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Alternative biomarkers for classification of latent tuberculosis infection status in pregnant women with borderline Quantiferon plus results

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ABSTRACT

Borderline interferon-gamma (IFN- γ) results (near the cut-off level 0.35 IU/ml) occur in QuantiFERON (QFT) assays. We investigated the performance of alternative biomarkers for classification of latent tuberculosis infection (LTBI) status in pregnant women with borderline QFT IFN- γ responses. Pregnant women (n = 96) were identified from a cohort study in Ethiopia, based on QFT-Plus IFN- γ results (QFT-low: <0.20 IU/ml, n = 33; QFT-borderline: 0.20–0.70 IU/ml, n = 31; QFT-high: >0.70 IU/ml, n = 32), including 12 HIV-positive individuals in each group and with 20 HIV-negative non-pregnant women from the same cohort with QFT IFN- γ <0.20 IU/ml as controls. Concentrations of 8 markers (IL-1ra, IL-6, IL-8, IP-10, MCP-1, MCP-2, osteopontin and resistin) were measured in whole blood QFT supernatants, stimulated separately with TB1 and TB2 antigens. K-nearest neighbor analysis (KNN) was used to classify participants with regard to likelihood of LTBI. Concentrations of MCP-2, IP-10 and IL-1ra were higher in QFT-borderline compared to QFT-low participants in both antigen stimulations (p < 0.001). KNN classification indicated high likelihood of LTBI in 13/31 (42%) women with QFT-Iborderline IFN- γ results. MCP-2, IP-10 and IL-1ra expressed in whole blood after TB antigen stimulation may be considered as alternative biomarkers for classification of LTBI status in pregnant women with borderline QFT IFN- γ results.

1. Introduction

Pregnancy is associated with increased incidence of active tuberculosis (TB) [1–3], and approximately 500 000 maternal deaths are annually attributed to TB, making this disease one of the leading causes of maternal mortality [4,5]. The risk of pregnancy-associated TB is especially high in HIV-positive women [6,7]. In addition, the immune modification that occurs during pregnancy can influence the performance of immune-based methods used to diagnose latent TB infection (LTBI), such as interferon- γ release assays (IGRAs). This effect is also most pronounced in HIV-positive individuals [8,9].

A modified IGRA, QuantiFERON-TB Gold Plus (QFT-Plus), has been developed in order to improve sensitivity in immunosuppressed

individuals. This assay is based on two sets of *Mycobacterium tuberculosis* (Mtb)-specific antigens; TB1, including early secretory antigenic target 6-kD protein and culture filtrate protein 10, which stimulate CD4⁺ T-cells, and TB2, which also contains short peptides derived from these antigens, with the ability to induce both CD4⁺ and CD8⁺ T-cell responses [10,11]. Similar to previous versions of the QuantiFERON assay, QFT-Plus is based on measurement of interferon- γ (IFN- γ) in plasma after whole blood antigen stimulation.

One problem in relation to the use of IGRAs concerns the definition of cut-off levels for positive and negative results. Although a binary threshold level of 0.35 IU/ml is recommended by the manufacturer [12], it has been noted that results around this cut-off level are subject to variability on repeated testing [13,14]. This has led researchers to study

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variability of QFT results in a borderline range (or uncertainty zone) near 0.35 IU/ml, usually defined as 0.20–0.70 IU/ml [13,15]. Interpretation of borderline QFT results remains unclear. Importantly, the reason for borderline results varies, depending on the pre-test risk of TB infection, technical performance, and the presence of immunosuppression in the individual tested [16–18]. Whereas most borderline results in persons living in low-endemic areas probably reflect false-positive reactions [19], such results may represent true TB infection in immunocompromised persons in TB-endemic settings. We have observed high proportions of borderline QFT IFN- γ results (0.20–0.70 IU/ml), among pregnant women in Ethiopia (7.6% among HIV-negative and 29% among HIV-positive women, respectively) [20]. Consequently, IFN- γ based assessment of response to Mtb antigens could be unreliable for individuals with physiological or pathological immunosuppression.

Based on this, we hypothesized that the characterization of expression patterns of a broader range of markers, i.e. cytokines and inflammatory molecules, in response to TB antigen stimulation could identify alternative biomarkers that might be used separately or in combinations to elucidate LTBI status in pregnant women. Here we present data on Mtb-induced immune responses in whole blood of pregnant women living in a TB-endemic setting, with special focus on individuals with borderline IFN- γ results in the QFT-Plus assay.

2. Materials and methods

2.1. Study participants

Participants for this study were identified from a prospective cohort of women recruited during pregnancy at three public antenatal care clinics in the city of Adama, Ethiopia. The overall purpose of this cohort is to study different aspects of Mtb infection in relation to pregnancy [21]. Pregnant women attending their first antenatal care visit were included in the cohort after providing written informed consent. Recorded characteristics of the study participants included age, gestational age, parity and mid-upper arm circumference (MUAC; for assessment of malnutrition) (Table 1). HIV serostatus was determined using rapid tests according to national guidelines [22], and for HIV-positive women, CD4⁺ T cell count, viral load (VL) and antiretroviral treatment (ART) data was collected.

Two morning sputum samples were obtained from participants with symptoms and/or signs suggestive of active TB, and from all HIVpositive women (regardless of clinical presentation) for bacteriological testing [20]. Blood was collected from all participants at inclusion and at 9 months after delivery for QFT-Plus testing.

For the current study, participants were selected from the cohort

based on their QFT IFN- γ results at the inclusion visit. Three categories of participants were selected: QFT-low (IFN- $\gamma < 0.20$ IU/ml); QFT-borderline (IFN- $\gamma 0.20-0.70$ IU/ml) and QFT-high (IFN- $\gamma \geq 0.70$ IU/ml). All women with QFT-borderline results and available plasma samples were included (both TB1 and TB2 IFN- γ responses were required to be within the same QFT IFN- γ category for inclusion). For each QFT-borderline participant one QFT-low and one QFT-high participant were randomly selected for inclusion. We aimed to include similar numbers of HIV-positive individuals in each category. Participants with current or previous active TB were excluded.

In order to control for effects of pregnancy *per se* on biomarker levels, we included samples obtained 9 months after delivery from women at low likelihood of having LTBI from the same cohort as negative controls. These women were HIV-negative, without confirmed nor suspected active TB at any time point, nor self-reported history of active TB, and with negative QFT result (IFN- γ <0.20 IU/ml) both during and after pregnancy.

2.2. QuantiFERON-TB Gold Plus assay

Venous blood was collected in lithium heparin tubes. Within 8 h of venipuncture, 1 ml of blood was transferred to each of the 4 QFT-plus incubation tubes (Nil, TB1, TB2 and mitogen). QFT-Plus testing (Qiagen, Carnegie, Australia) was performed according to the manufacturer's instructions. In brief, after mixing by inversion, samples were incubated at 37 °C for 18 h, followed by centrifugation for 15 min at 2000g, with storage of plasma supernatants at -20 °C.

IFN- γ concentration was measured by ELISA, with conversion of optical density to international units per milliliter (IU/ml) using the QFT-Plus analysis Software. The background (nil) corrected IFN- γ levels of TB antigens were recorded. Remaining aliquots of TB antigen stimulated supernatants were stored at $-80~^\circ\text{C}$ at Adama Public Health Research and Referral Laboratory and one aliquot of stored supernatants from each participant was transported frozen to Lund University, Sweden, for further analyses.

2.3. Analysis of cytokines and inflammatory markers

Based on a literature search for biomarkers shown to be associated with TB infection [23–26] we initially considered the following 20 markers for this study; granulocyte-macrophage-colony-stimulating factor (GM-CSF), interleukins (IL-2, IL-6, IL-8, IL-10, IL-13, IL-15, IL-17a, IL-1ra [receptor antagonist]), interferon gamma-induced protein 10 (IP-10), monocyte induced interferon- γ (MIG), interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), monocyte chemoattractant

Table 1

Study participant characteristics. Participants selected based on IFN-7 expression category in the Quantiferon-TB Plus assay.

QFT categories [®]	QFT-low	QFT-borderline	QFT-high	Controls
	n = 33	n = 31	n = 32	n = 20
Age* (years)	23 (21–27)	28 (25–30)	26 (23–30)	25 (24–28)
Gestational age* (weeks)	18 (14–20)	18 (14–20)	19 (16–21)	N/A
Parity*	1	1 2		1
MUAC (cm)*	24 (22–26)	24 (22–26)	24 (22–26)	27 (24–28)
HIV sero-positive	n = 12	n = 12	n = 12	
CD4 count*(Cells/mm ³)**	367 (297–664)	566 (291–766)	677 (450-860)	
	5/12	5/12	8/12	
Viral load*(Copies/ml)**	$1.1 imes10^4$ ($1.5 imes10^3$ – $4.4 imes10^4$)	$2 imes10^4$ (9 $ imes10^3$ –1.3 $ imes10^4$)	1065	
	1/12	0/12	1/12	
<150 RNA copies/ml	7/12	9/12	10/12	
ART status#				
On ART	10/12	8/12	11/12	
ART naïve	2/12	4/12	1/12	

 \mathbb{P} QFT-low (IFN- $\gamma < 0.20$ IU/ml), QFT-borderline (IFN- $\gamma 0.20-0.70$ IU/ml), QFT-high (IFN- $\gamma \ge 0.70$ IU/ml), Controls (non-pregnant HIV negative women with IFN- $\gamma < 0.20$ IU/ml). *median, except viral load result for QFT-high (exact result is shown). IQR (interquartile range) is shown in bracket. MUAC (mid-upper arm circumference). ** Subjects whose CD4 and Viral load results were missing at the time of enrolment. N/A (not applicable). #only applied for HIV-positive persons.

protein-1 and 2 (MCP-1 and 2), regulated on activation, normal T cell expressed and secreted (RANTES), resistin, osteopontin, platelet-derived growth factor-BB (PDGF-B), and macrophage inflammatory protein-1- β (MIP-1 β). The concentrations of these markers were analyzed using Magnetic Luminex assay (R&D Systems Inc., Minneapolis, MN) on the Bio-Plex 200 platform (Bio-Rad Laboratories Inc., Hercules, CA). Concentrations of these markers were measured in duplicate in supernatants of Mtb antigen (TB1 and TB2) stimulated and unstimulated (Nil) whole blood in the QFT-Plus assay.

In a pilot experiment the levels of GM-CSF, IL-10, IL-15, MIG, TNF- α , IFN- γ , IL-13, IL-17a, IL -2 and MIP-1 β were found to be below the assay detection limit with dilution 1:3. RANTES and PDGF-BB secretion did not show any TB-specific response. Therefore, these markers were omitted from further evaluation. The remaining 8 markers were included and analyzed in 5-plex (MCP-1, MCP-2, IL-6, resistin and osteopontin) and 3-plex (IP-10, IL-8 and IL-1ra) assay in 1:2 and 1:30 dilutions, respectively.

For samples in which marker concentrations were above or below the assay detection limit the concentrations of the higher and lower standard concentration were used, respectively. Internal controls were included throughout each run.

2.4. Statistical analysis

In order to detect TB-specific responses the background concentration of each marker detected in the negative control (Nil) supernatant was subtracted from the concentration of the marker in the supernatants after Mtb antigen (TB1 or TB2) stimulation. Continuous variables were determined by medians and interquartile ranges (IQR). Kruskal-Wallis followed by Dunn's multiple comparisons test was used for the three group comparisons. P-values <0.05 were considered significant. Receiver operating characteristic (ROC) analysis was performed separately for the biomarkers to assess their potential to identify different categories of QFT IFN- γ responses. From the ROC analysis, area under the curve (AUC), 95% confidence interval [CI] and p-values were obtained. Control subjects were not included for the ROC analysis.

In addition, we used two supervised machine learning classification algorithms, k-Nearest Neighbor (KNN) and k-mean clustering (KMC) analyses. These models were applied to investigate the distribution of women with QFT-borderline results with regard to their similarity with participants considered to be at high vs. low likelihood of having LTBI. For this purpose, two reference groups were used; non-pregnant HIV-negative women with IFN- $\gamma < 0.20$ IU/ml (low likelihood of LTBI), and pregnant HIV-negative women with IFN- $\gamma \ge 0.70$ IU/ml (high likelihood of LTBI). Separate KNN and KMC analyses were performed for biomarker levels obtained after TB1 and TB2 stimulation. Individuals with QFT-borderline results were categorized as high likelihood of LTBI if data from either TB1 or TB2 antigen showed clustering with this reference group. Both these analyses were repeated separately on HIV-positive and HIV-negative study participants.

K-Nearest Neighbor: In this analysis, data was divided into a training dataset and a test dataset. Women in the low and high likelihood LTBI reference groups were assigned as the training dataset, and all remaining study participants were assigned as the test dataset. The test cases were each classified by identifying the K-nearest neighbors in the n-dimensional space of the normalized cytokine levels in the test dataset, and classified based on whether the majority of the K-nearest neighbors was at high or low likelihood of LTBI. The KNN algorithm was repeated with several numbers of neighbors (K = 1, 3, 5, 7, 9).

K-mean clustering: In the n-dimensional space of normalized cytokine levels, high or low likelihood of LTBI centroids were defined to minimize the total Euclidian distance from the training cases to their respective centroid. Women in the low and high likelihood LTBI reference groups were used as pre-determined cluster centroids for reference in this analysis, and the test cases were classified based on the lowest Euclidian distance to either of these centroids. All data analysis was performed using Graph pad prism 8 and IBM SPSS statistics version 25.

2.5. Ethical consideration

This study received ethical approval from National Research Ethics Review Committee, Addis Ababa, Ethiopia and the Regional Ethical Review Board at Lund University, Sweden. Written informed consent was obtained from participants prior to enrolment.

3. Results

3.1. Study participant characteristics

In order to study alternative biomarkers for classification of LTBI status, 96 pregnant women were included. They were grouped according to QFT responses (33 QFT-low, 31 QFT-borderline, and 32 QFT-high). Twelve women in each of these groups were HIV-positive (Table 1). In addition, 20 HIV-negative women with QFT IFN- $\gamma < 0.20$ IU/ml sampled 9 months after delivery were included as controls. The distribution of age, gestational age, parity and MUAC did not to vary between the different groups (Table 1). Among HIV-positive women, CD4 count tended to be lower in QFT-low compared to QFT-high, although this difference was not statistically significant (Table 1).

3.2. Mtb specific cytokine expression patterns in pregnant women in different QFT categories

Concentrations of 8 cytokines were analyzed in supernatants after TB1 and TB2 antigen stimulation of whole blood, revealing specific expression patterns (Fig. 1 and Table S1). Median concentrations of MCP-2, IP-10 and IL-1ra were greater in QFT-borderline compared with QFT-low and controls after both TB1 and TB2 stimulation (p < 0.05 to p < 0.0001), with the exception of TB2-stimulated IL-1ra between QFT-borderline and controls (Fig. 1a–c).

In addition, median concentrations of MCP-2 in QFT-borderline were lower than those in QFT-high, after both TB1 and TB2 stimulation (p < 0.05 to p < 0.01) (Fig. 1a), while this was not seen for IP-10 nor for TB1stimulated IL-1ra. Moreover, after TB1 stimulation, resistin and MCP-1 levels were greater in QFT-borderline compared to QFT-low (p < 0.05; Fig. 1d and e). IL-8, IL-6 and osteopontin did not show significant differences between any of the QFT categories (Fig. 1f–h).

3.3. Cytokine expression patterns in different QFT categories with regard to HIV serostatus

To analyze the influence of HIV infection on cytokine expression, we analyzed these patterns in women subdivided according to HIV serostatus. Similar to the total population of studied women, median levels of MCP-2 and IP-10 in QFT-borderline were elevated compared to QFT-low in the subset of HIV-positive women, whereas no differences in IL-1ra levels were observed between QFT-borderline and QFT-low (Fig. 2 and Table S3). No differences in secretion of the cytokines were observed between QFT-high and QFT-borderline in HIV-positive women. Cytokine secretion patterns in HIV-negative women mirrored those observed in the total study population (Table S2).

3.4. Performance of selected Mtb cytokine responses for differentiation of QFT categories

ROC curve analysis was performed to evaluate the potential to distinguish different QFT categories for each of the 5 markers found to have differential expression, i.e. MCP-2, IP-10, IL-Ira, MCP-1 and resistin. The markers with the best capacity to distinguish QFT-borderline from QFT-low were: IP-10 (AUC: 0.98; 95% CI, 0.95–1 with TB1; AUC: 0.97; 95% CI, 0.94–1 with TB2), MCP-2 (AUC: 0.96; 95% CI, 0.96)



Fig. 1. Scatter plots showing expression patterns of eight markers in response to TB1 and TB2 antigen stimulations; QFT-high (IFN- $\gamma \geq 0.70$ IU/ml, n = 32, (filled circles)); QFT-borderline (IFN-γ 0.20-0.70 IU/ ml, n = 31, (open circles)); QFT-low (IFN- γ < 0.20 IU/ml, n = 33 (filled triangles)); controls (IFN- $\gamma < 0.20$ IU/ml, n = 33 nonpregnant and HIV-, (open triangles)). Kruskal-Wallis test followed by Dunn's multiple comparisons was used for comparison between groups and median (IQR) was calculated for each markers. *p < 0.05, **p< 0.01, ***p < 0.001 and ****p < 0.0001indicated in the graph for each group difference using the bracket. Abbreviations: a) MCP-2: Monocyte chemoattractant protein-2 b) IP-10: IFN-γ inducible protein 10 c) IL-1ra: interleukin 1receptor antagonist e) MCP-1: Monocyte chemoattractant protein-1 f) IL-8: interleukin 8 g) IL-6: interleukins 6.

0.91–1 with TB1; AUC: 0.97; 95% CI, 0.93–1 with TB2) and IL-1ra (AUC: 0.87; 95% CI, 0.77–0.96 with TB1; AUC: 0.85; 95% CI, 0.74–0.95 with TB2) (all P < 0.0001; Table 2). These 3 markers also had relatively high AUC (ranging from 0.75 to 0.89) when comparing QFT-high and QFT-borderline (all p < 0.0001; Table 2). Moreover, MCP-2, IP-10, IL-Ira, MCP-1, and resistin had the greatest AUCs (ranging from 0.99 to 0.80) in differentiating QFT-high from QFT-low for both Mtb antigens (Table 2).

3.5. Combination of markers classifying women with QFT borderline results with regard to likelihood of LTBI

In order to differentiate women with QFT-borderline IFN- γ results with regard to their likelihood of LTBI, we performed two types of

machine learning classification analyses, KNN and KMC, and evaluated the robustness of these models in regard to categorization of defined training and centroid groups, respectively. For these analyses, we included both results from all 8 markers analyzed, as well as results of the 3 cytokines found to have the greatest discriminatory capacity when tested separately (IP-10, MCP-2 and IL-1ra).

Initially we performed KNN analysis and based the categorization on two reference training groups for low and high likelihood of LTBI. KNN analysis was also conducted using different numbers of nearest neighbors (k = 1, 3, 5, 7 and 9) for the 3 and 8-cytokine combinations, respectively. In these analyses, KNN analysis using 7 nearest neighbors (k = 7) with the 3-cytokine combination showed the best classification of the training dataset in expected categories, 19/20 in the low and 18/ 20 in the high likelihood of LTBI, respectively (Table 3).



Fig. 2. Scatter plots showing the levels of five markers (IP-10, MCP-2, IL-1ra, MCP-1 and resistin) in response to the two TB antigens in HIV-positive and HIV-negative women, comparing the three QFT categories; QFT-low (IFN- $\gamma < 0.20$ IU/ml, n=12), QFT-borderline (IFN-y 0.20-0.70 IU/ml, n=12) and QFT-high (IFN- $\gamma \ge 0.70$ IU/ml, n=12) in TB1 and TB2. Comparison between QFT categories was performed using the Kruskal Wallis test followed by Dunn's multiple comparisons test. P values are indicated separately in Table S2 for HIV-negative and Table S3 for HIV-positive pregnant women. The middle line represents median concentration of each marker responses to TB antigen stimulations. Abbreviations: IP-10: IFN-y inducible protein 10; MCP-2: Monocyte chemoattractant protein-2; IL-1ra: interleukin 1receptor antagonist; MCP-1: Monocyte chemoattractant protein-1.

Based on this, we used the combination of 3 cytokines in KNN with k = 7. With this approach, 13/31 (42%) of the QFT-borderline individuals classified with the high likelihood of LTBI training group in TB1 and/or TB2 stimulations (Table 3). IFN- γ responses in the QFT Plus assay among women with QFT borderline results and LTBI status classification after KNN analysis with K = 7 are presented in Table S7. These results show that all of the 13 women that classified with the high LTBI likelihood training group had IFN- γ results within the high QFT borderline range (0.35–0.70 IU/ml) in TB1 and/or TB2 stimulation (Table S7), supporting the correct LTBI classification of these women using the IFN- γ threshold

0.35 IU/ml. However, 11/18 (61%) women with IFN- γ results within the high QFT borderline range (0.35–0.70 IU/ml) in TB1 and/or TB2 stimulation classified with the low likelihood training group.

The second best training group classification was obtained using KNN analysis including combination of 8 cytokines and K = 3 (Table S4). In this analysis, 10/31 (32%) of QFT-borderline women were categorized with the high likelihood of LTBI training group in TB1 and/or TB2 antigen stimulations. KNN using k = 1, 3, 5 or 9 with the 3-cytokine combination and KNN using k = 1, 5, 7 and 9 with the 8-cytokine combination resulted in lower numbers of the training group

Table 2

ROC curve analyses for differentiation between	OFT categories among pregnant wo	men with high low and borderline ()	FT interferon-y results
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QFT Groups ^a		Low vs High			Low vs Borderline			Borderline vs High	
Markers	AUC	95% CI	Р	AUC	95% CI	Р	AUC	95% CI	Р
MCP-2									
TB1-nil	0.98	0.96-1.00	0.0001	0.96	0.91-1.00	0.0001	0.88	0.79-0.96	0.0001
TB2-nil	0.99	0.98 - 1.00	0.0001	0.97	0.93-1.00	0.0001	0.89	0.81-0.98	0.0001
IP-10									
TB1-nil	0.98	0.94-1.00	0.0001	0.98	0.95-1.00	0.0001	0.85	0.74-0.95	0.0001
TB2-nil	0.98	0.95-1.00	0.0001	0.97	0.94-1.00	0.0001	0.82	0.71-0.93	0.0001
IL-1ra									
TB1-nil	0.91	0.82-0.98	0.0001	0.87	0.77-0.96	0.0001	0.75	0.63-0.88	0.001
TB2-nil	0.92	0.84-1.00	0.0001	0.85	0.74-0.95	0.0001	0.77	0.65-0.88	0.0001
MCP-1									
TB1-nil	0.83	0.73-0.94	0.0001	0.73	0.63-0.87	0.003	0.68	0.54-0.82	0.01
TB2-nil	0.76	0.64-0.88	0.002	0.66	0.53-0.79	0.02	0.64	0.49-0.78	0.06
Resistin									
TB1-nil	0.80	0.69-0.90	0.0001	0.75	0.63-0.87	0.001	0.61	0.47-0.75	0.15
TB2-nil	0.87	0.71-0.92	0.0001	0.65	0.51-0.79	0.04	0.67	0.53-0.80	0.02
IL-8									
TB1-nil	0.67	0.54-0.80	0.02	0.58	0.44-0.72	0.26	0.58	0.44-0.73	0.25
TB2-nil	0.61	0.47-0.76	0.11	0.52	0.38-0.67	0.74	0.57	0.42-0.71	0.35
IL-6									
TB1-nil	0.58	0.44-0.72	0.26	0.56	0.41-0.70	0.43	0.52	0.37-0.66	0.83
TB2-nil	0.51	0.37-0.66	0.85	0.53	0.39-0.68	0.59	0.53	0.39-0.69	0.64
Osteopontin									
TB1-nil	0.62	0.48-0.76	0.10	0.62	0.48-0.76	0.08	0.55	0.40-0.69	0.50
TB2-nil	0.59	0.45–0.73	0.23	0.55	0.40-0.69	0.51	0.55	0.41-0.70	0.47

^a High (QFT IFN- $\gamma \ge 0.70$ IU/ml); Borderline (QFT IFN- $\gamma 0.20-0.70$ IU/ml); Low (QFT IFN- $\gamma < 0.20$ IU/ml); controls (QFT IFN- $\gamma < 0.20$ IU/ml non-pregnant and HIV⁻).

^b Abbreviations: MCP-2: Monocyte chemoattractant protein-2, IP-10: IFN-γ inducible protein 10, IL-1ra: interleukin 1receptor antagonist, MCP-1: Monocyte chemoattractant protein-, IL-8: interleukin-8, IL-6: interleukins 6, AUC: Area under the curve.

Table 3

K-nearest neighboring analysis (K = 7) for classification of women with different categories of QFT IFN-γ responses into low and high likelihood of LTBI using three cytokines (IP-10, MCP-2 and IL-1ra).

	Low likelihood of $LTBI^{b}$			High likelihood of LTBI ^c			
QFT-Groups ^a	TB1 and TB2	TB1 only	TB2 only	TB1 and TB2	TB1 only	TB2 only	Total (n)
Controls (low training group)	19	0	1	0	1	0	20
QFT-low	21	0	0	0	0	0	21
QFT-low HIV+	12	0	0	0	0	0	12
QFT-borderline	11	1	1	6	1	1	19
QFT-borderline HIV+	7	0	1	4	1	0	12
QFT-high (high training group)	2	0	0	18	0	0	20
QFT-high HIV+	2	0	0	10	0	0	12
Total	74	1	3	38	3	1	116

 $^{a} \text{ Controls (IFN-} \gamma < 0.20 \text{ IU/ml}, \text{ non-pregnant and HIV-}), \text{ QFT-low (IFN-} \gamma < 0.20 \text{ IU/ml}), \text{ QFT-borderline (IFN-} \gamma 0.20 - 0.70 \text{ IU/ml}), \text{ QFT-high (IFN-} \gamma \geq 0.70 \text{ IU/ml}).$

 $^{\rm b}\,$ Non-pregnant HIV-negative women with QFT IFN- $\gamma < 0.20$ IU/ml.

^c Pregnant HIV-negative women with IFN- $\gamma \ge 0.70$ IU/ml.

individuals classified in expected categories (see Tables S8 and S9), and were therefore not pursued further.

In addition to the KNN algorithm, we explored KMC analysis for determination of likelihood of LTBI among women with borderline QFT IFN-γ results. In this analysis, all 20 subjects in the low reference group clustered with the expected centroid using the 3-cytokine combination (Table S5). In KMC with the 3-cytokine combination we found that 7/31 (23%) QFT borderline subjects clustered with the high-likelihood of LTBI centroid in TB1 and/or TB2 antigen stimulations. KMC analysis based on all 8 cytokines showed that 13/31 (42%) of the QFT borderline subjects clustered with the high-likelihood LTBI centroid, with TB1 and/or TB2 stimulation (Table S6).

Comparing results of the KNN and KMC analyses, a higher number of subjects in the training groups classified according to their expected categorization using KNN than with KMC (Tables 3, S4, S5 and S6). Moreover, a higher proportion of TB1 and TB2 concordant results were observed when performing the classification with the 3-cytokine,

compared with the 8-cytokine, combination in both KNN and KMC.

4. Discussion

In this study, which was based on Mtb antigen-stimulated whole blood from pregnant women living in a TB-endemic setting, we evaluated several cytokines separately and in combination, for their capacity to characterize pregnant women with borderline IFN- γ results in the QFT-Plus assay. We show that three markers detected in QFT supernatants (IP-10, MCP-2 and IL-1ra) distinguished women with borderline QFT IFN- γ results from those with IFN- γ levels below the QFT borderline range (<0.20 IU/ml), and that 42% of women with borderline results were classified as having high likelihood of LTBI, using an algorithm based on a combination of these three cytokines.

In the development of the QFT-Plus assay, the problem of reduced sensitivity in immunosuppressed individuals has been addressed by addition of modified Mtb antigens that stimulate CD8 T cells. During pregnancy, T helper (Th) 1 pro-inflammatory responses shift towards Th2 anti-inflammatory responses; Th17 responses are also attenuated, with a corresponding increase in T-regulatory cell activity [27,28]. This physiological immune modification may disturb the control of bacterial replication in women with LTBI and promote development of active TB. For this reason, attention to TB infection in pregnant women is important. Pregnancy-related immune modification can furthermore influence the performance of diagnostic assays based on IFN- γ secretion [8,9]. IFN- γ is a well-recognized marker of cell-mediated immune activation, and is predominantly produced by Th1 cells, which have a key role in immune protection against TB. Therefore, IFN- γ has been used as a readout marker for immune-based TB diagnostic assays. However, the capacity of Th1 cells to produce IFN- γ - after Mtb stimulation is reduced in persons with immunosuppression (including pregnant women), who for the same reason have increased risk of progression to active TB.

In this study, we have explored the concept of using alternative cytokine markers to characterize borderline IFN- γ responses after Mtb antigen stimulation in pregnant women.

We found MCP-2, IP-10 and IL-1ra levels to be significantly higher in the QFT-borderline and QFT-high categories compared to the QFT-low category, regardless of HIV serostatus, suggesting that these markers could be used to assess LTBI status in women with QFT-borderline IFN- γ results. These markers also showed the highest discriminatory performance comparing the different QFT IFN- γ categories in ROC analyses. Similar to our previous observations for IFN- γ [20], we observed that TB1 and TB2 stimulation resulted in similar cytokine secretion patterns for most of the alternative markers analyzed in the different QFT IFN- γ categories.

With regard to IP-10, our results are in agreement with other reports, showing that this chemokine is expressed in a higher magnitude than IFN- γ , and that its expression after Mtb antigen stimulation is less dependent on cell-mediated immune capacity [29]. Our findings add support to previous data suggesting that IP-10 could be an alternative marker for IGRA reactivity, with higher sensitivity than IFN- γ in immunocompromised subjects [30–32]. This could be due to the fact that IP-10 is primarily secreted by monocytes and macrophages, in contrast to IFN- γ , which is mainly secreted by activated T cells. Furthermore, IP-10 secretion is triggered by multiple cytokines, including IFN- γ , TNF- α , as well as IFN- α/β , IL-2, IL-17, IL-27 and IL-1 β [23].

Similar to IP-10, but expressed in lower levels, MCP-2 has also been investigated as a potential marker for both active and latent TB [24]. MCP-2 is a proinflammatory chemokine, acting as a chemoattractant for granulocytes, monocytes and T-cells to the site of Mtb infection through various chemokine receptors [33]. IL-1ra is an internal inhibitor of IL-1 and secreted by macrophages; elevated plasma levels of this cytokine have been reported in patients with active TB [34]. It is therefore possible that the increased expression of IL-1ra may reflect incipient reactivation of LTBI in pregnant women.

The finding of higher levels of these 3 cytokines after whole blood TB antigen stimulation in women with QFT-borderline results compared to those with QFT-IFN- γ <0.20 IU/ml could imply that QFT-borderline individuals displaying this profile have LTBI. In order to explore this further, we performed classification analyses using machine-learning algorithms to determine how expression patterns of these markers classified QFT-borderline individuals with regard to their likelihood of having LTBI. For this purpose, we performed both KNN and KMC analyses, based on the combination of 3 (MCP-2, IP-10 and IL-1ra) as well as 8 cytokines (IL-1ra, IL-6, IL-8, IP-10, MCP-1, MCP-2, osteopontin and resistin). In the absence of a diagnostic gold standard for LTBI, we used two reference groups, considered to be at low and high likelihood of having LTBI, respectively. The proportions of QFT-borderline individuals clustering with the high LTBI likelihood group depended on the variant of the KNN and KMC analyses used, and if classification was based on 3 or 8 cytokines. The KNN variant using the 3-cytokine combinations with 7 nearest neighbors yielded the most robust analysis, and

indicated that 42% of women with QFT-borderline results were at high likelihood of LTBI; interestingly, all of these had IFN- γ levels within the higher borderline range.

Still, a majority of women with QFT-borderline results classified with the low likelihood of LTBI group. This distribution is in line with the QFT IFN- γ responses, among which 55% (17/31) were within the low borderline range (IFN- γ 0.20–0.34 IU/ml), regardless of type of antigen stimulation (Table S7). However, among the 18 women with OFTborderline results classified with the low LTBI likelihood group, 11 (61%) had IFN- γ levels in the higher borderline range. Taken together, these findings indicate that the QFT-Plus cut-off of 0.35 IU/ml has satisfactory sensitivity for LTBI classification in pregnant women, whereas the use of this threshold level might lead to over diagnosis of LTBI in some cases. We did not identify any difference in this distribution in the subset of HIV-positive women, suggesting that the 0.35 IU/ml cut-off level may be adequate also in this population. Yet, this finding has to be interpreted with some caution, since the majority of HIVpositive individuals in our study received long-term ART and did not have advanced immunosuppression. We could therefore not assess the performance in ART-naïve HIV-positive persons. In summary, our data show that analysis using a combination of cytokine markers may help clarify the LTBI status of pregnant women with borderline OFT-Plus IFN- γ results. In this regard, IP-10, MCP-2 and IL-1ra appear to be the most promising alternatives, especially when used in combination. Further studies are needed to define cut-off levels for determination of LTBI status using such markers, as well as validation studies before this strategy can be considered for clinical use.

To our knowledge, the concept of investigating several potential alternative biomarkers for LTBI diagnosis using the two Mtb antigen formulations in the QFT-Plus assay has not previously been explored. In particular, data is lacking on the performance of immune markers for LTBI in pregnant women. Our study was based on participants in a prospective cohort, representing women living in a TB-endemic setting. Furthermore, 9.3% of these women were HIV positive. Our findings therefore suggest that alternative cytokine markers, particularly IP-10 and MCP-2, could increase sensitivity for LTBI detection in individuals with suppressed cellular immune function, such as HIV infection and pregnancy.

Certain limitations for the current study should be mentioned. We only analyzed immune responses in women of reproductive age, and did not include men, nor women in other age categories for reference. Comparison with responses in persons with active TB could have been considered, as a reference for confirmed TB infection. However, since negative QFT IFN-γ results can occur in patients with active TB [35,36] we chose not to include such cases as controls. Given the cross-sectional design of this study, we were unable to assess whether the markers identified predict the risk of LTBI reactivation. Several of the markers initially considered were found to have low concentrations in the QFT supernatants tested in a pilot experiment, and were therefore not included. In several studies (mainly performed in settings with low TB prevalence), individuals with IFN-y values close to the threshold level (0.35 IU/ml) show negative results upon retesting [13,37]. Our study design did not allow for direct re-testing of women with borderline results, but follow-up testing after the post-partum period is part of the protocol and currently ongoing. Other methods, such as the Flow-cytometric Assay for Specific Cell-mediated Immune-response in Activated whole blood [38], in which long term incubation boosts the response to Mtb antigens, might have been useful for further investigation of borderline IFN-y results.

5. Conclusions

The concentrations of several cytokines displayed differential expression patterns after Mtb antigen stimulation in the QFT-Plus assay among pregnant women with different types of IFN- γ response. In particular, MCP-2, IP-10 and IL-1ra differentiated women with QFT-

borderline IFN- γ results from those with low IFN- γ results, irrespective of HIV serostatus, suggesting that these markers could be used to further investigate individuals with QFT-borderline results. KNN classification analysis based on a combination of these markers categorized 42% of pregnant women with QFT IFN- γ borderline results as having high likelihood of LTBI. These findings suggest that analysis of combinations of cytokines could be used to assess LTBI status in pregnant women with QFT IFN- γ borderline results.

Declaration of competing interest

QuantiFERON-TB Gold Plus kits and tubes were donated by Qiagen. This sponsor had no impact on the study design, data analysis, decision to publish or writing the manuscript. The authors have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2020.101984.

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