.01$).

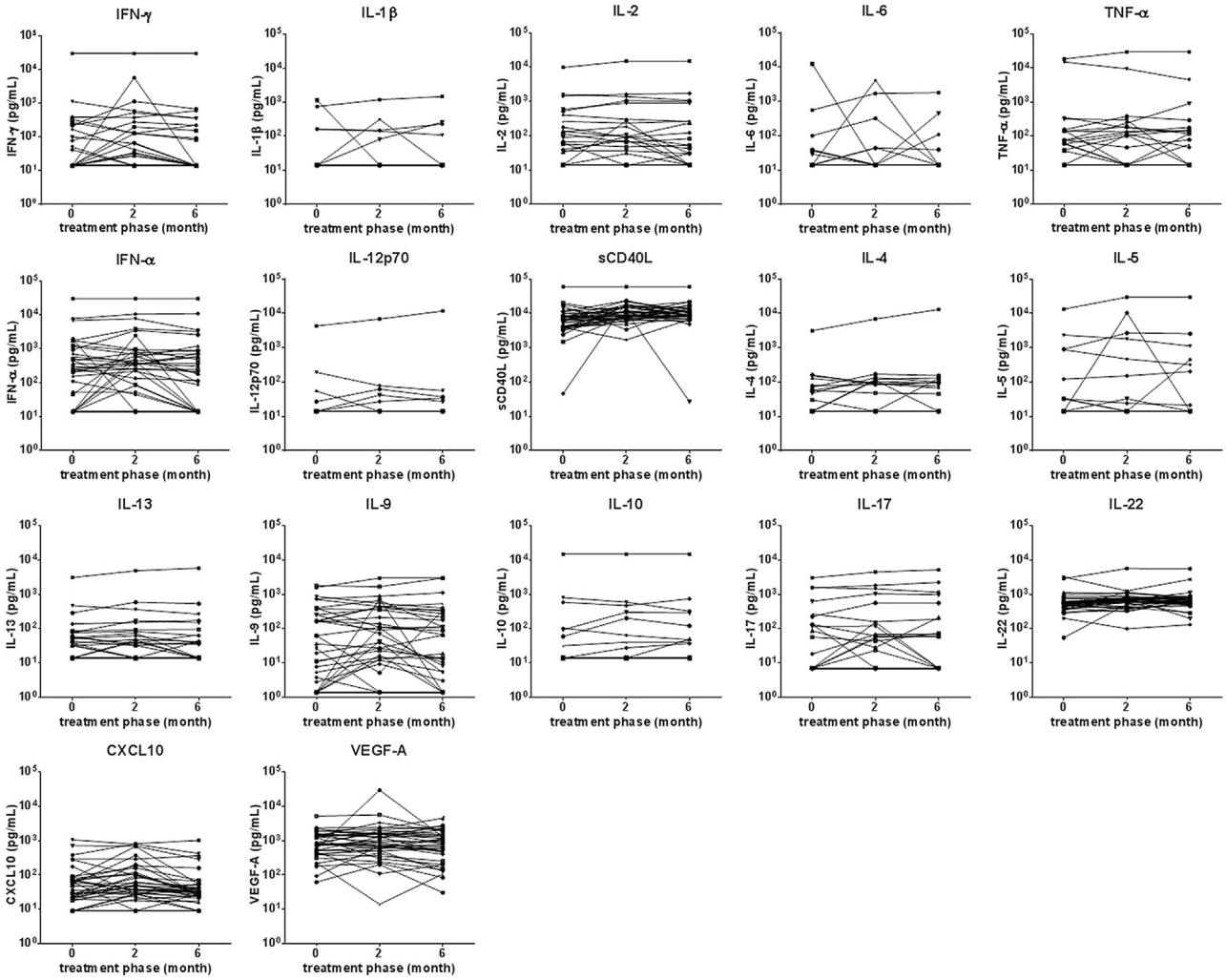

Figure 4 Concentration changes of serum cytokines in TB patients during anti-TB treatment. Concentrations of serum cytokines were measured in 38 TB patients at pre-treatment, after 2 months of treatment, and post-treatment (6 months). Most of the cytokine concentrations were not altered during anti-TB treatment. However, 29 out of the 38 TB patients showed increased serum sCD40L concentrations after 2 months of treatment ($P < 0.001$).

CD40L (CD154) is a co-stimulatory molecule that plays a role in enhancing cell-mediated immunity to intracellular pathogens by inducing IL-12, which subsequently generates Th1-type cytokines through interactions with CD40 on macrophages or dendritic cells.[24] Defective CD40L expression in PBMCs from TB patients contributes to decreased IFN-γ production by PBMCs.[25] Significantly higher levels of plasma sCD40L is present in plasma from TB patients in the fifth week of anti-TB treatment compared to pre-treatment,[7] which is consistent with our findings. However, sCD40L responses did not change significantly in response to *M. tb* antigens. It has been suggested that the IGRA is not appropriate as a monitoring tool for anti-TB treatment due to the substantial proportion of patients with positive QFT-IT (46%) and T-SPOT.TB® (79%) results after TB treatment.[26] There was no difference in IP-10 levels of QFT-IT plasma between pre- and post-treatment whereas significant changes in IP-10 release were observed in response to RD1 selected peptides (ESAT-6 and CFP-10).[27] Our study also showed no significant change in IP-10 levels of QFT-IT plasma between baseline and post-treatment. Meanwhile, both the magnitude of IFN-γ responses and the proportion of the responders showing high IFN-γ production (>1000 pg/mL) were significantly reduced post-treatment ($P < 0.001$). Rapid decreases in TNF-α and IL-2 responses and the percentage of responders correlated with *M. tb* sputum conversion in culture after 2 months of treatment. These results suggest that screening levels of serum sCD40L together with *M. tb* antigen-specific IFN-γ, TNF-α, and IL-2 responses may help evaluate drug efficacy, particularly the early therapeutic effect, in TB patients. However, our findings of *M. tb* antigen-specific IFN-γ, TNF-α, and IL-2 responses should be further tested considering the limited samples sizes at 2 months ($n = 14$) and 6 months ($n = 9$) follow-up time points.

Any association was not observed between the patients who had consistently higher levels of analytes in their sera versus plasma versus culture positivity. There was no correlation between cytokine signatures and the M. tb

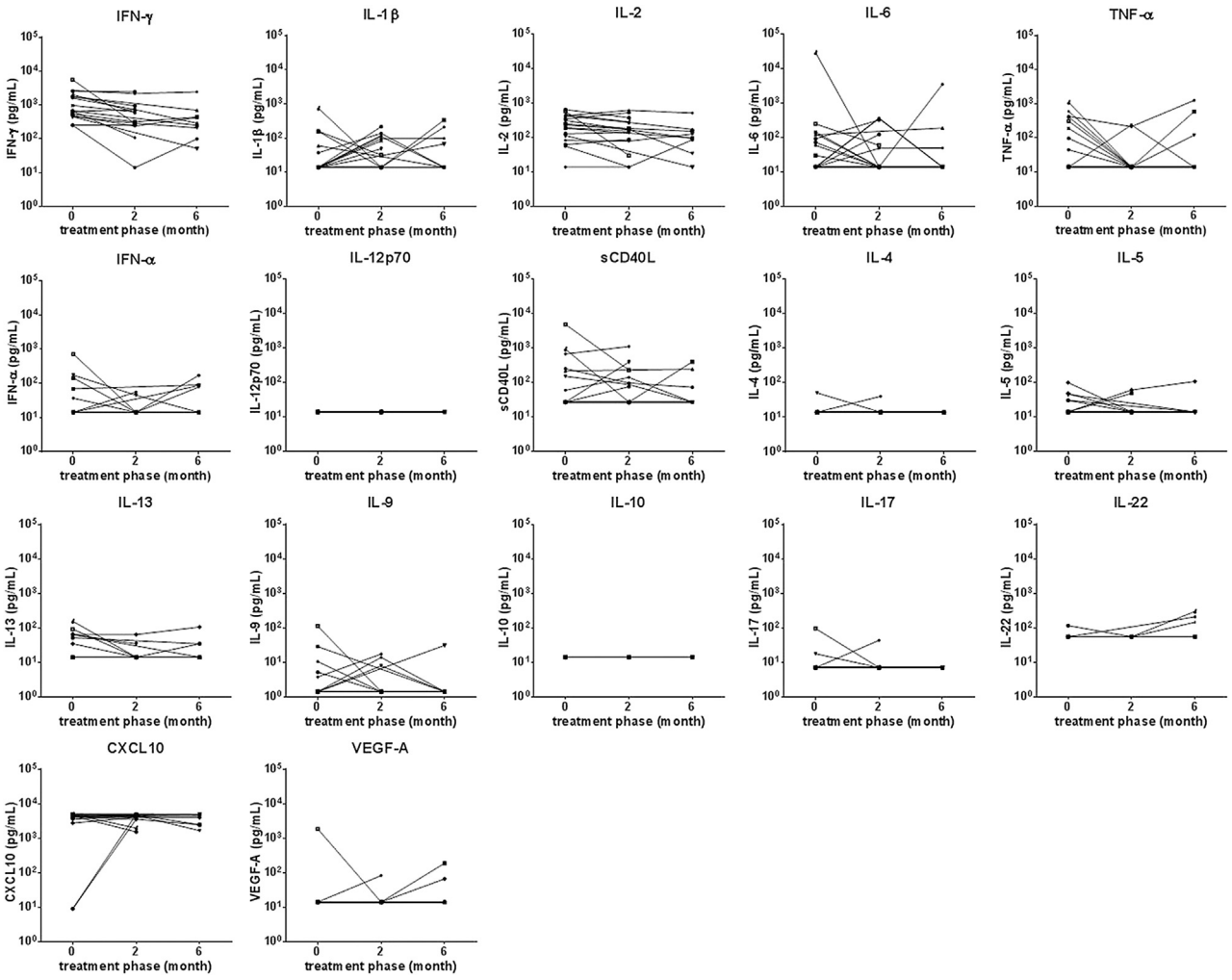

Figure 5 Changes in cytokine responses to *M. tb* antigens in TB patients during anti-TB treatment. QFT-IT plasma cytokine responses were followed up in 21 TB patients at pre-treatment, 14 patients after 2 months of treatment, and nine patients after 6 months of treatment. The IFN-γ and IL-2 responses were gradually reduced during treatment, whereas TNF-α responses were rapidly reduced after 2 months of treatment ($P < 0.05$) compared to other cytokine responses. Decreased plasma IFN-γ responses were found in all patients after 6 months of treatment ($P < 0.001$).

family (Beijing versus Non-Beijing) identified in the TB patients ($P > 0.05$, data not shown). Additionally, there was no evidence of significant differences between cytokine signatures and the NTM species identified ($P > 0.05$, data not shown). However, these results will likely hold true in future studies with larger sample sizes.

In conclusion, serum VEGF-A is the most informative marker for distinguishing active TB from LTBI, and a panel of serum IL-2, IL-9, IL-13, IL-17, TNF-α and sCD40L levels may contribute to more accurate and rapid differential diagnosis between active TB and NTM disease. Serum sCD40L levels and *M. tb* antigen-specific IFN-γ, TNF-α, and IL-2 responses could be a biomarker associated with treatment responses when combined with *M. tb* clearance in sputa cultures. Measurement of multiple analytes in serum or QFT-IT plasma could speed up diagnosis and may be utilised as a surrogate marker. In addition, it would greatly benefit the development of diagnostics to differentiate between active TB versus LTBI or active TB versus NTM disease.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jinf.2014.10.019.

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