Clinical Infectious Diseases

MAJOR ARTICLE

QuantiFERON Supernatant-Based Host Biomarkers Predicting Progression to Active Tuberculosis Disease Among Household Contacts of Tuberculosis Patients

Evangeline Ann Daniel,[1,2,©] Kannan Thiruvengadam,[1] Anuradha Rajamanickam,[3] Padmapriyadarsini Chandrasekaran,[1] Sathyamurthi Pattabiraman,[1] Brindha Bhanu,[1] Amsaveni Sivaprakasam,[1] Mandar Paradkar,[4,5] Vandana Kulkarni,[4,5] Rajesh Karyakarte,[6] Shri Vijay Bala Yogendra Shivakumar,[5] Vidya Mave,[4,5,7] Amita Gupta,[7] Subash Babu,[3] and Luke Elizabeth Hanna[1]

[1]National Institute for Research in Tuberculosis, Indian Council of Medical Research (ICMR), Chennai, India; [2]University of Madras, Chennai, India; [3]International Centre for Excellence in Research-National Institute for Research in Tuberculosis, Indian Council of Medical Research (ICMR), Chennai, India; [4]Byramjee Jeejeebhoy Government Medical College, Johns Hopkins Clinical Research Site, Pune, India; [5]Johns Hopkins Center for Infectious Diseases in India, Pune, India; [6]Byramjee Jeejeebhoy Government Medical College and Sassoon General Hospitals, Pune, India; and [7]John Hopkins University School of Medicine, Baltimore, Maryland, USA

Background. The positive predictive value of tuberculin skin test and current generation interferon gamma release assays are very low leading to high numbers needed to treat. Therefore, it is critical to identify new biomarkers with high predictive accuracy to identify individuals bearing high risk of progression to active tuberculosis (TB).

Methods. We used stored QuantiFERON supernatants from 14 household contacts of index TB patients who developed incident active TB during a 2-year follow-up and 20 age and sex-matched non-progressors. The supernatants were tested for an expanded panel of 45 cytokines, chemokines, and growth factors using the Luminex Multiplex Array kit.

Results. We found significant differences in the levels of TB-antigen induced production of several analytes between progressors and non-progressors. Dominance analysis identified 15 key predictive biomarkers based on relative percentage importance. Principal component analysis revealed that these biomarkers could robustly distinguish between the 2 groups. Receiver operating characteristic analysis identified interferon-γ inducible protein (IP)-10, chemokine ligand (CCL)19, interferon (IFN)-γ, interleukin (IL)-1ra, CCL3, and granulocyte-macrophage colony-stimulating factor (GM-CSF) as the most promising predictive markers, with area under the curve (AUC) ≥90. IP-10/CCL19 ratio exhibited maximum sensitivity and specificity (100%) for predicting progression. Through Classification and Regression Tree analysis, a cutoff of 0.24 for IP-10/CCL19 ratio was found to be ideal for predicting short-term risk of progression to TB disease with a positive predictive value of 100 (95% confidence interval [CI] 85.8–100).

Conclusions. The biomarkers identified in this study will pave way for the development of a more accurate test that can identify individuals at high risk for immediate progression to TB disease for targeted intervention.

Keywords. QuantiFERON supernatants; tuberculosis; biomarkers; progression.

INTRODUCTION

Recent estimates suggest that one-fourth of the global population [1] and almost 40% of the population in tuberculosis (TB) endemic countries are latently infected with TB [2]. However, only 5%–10% of this population eventually progress to active TB disease [3] and identifying this high-risk group and precisely targeting them for prophylactic treatment is key to achieving the TB elimination targets set forth by the World Health Organization (WHO).

The currently available diagnostic tests for latent TB infection (LTBI) viz. the interferon gamma release assays (IGRAs) and tuberculin skin test (TST) have a low positive predictive value (PPV) of 2.7% and 1.5%, respectively [4], and thus, the number of TST or IGRA-positive individuals who will require treatment (numbers needed to treat [NNT]) so as to prevent progression of TB in a single individual is very high. In a scenario of non-availability of proper predictors and very high estimated prevalence of LTBI, a panel of easily measurable biomarkers or bio-signatures with high diagnostic accuracy for predicting risk of disease progression is one of the foremost priorities for global TB control.

Host immunological markers including cytokines and chemokines serve as a double-edged sword in balancing between protection and pathology of TB [5]. Stimulation of whole blood with TB antigen results in significant enrichment of the TB-specific cytokine response, and hence the use of QuantiFERON supernatants would enable more accurate measurement of TB-specific responses. However, there have been

Received 07 June 2022; editorial decision 21 December 2022; published online 30 December 2022

Correspondence: L. E. Hanna, Department of Virology and Biotechnology, ICMR-National Institute for Research in Tuberculosis, No 1, Mayor Sathyamoorthy Rd, Chetpet, Chennai 600 031, Tamil Nadu, India (hannatrc@yahoo.com).

Clinical Infectious Diseases® 2023;76(10):1802–13

https://doi.org/10.1093/cid/ciac979

no studies thus far on the identification of soluble biomarkers that can predetermine progression from latent to active TB in QuantiFERON supernatants. In the present study, we measured the levels of unstimulated and TB-antigen stimulated cytokines, chemokines and growth factors in healthy household contacts (HHCs) of TB patients who progressed to active TB versus those who did not, to elucidate the differential immune profile between the two groups and to identify predictors for progression to infectious TB disease.

METHODS

Ethical Approval

The parent protocol of this study had the approval of the respective Institutional Ethics Committees of ICMR-National Institute for Research in Tuberculosis (ICMR-NIRT), Chennai, India, Byramjee Jeejeebhoy Government Medical College (BJGMC), Pune, India and Johns Hopkins University (JHU), Baltimore, Maryland USA.

Study Cohort

A cohort of HHCs of newly diagnosed pulmonary TB (PTB) patients was established at 2 sites, ICMR-NIRT and BJGMC, India, in collaboration with JHU, USA, as part of the Cohort for Tuberculosis Research by the Indo-US Medical Partnership (C-TRIUMPH) study, and the enrolled participants were followed up between August 2014 and December 2017. Details of the C-TRIUMPH study design and implementation have been described previously [6]. The definitions used for classifying study participants are provided in Table 1. All HHCs underwent clinical and laboratory assessment for TB at baseline and during each follow-up visit. TST and IGRA were performed at baseline and repeated at every visit if the previous test was negative. An HHC was confirmed to have active TB disease if they tested positive by TB culture or GeneXpert/MTB Rif.

Diagnostic Tests for Active TB

Sputum samples were collected from all the participants and subjected to GeneXpert MTB/RIF assay, followed by Löwenstein-Jensen (LJ) media and mycobacterial growth indicator tube (MGIT) liquid culture. Samples testing positive for *Mtb* were categorized as confirmed TB cases.

Table 1. Definitions Used for Stratifying Study Participants

Classification	Definition
HHCs	Adults and children residing in the same house with a TB patient during 3 months before TB diagnosis in the index case.
Progressors	HHCs who developed TB any time after 2 months of TB diagnosis in the index case. TB diagnosis was based on CXR, positive sputum smear, and culture.
Non-progressors	HHCs who remained healthy and did not develop TB during the follow-up period of 2 years. negative for symptom screen, CXR, TST, and IGRA.

Abbreviations: CXR, chest X-ray; HHC, healthy household contact; IGRA, interferon gamma release assay; TB, tuberculosis; TST, tuberculin skin test.

QuantiFERON-TB Gold-in Tube (QFT-GIT) Assay

Whole blood was collected and incubated in stimulated (TB antigen), positive control (mitogen) and unstimulated (Nil) tubes and processed as per the manufacturer's instructions (QIAGEN, Germany). After incubation, supernatants were harvested to measure the interferon (IFN)-γ response (IU/mL). Individuals were considered QFT-positive or negative based on the analysis using the QFT-GIT analysis software (version 2.62). The remaining QuantiFERON supernatants were immediately stored at −80°C for further analysis.

Multiplex Cytokine Assay

Circulating levels of cytokines, chemokines and growth factors were quantified in stored unstimulated and TB antigen-stimulated supernatants using the Human XL Cytokine Magnetic Luminex Performance Assay 45-plex Fixed Panel (R&D systems) according to the manufacturer's protocol. The 45-plex panel included IFN-α, IFN-β, IFN-γ, interleukin (IL)-1α, IL-1ra, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL12p70, IL-13, IL-15, IL-17A, IL-17E, IL-33, tumor necrosis factor (TNF)-α, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), chemokine (C-X-C motif) ligand (CXCL)1, CXCL2, IL-8/CXCL8, CXCL10/IP-10, CCL2, CCL3, CCL4, CCL5/ RANTES (Regulated on Activation, Normal T Expressed and Secreted), CCL11/Eotaxin, CCL19, CCL20, CX3CL1/Fractalkine, CD40 Ligand, epidermal growth factor (EGF), fibroblast growth factor (FGF) basic, FLT-3L, Granzyme B, programmed death ligand (PDL)1, platelet-derived growth factor (PDGF)-AA, PDGF-AB/BB, transforming growth factor (TGF)α, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and vascular endothelial growth factor (VEGF). The analyte levels were background-corrected before analysis by subtracting the concentration of the analyte in the unstimulated supernatants from their corresponding concentration in TB antigen-stimulated supernatants.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 9.2.0 [7] and R software [8]. Mann–Whitney test was used to compare between progressors and non-progressors, and the *P* values were adjusted for family-wise error rate (FWER) across the analytes by applying Bonferroni correction. Kruskal–Wallis test followed by Dunn's multiple comparison for post hoc correction was used for comparing between the progressor, non-progressor, and index TB groups. The same test was used to compare between non-progressors and progressors stratified based on the time duration to TB breakdown. $P < .05$ was fixed as the threshold of significance.

Receiver operating characteristic (ROC) analysis was performed to evaluate the diagnostic potential of each biomarker in terms of sensitivity, specificity, positive and negative predictive value. Because each of the 45 analytes are intercorrelated, we performed a dominance analysis to rank the best predictive analytes based on relative importance. The discriminatory power of the potential predictive biomarkers was evaluated by principal component analysis (PCA). To determine the cutoffs for the biomarkers that distinguished between progressors and non-progressors, classification and regression tree (CART) models were employed.

RESULTS

Selection of Screening Cohort

Among the 1051 HHCs enrolled and followed up between August 2014 and December 2017, 20 (1.9%) developed active TB during a 2-year follow-up and were identified as "Progressors." Six of the progressors did not have QuantiFERON supernatants and were therefore excluded. The duration from enrolment to diagnosis with active TB ranged from 3 to 21 months. An equal number of HHCs who did not develop active TB during the entire period of follow-up ("non-progressors") matched for age and sex with the

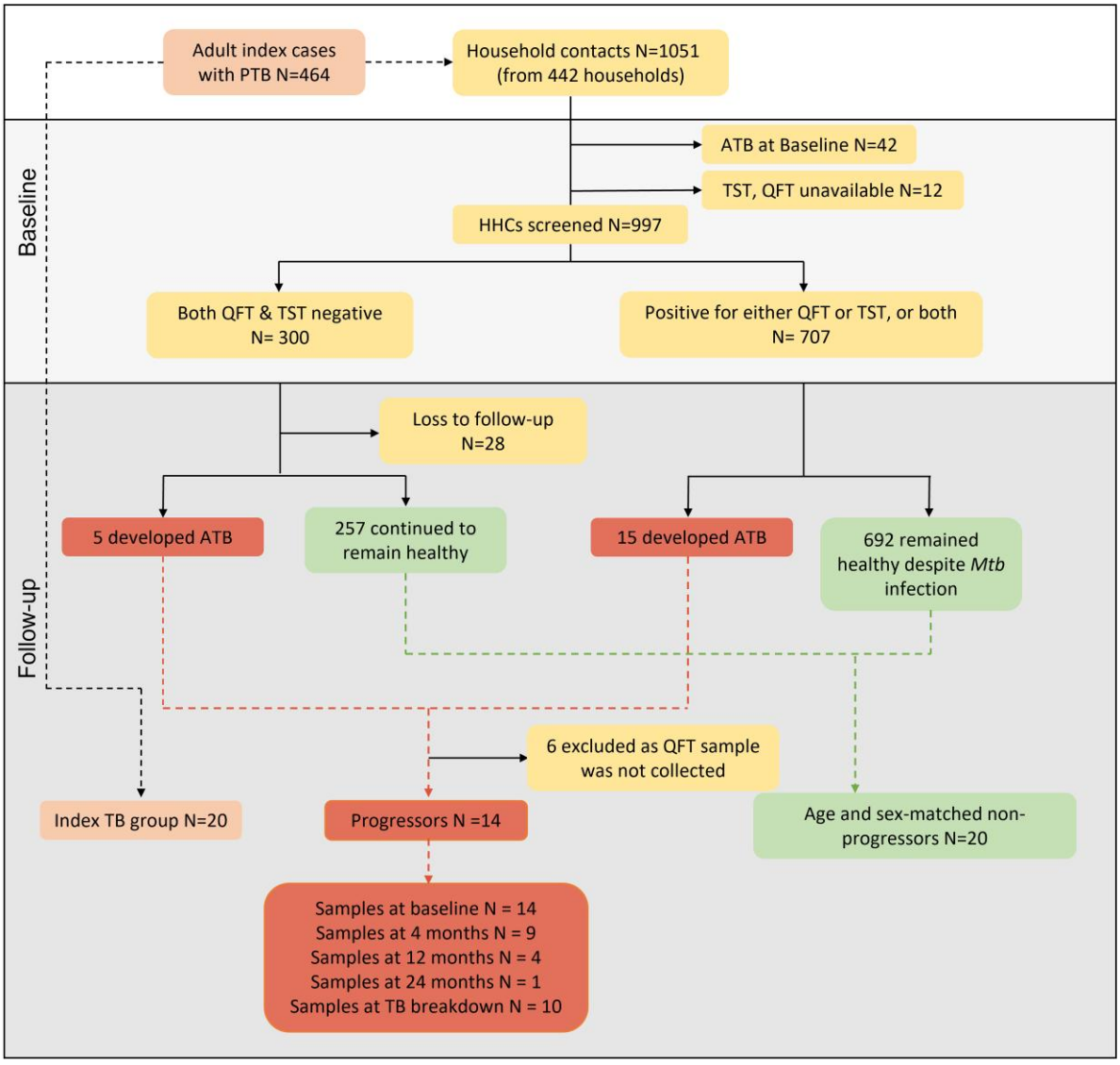

Figure 1. Participant selection from 2 sites of the C-TRIUMPH cohort study: A total of 1051 adults and children were recruited in the C-TRIUMPH study. Participants were classified based on their baseline *Mtb*-infection status as positive for QFT (≥0.35 IU/mL) and/or positive for TST (induration diameter ≥5 mm) and negative for both. Among these, participants who went on to develop TB during follow-up were identified as progressors, and those who remained healthy were defined as non-progressors. Progressors were matched to non-progressors for age and gender. Abbreviations: ATB, active tuberculosis; C-TRIUMPH, Cohort for Tuberculosis Research by the Indo-US Medical Partnership; HHC, healthy household contacts; *Mtb, Mycobacterium tuberculosis*; PTB, pulmonary tuberculosis; QFT, QuantiFERON-TB Gold; TST, tuberculin skin test.

Table 2. Clinical and Demographic Characteristics of Participants

Patient Characteristics	Progressors (n = 14)	Non-Progressors (n = 20)	Index TB (n = 20)
Age in years, median (IQR)	32 (24–38)	31 (22–37)	27 (20–38)
Sex, n (%)			
Male	5 (36)	7 (35)	10 (50)
Female	9 (64)	13 (65)	10 (50)
HIV status, n (%)			
Positive	1 (7)	0 (0)	2 (10)
Negative	13 (93)	20 (100)	18 (90)
History of diabetes, n (%)			
Yes	0 (0)	0 (0)	3 (15)
No	14 (100)	20 (100)	16 (80)
N/A	…	…	1 (5)
IGRA result, n (%)			
Positive	9 (64)	12 (60)	…
Negative	5 (36)	8 (40)	…
Patient categorization			
Culture positive	6	…	15
Xpert positive	1	…	14
Both positive	4	…	13
AFB smear positive	1	…	11
Positive by culture, Xpert and smear	0	…	10

Abbreviations: AFB, acid-fast bacillus; HIV, human immunodeficiency virus; IGRA, interferon gamma release assay; IQR, interquartile range; N/A, not applicable.

progressors were identified as controls. The quantitative outcome measures of progressors and non-progressors through QFT-GIT assay are provided in Supplementary Table 1. Twenty index TB cases were included in the active TB group. The overall study design and participant selection are shown in Figure 1. The clinical characteristics and demographic details of the study cohort are provided in Table 2.

Levels of Cytokines, Chemokines and Growth Factors in QuantiFERON Supernatants of Progressors and Non-progressors

We measured levels of Type 1 and Type 2 IFNs, pro and anti-inflammatory cytokines, CC and CXC chemokines and growth factors in the Nil and TB antigen-stimulated QuantiFERON supernatants of progressors, non-progressors and active TB cases. Significantly elevated levels of IFN-α, IFN-β, IL-4, IL-5, IL-6, IL-15, G-CSF, GM-CSF, IL-1β, CCL3, CCL4, CCL11, IP-10, and TGF-α, and decreased levels of IFN-γ, IL-1α, IL-1ra, IL-2 and CCL19 were identified in progressors as compared to non-progressors. The median levels (pg/mL) of all the analytes in antigen-stimulated QuantiFERON supernatants of progressors, non-progressors and index TB cases are shown in Figures 2–4 and Table 3.

Levels of Cytokines, Chemokines and Growth Factors in QuantiFERON Supernatants of Progressors and Non-progressors at Different Time-points Prior to TB Breakdown

We further stratified the progressors based on the time duration between enrolment and TB breakdown as <6 months, 6–10 months and >10 months. IP-10, CCL19, CCL3, CCL11, IFN-γ, IFN-β, IL-1α, and IL-1ra were found to be significantly different ($P < .05$) between the progressors and non-progressors as early as 21 months prior to the development

Table 3. Median Levels of Analytes (pg/mL) in *Mtb* Antigen-stimulated QuantiFERON Supernatants of Progressors and Non-progressors

Analyte	Non-Progressors	Progressors	*P* Value
CCL19	1159.1 (703–1648)	268.5 (33.7–629)	<.0001
IP-10	160.1 (68.7–226.1)	725.9 (433.1–1008.3)	<.0001
IFNγ	535.4 (439.1–761.4)	372.6 (94.2–491.2)	<.0001
IL-1ra	3147.5 (2013.2–5972.8)	1118.7 (196.1–2405.5)	<.0001
GM-CSF	103.4 (42.4–153.1)	170.1 (120–538.3)	<.0001
CCL3	143.5 (59–217.5)	346.6 (126.3–473.3)	<.0001
CCL11	191.9 (111.2–399)	485 (204.2–2433)	<.0001
IFNβ	25.3 (16–35.3)	44.4 (23.4–263.3)	<.0001
IL-1α	397.1 (326.7–507.5)	218.4 (57.2–391.5)	<.0001
IL-2	258.7 (164.1–428.5)	168.5 (0–305.8)	.0003
IL-5	52.1 (30.6–122)	107.5 (22.3–253.3)	.0003
IFN-α	244.8 (163.3–391.5)	389.9 (132.8–547.9)	.0005
IL-6	123.3 (87–198.5)	195.5 (89–408.5)	.0009
IL-4	134.6 (120.2–155.3)	155.5 (111.1–297.5)	.0012
IL-1β	152.4 (72–380.8)	251.7 (108.4–409.3)	.0043
TGF-α	167.6 (103.3–358.4)	247.8 (113–850.7)	.0065
CCL4	765.1 (442–1042)	1003.5 (520.9–2296.9)	.0072
G-CSF	115.8 (59.7–160.3)	136.6 (86.4–566)	.0088
IL-15	76.6 (53.7–120.5)	81.1 (59.2–423.5)	.0450
CCL5	3783.3 (2028–6171)	3404.2 (757.5–5396)	.0626
Granzyme B	113.8 (51.3–197.8)	80.8 (35.1–175.6)	.0658
VEGF	231.4 (124.9–482.2)	384.2 (31.6–702)	.0660
TRAIL	213.5 (115.2–342.9)	182.5 (50.6–389.6)	.1141
TNFα	217.1 (68.7–292.3)	178.3 (55–328.8)	.1254
IL-17A	391.1 (208–570.8)	604.6 (139.5–902.9)	.1314
GROβ	1878.3 (1048.7–2671.1)	2310.8 (921.5–4190.9)	.1505
CX3CL1	591.4 (423.8–755.3)	538.4 (84.2–760.9)	.1572
IL-7	96.3 (44.6–137.1)	114.5 (48.3–168.4)	.1643
IL-8	115.2 (37.7–221.4)	106.6 (29.3–172.8)	.1791
IL-13	197.6 (192.8–217.4)	208.7 (135.3–348.7)	.2024
CD40L	1476.4 (1413.8–2334.9)	1480.7 (1278.2–1915.3)	.2030
IL-3	263.5 (117.8–428.6)	242.3 (57.9–467.6)	.2287
MCP1	383.8 (232.7–537.3)	425.1 (136.9–1853.7)	.2293
IL-33	99.1 (62.8–186)	91.7 (26.3–152.8)	.2339
FLT3L	367.1 (260.9–885.6)	333.7 (185.2–891.9)	.2385
GROα	370.8 (159.2–1089.7)	378.7 (113.9–1023.4)	.2835
PDL1	156.2 (117.3–211.8)	263.3 (81.7–536.5)	.3456
IL-12p70	288.2 (200.6–497.4)	384.2 (76.8–674.5)	.3517
IL-17E	149 (100.3–270.4)	134.9 (58.5–233.3)	.3767
PDGF-AB/BB	8500.3 (393.6–217 705.8)	9387.7 (239.7–48 591.8)	.5557
FGF basic	81.7 (39.7–147.4)	84.3 (24.4–277)	.6289
CCL20	133.5 (93–176)	109.6 (69.6–758.6)	.6373
PDGF-AA	3457.5 (163.1–8165.7)	4231.1 (498.6–7431.3)	.7237
EGF	233.3 (103.3–681.3)	277.5 (82.5–516.5)	.8597
IL-10	149.5 (112.4–404.8)	257.8 (0.5–593.6)	.9249

Mann–Whitney test was used to compare the 2 groups. *P* values were adjusted for family-wise error rate (FWER) across the 45 analytes by applying Bonferroni correction.

Abbreviations: CCL, chemokine ligand; EGF, epidermal growth factor; FGF, fibroblast growth factor; IFN, interferon; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; *Mtb*, *Mycobacterium tuberculosis*; PDGF, platelet-derived growth factor; TGF, transforming growth factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; VEGF, vascular endothelial growth factor.

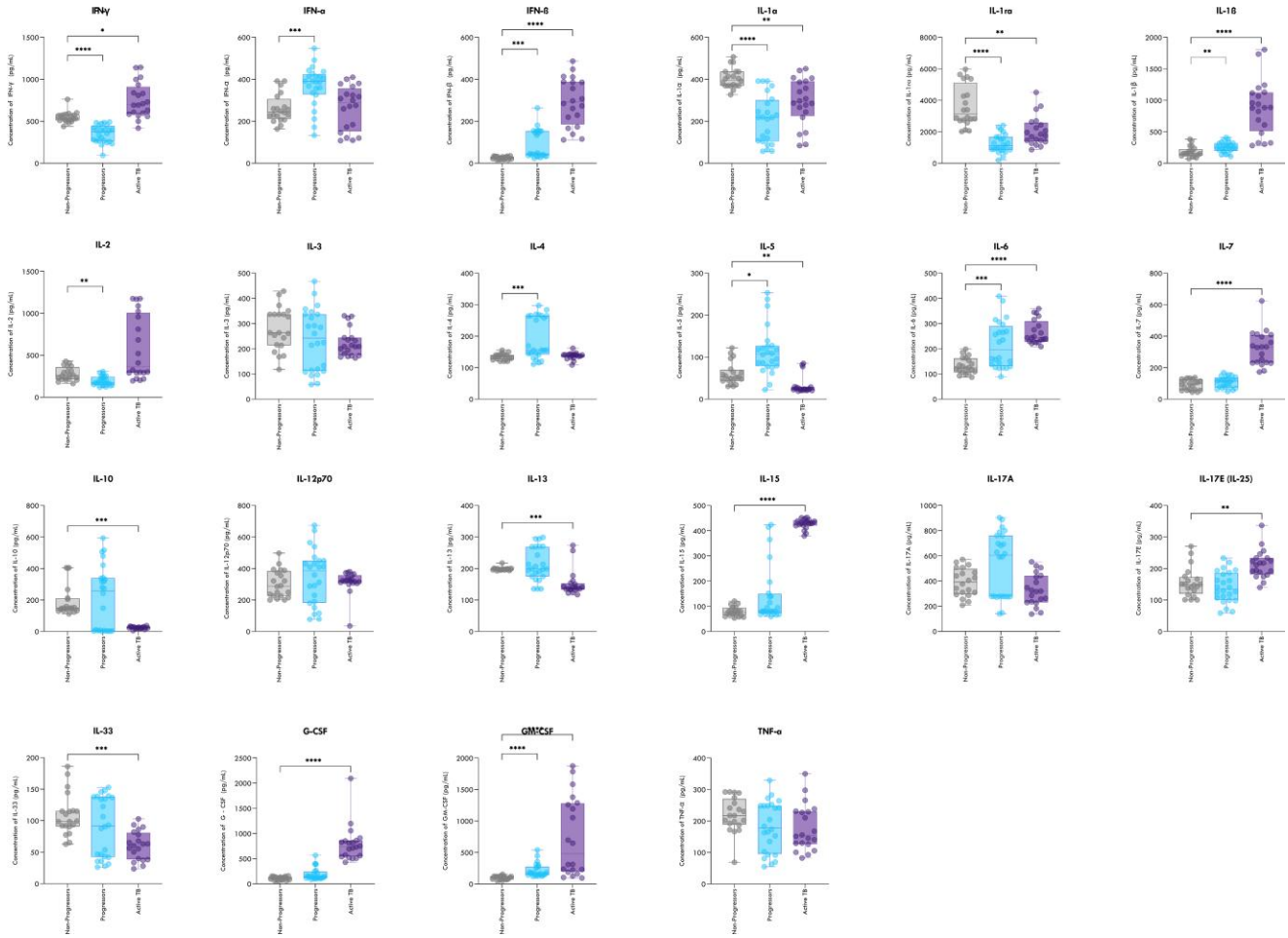

Figure 2. Median levels of cytokines (pg/mL) in antigen stimulated QuantiFERON supernatants of progressors, non-progressors, and index TB cases: Significant differences between groups were calculated using Kruskal-Wallis test coupled with Dunn's correction for multiple comparison and expressed as: * ($P < .05$), ** ($P < .01$), *** ($P < .001$), **** ($P < .0001$). Abbreviations: G-CSF, granulocyte colony-stimulating factor; IL, interleukin; IFN, interferon; TB, tuberculosis; TNF, tumor necrosis factor.

of active TB disease (Figure 5). IFN-α levels increased significantly 10 months prior to TB breakdown. On the other hand, levels of GM-CSF, IL-5, IL-6, and IL-18 increased significantly near the time of breakdown with active TB (<6 months) while IL-2 levels declined significantly (Figure 6).

Performance of the Biomarkers in Predicting Development of Active TB

The performance of the significantly deregulated biomarkers in predicting progression to active TB was assessed by constructing ROC curves to determine the sensitivity, specificity and area under the curve (AUC) with 95% confidence intervals (CI). Among the evaluated biomarkers, IP-10, CCL19, IFN-γ, IL-1ra, CCL3 and GM-CSF showed potential as promising predictive markers with AUC ≥90. IP-10 and CCL19 had the best diagnostic performance, with AUC = 100 (95% CI: 92–100) and sensitivity and specificity of 100%. IFN-γ exhibited a sensitivity of 100% (95% CI: 85.8–100) but specificity of 90% (95% CI: 68.3–98.8) with AUC = 95.5 (95% CI: 84.5 to 99.4). CCL3 had a sensitivity of 91.7% (95% CI: 73–99), specificity of 100% (95% CI: 84.6–100) and AUC = 95.5 (95% CI: 84.5 to 99.4). GM-CSF had a sensitivity of 91.7% (95% CI: 73–99) and specificity of 90% (95% CI: 68.3–98.8) with AUC = 90.9 (95% CI: 78.3–97.5). Cutoffs were determined for all analytes to assess their overall discriminatory performance between progressors and non-progressors (Table 4 and Supplementary Table 2) as well as their performance after stratification as <6 months and >6 months prior to TB activation to assess their predictive ability for short and long term risk (Supplementary Tables 3 and 4). IP-10, CCL19, CCL11, CCL3, IL-17A and TNF-α were identified as short-term risk predictors, whereas IFN-β, IL-1ra, GM-CSF and IL-1α performed well as long-term risk predictors.

Dominance Analysis

Dominance analysis was performed to identify the most promising biomarkers that could clearly discriminate between

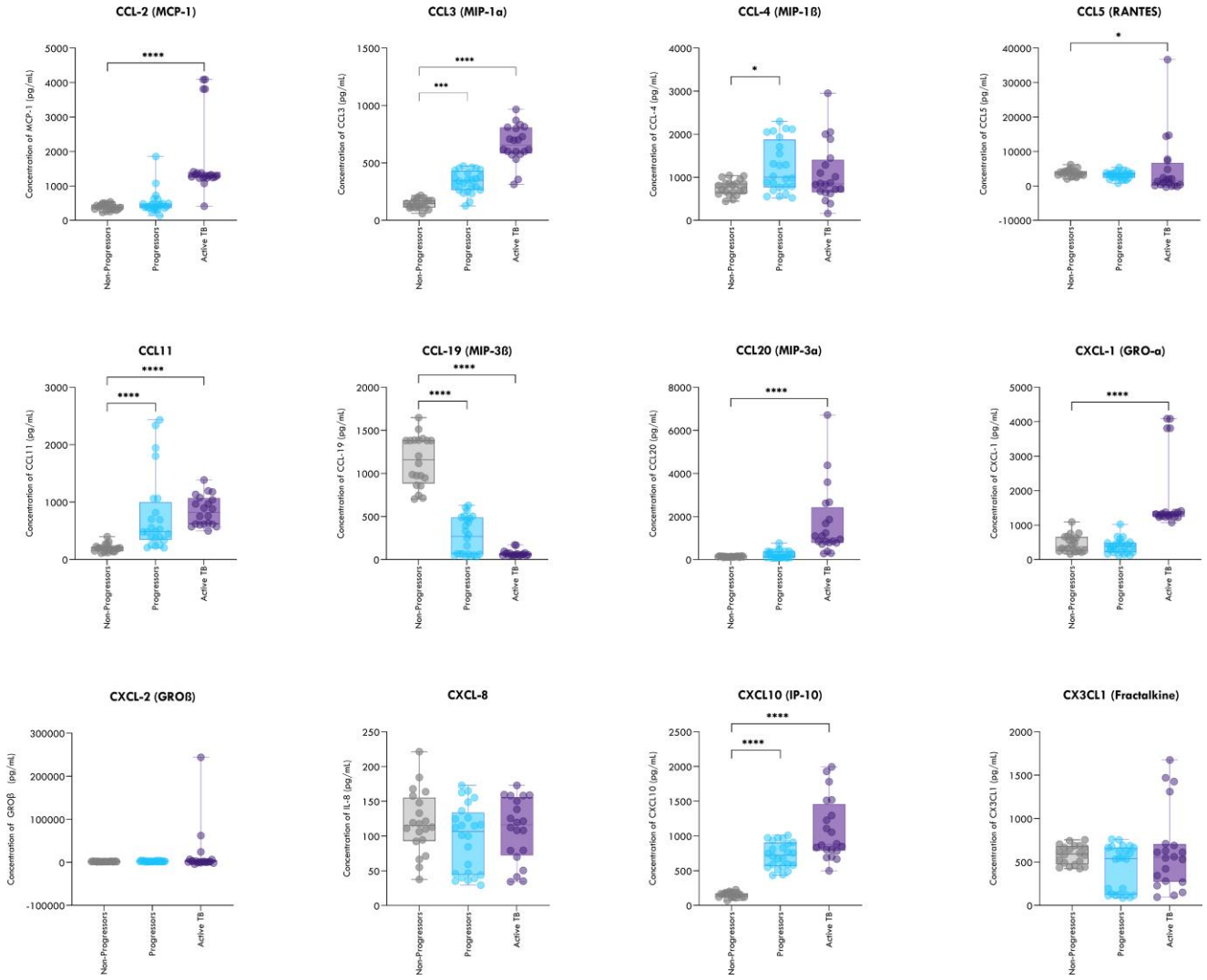

Figure 3. Median levels of chemokines (pg/mL) in *Mtb* antigen-stimulated QuantiFERON supernatants of progressors, non-progressors and index TB cases: Significant differences between groups were calculated using Kruskal–Wallis test coupled with Dunn's correction for multiple comparison and expressed as: * ($P < .05$), ** ($P < .01$), *** ($P < .001$), **** ($P < .0001$). Abbreviations: CCL, chemokine ligand; CXCL, chemokine (C-X-C motif) ligand; MCP, *monocyte chemoattractant protein; Mtb, Mycobacterium tuberculosis*; RANTES, Regulated on Activation, Normal T Expressed and Secreted; TB, tuberculosis.

progressors and non-progressors. For the 45 biomarkers, 70 369 000 000 000 regressions were computed, and 15 key predictive markers were narrowed down based on their relative importance (R^2). For the 15 key predictors, 32 767 regressions were performed to identify the best set of biomarkers. Based on the percentage of relative importance, IP-10, CCL19, IL-1ra, CCL3, IFN-γ, CCL11, GM-CSF, IFN-α, IL-6, CCL4, IL-1α, IFN-β, IL-2, IL-5 and IL-4 were identified as the best predictors (Figure 7).

We performed PCA on the 15 significantly different analytes identified by dominant analysis to assess the discriminatory power of these biomarkers in distinguishing progressors from non-progressors (Figure 8). PCA clearly demonstrated the ability of these markers to differentiate both the 2 groups with perfect bifurcation.

IP10/CCL19 Ratio as a Useful Biomarker for Predicting Progression to Active Tuberculosis

Because most cytokines are not specific for TB and can be found in various other inflammatory and autoimmune diseases, a single biomarker like IFN-γ or IP-10 cannot accurately predict progression. As observed in Figure 3, the progressor group had significantly higher levels of IP-10 and decreased levels of CCL19 when compared to the non-progressor group. The diagnostic performance of these two biomarkers is also superlative (Table 4). Thus, IP-10/CCL19 ratio stands out as a

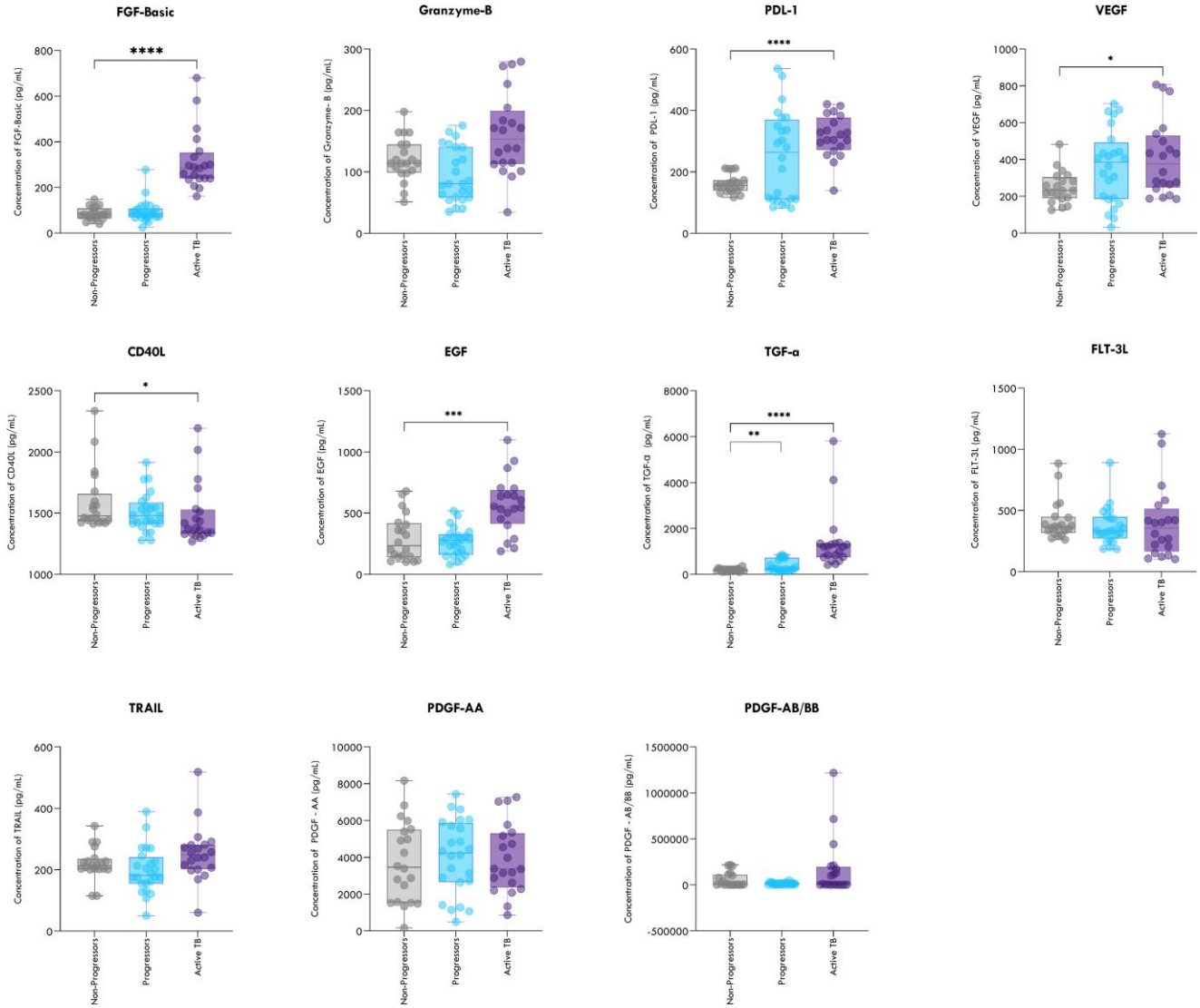

Figure 4. Median levels of growth factors (pg/mL) in *Mtb* antigen-stimulated QuantiFERON supernatants of progressors, non-progressors and index TB cases: Significant differences between groups were calculated using Kruskal-Wallis test coupled with Dunn's correction for multiple comparison and expressed as: * ($P < .05$), ** ($P < .01$), *** ($P < .001$), **** ($P < .0001$). Abbreviations: EGF, epidermal growth factor; FGF, fibroblast growth factor; *Mtb, Mycobacterium tuberculosis*; PDGF, platelet-derived growth factor; TB, tuberculosis; TGF, transforming growth factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; VEGF, vascular endothelial growth factor.

highly potential risk signature for predicting progression to active infectious TB.

CART Analysis to Determine Cutoff for the Candidate Predictive Biomarkers and Biomarker Combinations

In order to derive a cutoff for the biomarkers that clearly distinguished progressors from non-progressors, CART models were utilized. We used all the biomarker data for constructing the CART and subsequently selected the biomarkers and their combinations that classified the groups with high accuracy (Figure 9). The CART model identified IP-10/CCL19 ratio at a cutoff value of 0.24 pg/mL, as the most promising risk predictor (Figure 9*A*). Individually, IP-10 presented a cutoff value of 226.06 pg/mL, and CCL19 gave a cutoff value of 628.98 pg/mL (Figure 9*B* and 9*C*). Because the above models exhibited 100% accuracy, we performed further pruning by removing IP-10 and CCL-19 to determine the cut-offs for other potential predictive biomarker combinations. This revealed a combination of CCL3 having a threshold of 217.5 pg/mL and IL-2 with a threshold of 163.9 pg/mL, sensitivity of 95.2%, and specificity of 100% as the next best combination (Figure 9*D*). The combination of IFN-γ with a cutoff of 491.2 pg/mL and CCL11 with a cutoff of 202.43 pg/mL gave a sensitivity of 100% but specificity of 90% (Figure 9*E*). Thus, CART analysis demonstrated that IP-10/CCL19 ratio (cutoff: 0.24 pg/mL) and combinations of CCL3 and IL-2,

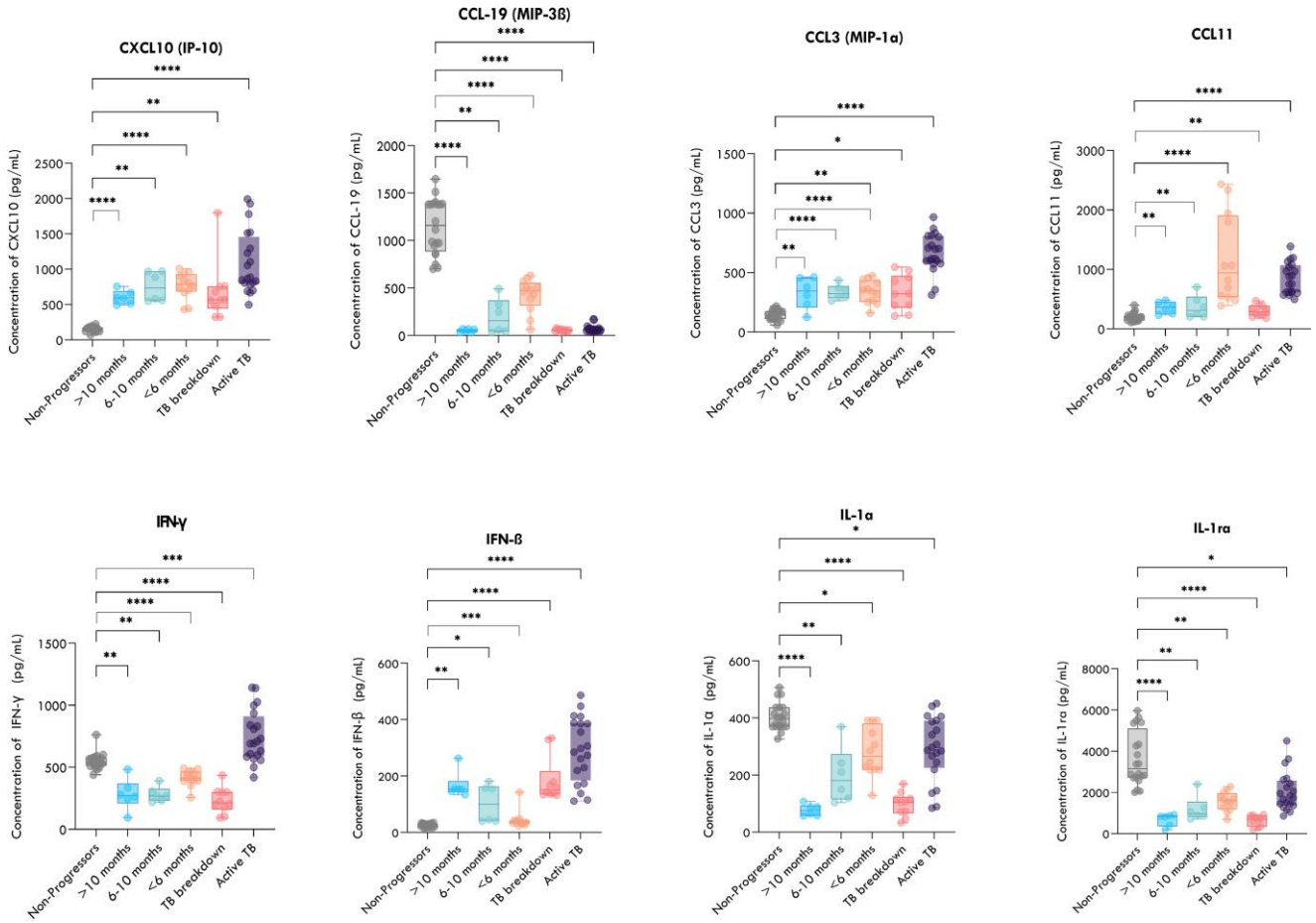

Figure 5. Median levels of analytes (pg/mL) in *Mtb* antigen-stimulated QuantiFERON supernatants of progressors, non-progressors and active TB groups: Progressors were further stratified based on time duration to TB activation. The above analytes were statistically significant at all time points studied, between the progressor and non-progressor groups. Significant differences between groups were calculated using Kruskal–Wallis test coupled with Dunn's correction for multiple comparison and expressed as: * ($P < .05$), ** ($P < .01$), *** ($P < .001$), **** ($P < .0001$). Abbreviations: CCL, chemokine ligand; CXCL, chemokine (C-X-C motif) ligand; IFN, interferon; IL, interleukin; MIP, macrophage inflammatory protein *Mtb, Mycobacterium tuberculosis*; TB, tuberculosis.

and IFN-γ and CCL11 could be promising predictive biomarkers for progression from LTBI to active TB.

DISCUSSION

Though IGRAs bear poor prognostic ability, the IGRA test can be usefully exploited to look for additional biomarkers, because stimulation of T cells with *Mycobacterium tuberculosis* (*Mtb*) antigens results in the upsurge of a complex network of cytokines and chemokines that represent TB-specific immune responses. In this study, we undertook an exploratory analysis of soluble biomarkers in QuantiFERON supernatants capable of predicting progression to TB disease and found significant difference in the levels of 19 of the 45 analytes in progressors as compared to non-progressors. Many of these analytes have previously been reported by others to be able to detect active TB disease [9]. Our findings of a similar profile in high-risk individuals up to 21 months prior to clinical diagnosis is very interesting. IP-10/CCL19 ratio demonstrated maximum sensitivity and specificity for predicting TB risk. IP-10 is a strong chemoattractant that causes necrosis of tuberculous granuloma by recruiting various T cells. Higher levels of IP-10 in progressors indicate the inhibition of angiogenesis and subsequent cessation of granuloma. Studies in murine lung models have documented the potential role of CCL19 in homing CCR7 expressing T cells and dendritic cells during the organization of lymphoid structures [10, 11]. Reduced CCL19 levels observed in progressors signify an impairment in granuloma formation leading to disease progression. Furthermore, we also observed significant upregulation in IFN-αβ levels and downregulation in IFN-γ levels in the progressor group. Increased levels of type I IFNs have been observed in *Mtb*-infected macrophages, and this is known to have an adverse impact on infection control [12]. On the contrary, type II IFNs (IFN-γ) have a protective response against *Mtb* [13]. Previous studies have observed

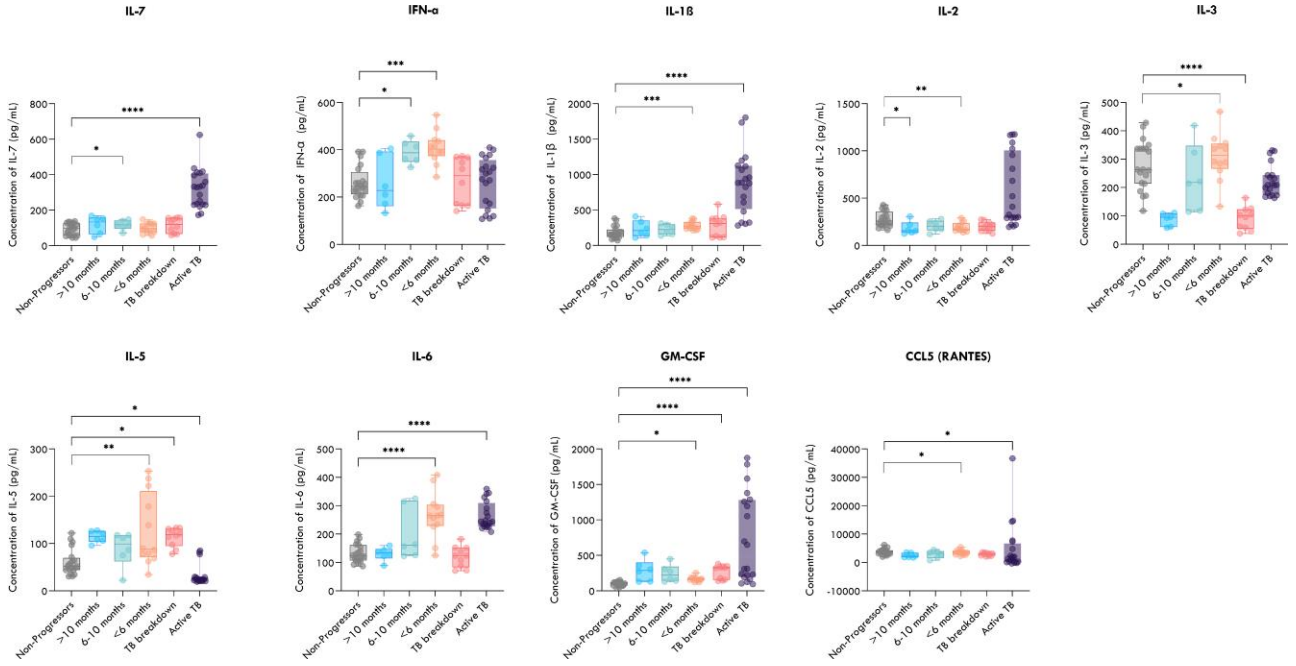

Figure 6. Median levels of analytes (pg/mL) in *Mtb* antigen-stimulated QuantiFERON supernatants of progressors that are significantly different <10 months before TB breakdown: Significant differences between groups were calculated using Kruskal–Wallis test coupled with Dunn's correction for multiple comparison and expressed as: * ($P < .05$), ** ($P < .01$), *** ($P < .001$), **** ($P < .0001$). Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; *Mtb*, *Mycobacterium tuberculosis*; RANTES, Regulated on Activation, Normal T Expressed and Secreted; TB, tuberculosis.

that *Mtb* infection is more likely in mice with disrupted IFN-γ production as compared to wild-type mice [14]. In a recent study, 20 IFN-γ related genes were reported to be downregulated in progressors after TB-specific antigenic stimulation [15]. These observations are concordant with our findings, suggesting that individuals with attenuated type II IFN response are more likely to develop active TB. After grouping the samples based on the time to TB breakdown as <6 months, 6–10 months, and >10 months, we could find that median levels of IP-10, CCL19, CCL3, CCL11, IFN-γ, IFN-β, IL-1α, and IL-1ra were significantly different at all time points studied and could very well reflect progression.

Earlier studies have found that IGRAs lack predictive accuracy, and this is evident from our study as well, as 5 of the 14 progressors (36%) had a negative IGRA and yet went on to progress to active TB. Many studies have addressed the question of whether higher IFN-γ values in IGRA and QFT conversion is a reflection of the risk of subsequent progression to active TB disease, but the results have been inconsistent. Although studies in children found high IFN-γ values (>3.5 IU/mL [4] and >4 IU/mL [16]) to be associated with increased progression rates, studies in adults [17, 18] did not find such an association. Our study also did not find higher IFN-γ levels in progressors reinstating the poor predictive value of IGRA.

It should be noted here that TB is a dynamic spectrum of disease rather than a classical binary state of latent and active TB. Earlier transcriptomics studies identified predictive gene signatures in individuals who developed incipient TB [15, 19–22], but the performance of these signatures was suboptimal in validation studies [23–25] implying that these signatures might reflect early bacterial replication (subclinical TB). In our study, we excluded individuals who developed TB within 2 months of TB diagnosis in the index case so as to ensure that the identified predictive markers corroborate immune alterations that are indicative of impending progression of infection and are not just markers of subclinical infection.

Host biomarker studies have been at the center of interest for TB immunodiagnostics due to their utility for development of a point-of-care assay. High IL-4 levels with concomitantly lower IFN-γ levels in the plasma of unstimulated whole-blood culture have been previously reported in HHCs who eventually developed TB, 6 months post TB exposure [26]. Similar observations have been made in PBMCs stimulated with TB antigens [27–29]. We also found a similar profile in our study. IL-13 expression was detected in 6 out of 14 progressors 6–8 months prior to disease onset in another study [30]. Mpande *et al* reported difference in HLA-DR expression between IFN-γ^{+}TNF^{+} *Mtb*-specific T cells and total CD3^{+} T cells as a predictive biomarker for TB [31]. De Groote *et al* reported significantly altered levels of IL-2, *monocyte chemoattractant protein* (MCP)-2 and IP-10, rather than IFN-γ in QFT supernatants in individuals with LTBI as compared to

Table 4. Accuracy of Biomarkers in Predicting Risk of Progression to Tuberculosis Disease

Biomarker[a]	Cutoff	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	AUC (95% CI)
CCL19	<628.98	100 (85.8–100)	100 (83.2–100)	100 (85.8–100)	100 (83.2–100)	100 (92–100)
IP-10	>226.06	100 (85.8–100)	100 (83.2–100)	100 (85.8–100)	100 (83.2–100)	100 (92–100)
CCL11	>204.20	100 (85.8–100)	75 (50.9–91.3)	82.8 (64.2–94.2)	100 (78.2–100)	88.6 (75.4–96.2)
IFN-γ	<491.20	100 (85.8–100)	90 (68.3–98.8)	92.3 (74.9–99.1)	100 (81.5–100)	95.5 (84.5–99.4)
IL-1ra	<2405.48	100 (85.8–100)	85 (62.1–96.8)	88.9 (70.8–97.6)	100 (80.5–100)	93.2 (81.3–98.6)
CCL3	>234.20	91.7 (73–99)	100 (83.2–100)	100 (84.6–100)	90.9 (70.8–98.9)	95.5 (84.5–99.4)
GM-CSF	>128.73	91.7 (73–99)	90 (68.3–98.8)	91.7 (73–99)	90 (68.3–98.8)	90.9 (78.3–97.5)
IFN-α	>326.45	79.2 (57.8–92.9)	80 (56.3–94.3)	82.6 (61.2–95)	76.2 (52.8–91.8)	79.5 (64.7–90.2)
IL-6	>227.00	50 (29.1–70.9)	100 (83.2–100)	100 (73.5–100)	62.5 (43.7–78.9)	72.7 (57.2–85)
CCL4	>862.18	66.7 (44.7–84.4)	80 (56.3–94.3)	80 (56.3–94.3)	66.7 (44.7–84.4)	72.7 (57.2–85)
IL-1α	<305.80	79.2 (57.8–92.9)	100 (83.2–100)	100 (82.4–100)	80 (59.3–93.2)	88.6 (75.4–96.2)
IFN-β	>36.76	79.2 (57.8–92.9)	100 (83.2–100)	100 (82.4–100)	80 (59.3–93.2)	88.6 (75.4–96.2)
IL-2	<180.94	62.5 (40.6–81.2)	95 (75.1–99.9)	93.8 (69.8–99.8)	67.9 (47.6–84.1)	77.3 (62.2–88.5)
IL-5	>75.29	83.3 (62.6–95.3)	80 (56.3–94.3)	83.3 (62.6–95.3)	80 (56.3–94.3)	81.8 (67.3–91.8)
IL-4	>141.14	79.2 (57.8–92.9)	80 (56.3–94.3)	82.6 (61.2–95)	76.2 (52.8–91.8)	79.5 (64.7–90.2)

Abbreviations: AUC, area under the curve; CI, confidence interval; CCL, chemokine ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; NPV, negative predictive value; PPV, positive predictive value.

[a]Only the key biomarkers predicting progression are listed. The accuracy of the remaining biomarkers is provided in Supplementary Table 2.

healthy controls, based on their analysis on SOMAscan platform [32], which however did not meet the target product profile (TPP) framed by WHO/FIND [22].

Our study identified IP-10/CCL19 ratio as a very promising predictive marker for active TB as it clearly achieves the optimal requirement of ≥90% sensitivity for biomarker-based non-

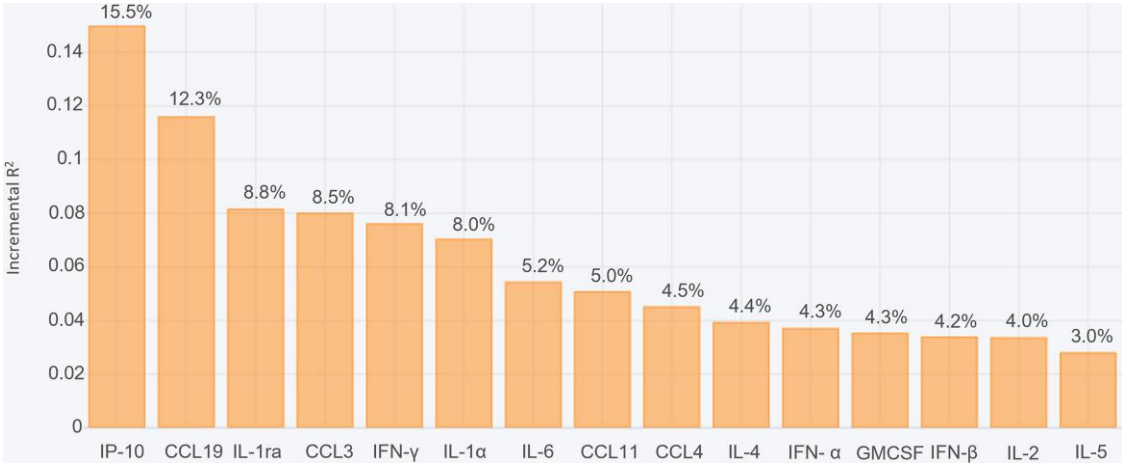

Figure 7. Incremental R^2 values for the 15 key biomarkers: Dominance analysis of the 45 analytes identified 15 key biomarkers based on relative importance (R^2). Abbreviations: CCL, chemokine ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin.

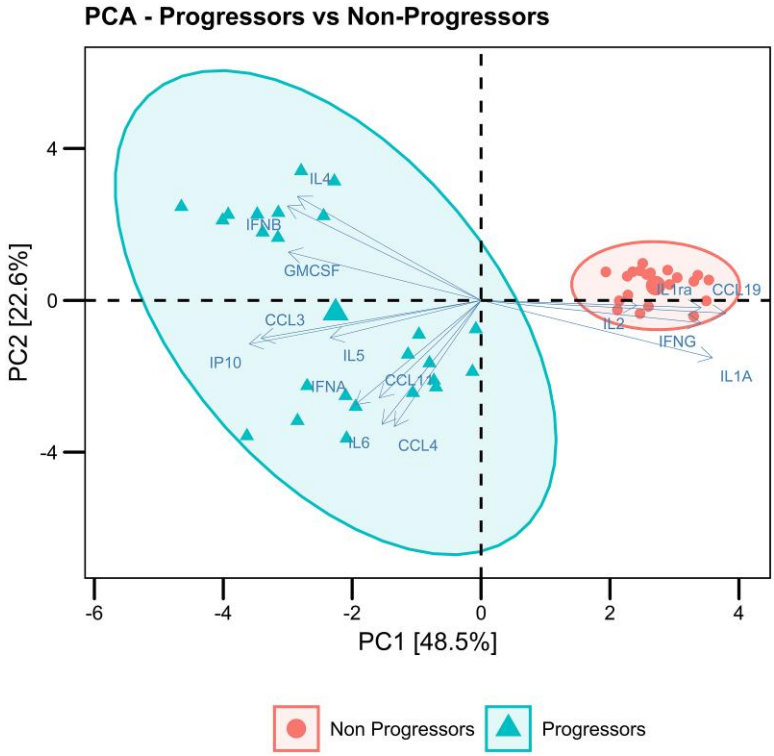

Figure 8. PCA plot of significantly different biomarkers between progressors and non-progressors: PCA shows that IP-10, CCL-19, IL-1ra, CCL3, IFN-γ, IL-2, IL-4, IL-1α, CCL4, IL-6, CCL11, IFN-α, IL-5, GM-CSF and IFN-β can clearly distinguish between progressors and non-progressors with no overlap. Abbreviations: CCL, chemokine ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; PCA, principal component analysis.

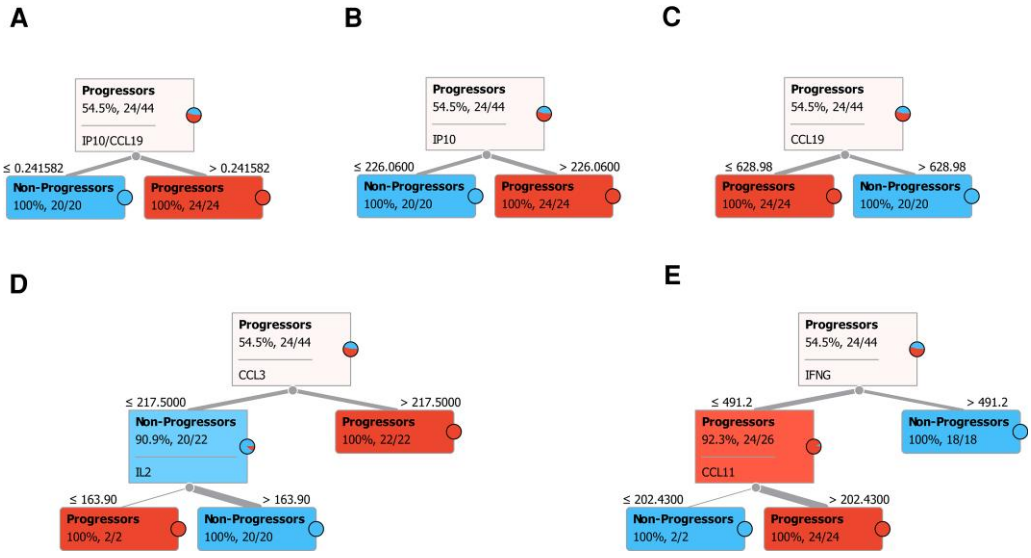

Figure 9. CART analysis to determine cut-offs for potential predictive biomarkers: CART models generated for IP-10/CCL19 ratio (*A*), IP-10 (*B*), and CCL19 (*C*) clearly identified progressors and non-progressors and did not require further pruning. The next best tree model generated was a combination of CCL3 and IL-2 (*D*) followed by IFN-γ and CCL11 (*E*). Abbreviations: CART, classification and regression tree; CCL, chemokine ligand; IFN, interferon; IL, interleukin; IP, interferon-γ inducible protein.

sputum tests to predict TB progression as per the TPP. Even with a low prevalence of TB progression, IP10/CCL-19 translates to very high PPV owing to the high specificity obtained. This predictive marker, if validated in multiple other cohorts, can have a major implication in TB control. Besides IP-10 and CCL19, we also found that combinations of CCL3 and IL-2 and IFN-γ and CCL11 could serve as potential biomarkers for prediction. Future studies can focus on validating these combinations of markers in their respective cohorts.

Though the major limitation of our study is the small sample size with variable progression time, its greatest strength lies in the inclusion of well-characterized, systematically followed-up HHCs at 6 monthly intervals, as well as clearly delineated controls. Another limitation of this analysis is that the study did not have adequate representation of people with human immunodeficiency virus or those with undernutrition to generalize the results across the population.

CONCLUSION

Our study identified soluble TB-specific biomarkers that can be usefully exploited as potential short-term risk predictors for tuberculosis. After validation in a larger cohort, our results can pave way for the development of a simple point-of-care test that can be widely used for identifying those at greatest risk for progression to active TB so as to target them for preventive therapy, and thereby contribute to the TB elimination goal.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Financial support. This work was supported by Indian Council of Medical Research (ICMR).[Grant number: 5/8/5/45/Adhoc/2022/ECD-1]

Potential conflicts of interest. The authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Houben RM, Dodd PJ. The global burden of latent Tuberculosis infection: a re-estimation using mathematical modelling. PLoS Med **2016**; 13:e1002152.
2. Cohen A, Mathiasen VD, Schön T, Wejse C. The global prevalence of latent tuberculosis: a systematic review and meta-analysis. Europ Resp J **2019**; 54: 1900655. doi:10.1183/13993003.00655-2019
3. World Health Organization. Latent tuberculosis infection: updated and consolidated guidelines for programmatic management; **2018**.
4. Diel R, Loddenkemper R, Nienhaus A. Predictive value of interferon-γ release assays and tuberculin skin testing for progression from latent TB infection to disease state: a meta-analysis. Chest **2012**; 142:63–75.
5. Orme IM, Robinson RT, Cooper AM. The balance between protective and pathogenic immune responses in the TB-infected lung. Nat Immunol **2015**; 16:57–63.
6. Gupte A, Padmapriyadarsini C, Mave V, et al. Cohort for Tuberculosis Research by the Indo-US Medical Partnership (CTRIUMPH): protocol for a multicentric prospective observational study. BMJ Open **2016**; 6:e010542.
7. GraphPad Prism. San Diego, California, USA. Available at: www.graphpad.com. Accessed 23 March 2022
8. R Core Team. R: a language and environment for statistical Computing, **2021**. Available at: https://www.R-project.org/. Accessed 15 April 2022
9. Sudbury EL, Clifford V, Messina NL, Song R, Curtis N. Mycobacterium tuberculosis-specific cytokine biomarkers to differentiate active TB and LTBI: a systematic review. J Infect **2020**; 81:873–81.
10. Kahnert A, Höpken UE, Stein M, Bandermann S, Lipp M, Kaufmann SH. *Mycobacterium tuberculosis* triggers formation of lymphoid structure in murine lungs. J Infect Dis **2007**; 195:46–54.
11. Khader SA, Rangel-Moreno J, Fountain JJ, et al. In a murine tuberculosis model, the absence of homeostatic chemokines delay granuloma formation and protective immunity. J Immunol **2009**; 183:8004–14.
12. Singhania A, Wilkinson RJ, Rodrigue M, Haldar P, O'Garra A. The value of transcriptomics in advancing knowledge of the immune response and diagnosis in tuberculosis. Nat Immunol **2018**; 19:1159–68.
13. Cooper AM. Cell mediated immune responses in tuberculosis. Annu Rev Immunol **2009**; 27:393–422.
14. Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG, Orme IM. Disseminated tuberculosis in interferon gamma gene-disrupted mice. J Exp Med **1993**; 178:2243–7.
15. Ruan Q-L, Yang Q-L, Gao Y-X, et al. Transcriptional signatures of human peripheral blood mononuclear cells can identify the risk of tuberculosis progression from latent infection among individuals with silicosis. Emerg Microbes Infect **2021**; 10:1536–44.
16. Andrews JR, Nemes E, Tameris M, et al. Serial QuantiFERON testing and tuberculosis disease risk among young children: an observational cohort study. Lancet Respir Med **2017**; 5:282–90.
17. Zellweger J-P, Sotgiu G, Block M, et al. Risk assessment of tuberculosis in contacts by IFN-γ release assays. A Tuberculosis Network European Trials Group study. Am J Respir Crit Care Med **2015**; 191:1176–84.
18. Haldar P, Thuraisingam H, Patel H, et al. Single-step QuantiFERON screening of adult contacts: a prospective cohort study of tuberculosis risk. Thorax **2013**; 68:240–6.
19. Zak DE, Penn-Nicholson A, Scriba TJ, et al. A prospective blood RNA signature for tuberculosis disease risk. Lancet **2017**; 387:2312–22.
20. Suliman S, Thompson EG, Sutherland J, et al. Four-gene pan-African blood signature predicts progression to tuberculosis. Am J Respir Crit Care Med **2018**; 197: 1198–208.
21. Duffy FJ, Thompson E, Downing K, et al. A serum circulating miRNA signature for short-term risk of progression to active tuberculosis among household contacts. Front Immunol **2018**; 9:661.
22. Penn-Nicholson A, Hraha T, Thompson EG, et al. Discovery and validation of a prognostic proteomic signature for tuberculosis progression: a prospective cohort study. PLoS Med **2019**; 16:e1002781.
23. Bayaa R, Ndiaye MDB, Chedid C, et al. Multi-country evaluation of RISK6, a 6-gene blood transcriptomic signature, for tuberculosis diagnosis and treatment monitoring. Sci Rep **2021**; 11:13646.
24. Leong S, Zhao Y, Ribeiro-Rodrigues R, et al. Cross-validation of existing signatures and derivation of a novel 29-gene transcriptomic signature predictive of progression to TB in a Brazilian cohort of household contacts of pulmonary TB. Tuberculosis **2020**; 120:101898.
25. Scriba TJ, Fiore-Gartland A, Penn-Nicholson A, et al. Biomarker-guided tuberculosis preventive therapy (CORTIS): a randomised controlled trial. Lancet Infect Dis **2021**; 21:354–65.
26. Hussain R, Talat N, Ansari A, Shahid F, Hasan Z, Dawood G. Endogenously activated interleukin-4 differentiates disease progressors and non-progressors in tuberculosis susceptible families: a 2-year biomarkers follow-up study. J Clin Immunol **2011**; 31:913–23.
27. Bhattacharyya S, Singla R, Dey AB, Prasad HK. Dichotomy of cytokine profiles in patients and high-risk healthy subjects exposed to tuberculosis. Infect Immun **1999**; 67:5597–603.
28. Smith SM, Klein MR, Malin AS, Sillah J, McAdam KP, Dockrell HM. Decreased IFN- gamma and increased IL-4 production by human CD8(+) T cells in response to *Mycobacterium tuberculosis* in tuberculosis patients. Tuberculosis (Edinb) **2002**; 82:7–13.
29. Ordway DJ, Costa L, Martins M, et al. Increased Interleukin-4 production by CD8 and gammadelta T cells in health-care workers is associated with the subsequent development of active tuberculosis. J Infect Dis **2004**; 190:756–66.
30. Sloot R, Schim van der Loeff MF, van Zwet EW, et al. Biomarkers can identify pulmonary tuberculosis in HIV-infected drug users months prior to clinical diagnosis. EBioMedicine **2015**; 2:172–9.
31. Mpande CAM, Musvosvi M, Rozot V, et al. Antigen-specific T-cell activation distinguishes between recent and remote tuberculosis infection. Am J Respir Crit Care Med **2021**; 203:1556–65.
32. De Groote MA, Higgins M, Hraha T, et al. Highly multiplexed proteomic analysis of QuantiFERON supernatants to identify biomarkers of latent tuberculosis infection. J Clin Microbiol **2017**; 55:391–402.