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Alison M Grossman Date

DETECTION OF TB INFECTION AMONG INDIAN INFANTS USING NON-INTERFERON GAMMA CYTOKINES

BY

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DEGREE TO BE AWARDED: MASTER IF PUBLIC HEALTH

DEPARTMENT OF EPIDEMIOLOGY

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Detection of TB Infection among Indian Infants Using Non-Interferon Gamma Cytokines

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2020

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Abstract

DETECTION OF TB INFECTION AMONG INDIAN INFANTS USING NON-INTERFERON GAMMA CYTOKINES

BY ALISON M GROSSMAN

Background: Infants who are infected with *Mycobacterium tuberculosi*s (Mtb) have a high risk of progression to tuberculosis (TB) disease compared to older children and adults[1]. Detection of Mtb infection among infants has been hampered by low sensitivity of Interferon-gamma (IFNγ) release assays (IGRAs) such as QuantiFERON® (QFT). Recent studies have explored the use of non-IFNγ cytokines to detect Mtb immune responses. Our objective was to assess the prevalence and consistency of Mtb-specific cytokine responses over the first year of life using QFT and Luminex among Indian infants, evaluate clinical and sociodemographic cofactors of TBI, and assess agreement with traditional IGRA results to detect TBI in infants.

Methods: HIV exposed uninfected (HEU) and HIV unexposed uninfected (HUU) were enrolled at birth and followed for one year. We performed QFT at 6 months and 12 months of life and measured non-IFNγ cytokine secretion in cryopreserved QFT supernatants using a Luminex assay after the study's completion. Prevalence of TBI was evaluated by Luminex and QFT. Agreement between Luminex and QFT was calculated using the kappa statistic and clinical cofactors of infant TBI were assessed by univariable linear regression.

Results: Thirteen (5.9%) of the 222 live-born infants were QFT positive, 6 (3.4%) at 6 months and 7 (4.2%) at 12 months. The Luminex panel detected an additional 74 possible infections (74/216, 34%) that were QFT negative, 49 (21%) at 6 months and 25 (11%) at 12 months. At 6 months there was no agreement ($\kappa = -0.009$) and slight agreement ($\kappa = 0.110$) at 12 months. Eight (3.5%) of the infants had sustained Luminex positive results. There was no association of TBI with maternal HIV exposure in contrast to prior studies. Weight-for-age z-score < -2 was associated with TBI using by Luminex at 6 months (RR = 0.29, $CI_{95\%}$: 0.12, 0.69).

Conclusion: Luminex detected additional Mtb specific immune responses that QFT could not. A combined QFT and Luminex test may be effective in the detection of TBI in infants whose mothers with TBI during pregnancy. Future studies are needed to evaluate the prognostic value of Mtb-specific non-IFNγ cytokines for progression to TB disease.

Detection of TB Infection among Indian Infants Using Non-Interferon Gamma Cytokines

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Introduction

In 2021, tuberculosis (TB) was a leading cause of death by a single infectious disease with an estimated 1.6 million deaths, of them 187,000 among HIV-positive people.[2] The COVID-19 pandemic essentially reversed a two-decade trend of decreasing TB deaths and was brought back to 2017 levels.[2] India has a higher than global estimated TB incidence of 210 cases per 100,000 and accounted for 32% of the global TB deaths.[3] Globally, it is estimated that almost a fourth of the world is infected with *Mycobacterium tuberculosis* (Mtb), the bacteria that causes TB disease. [4,5] India's estimated prevalence of infection is almost twice the global prevalence at over 40%.[4,5] Mtb infection can last for a lifetime without symptoms and in a perpetual stage of dormancy, with a 10% lifetime risk of progression to disease in the general population, the host possibly never knowing they are infected. [2,6]

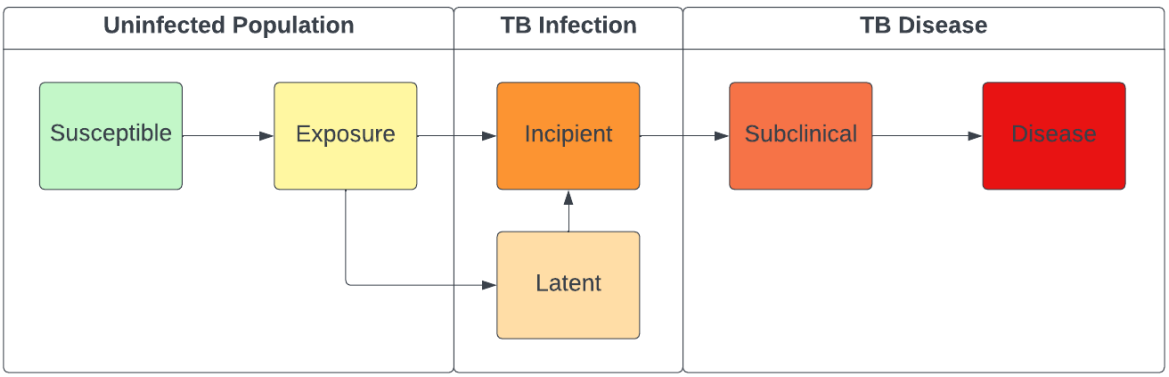
Childhood TB is of particular concern as of the estimated 1.2 million pediatric cases, a third are from India.[5,7] Logistical difficulties plague pediatric TB and Mtb infection diagnostics and reporting, making the true burden of TB and Mtb infection a mystery, leading to undiagnosed disease, lifelong disability, and death. [2,8,9] What could have been treated may lead to severe disease or death because of the failures of testing capacity and diagnostic standards. As children in their first year of life are at the greatest risk of Mtb infection, primary progressive TB, and TB related death, the improvement Mtb infection diagnostic methods is vital.[1] We aim to use a multiplex assay to detect the incidence of Mtb antigen specific Th9/Th17/Th22 cytokine immune responses among Indian infants over the first year of life to understand the prevalence of non-IFN gamma immune responses to Mtb in infancy, and evaluate their correlation with traditional IGRA results to detect Mtb infection.

Overview of Tuberculosis

The natural history of tuberculosis is complex with a highly variable and unpredictable incubation period. Six different stages of tuberculosis are recognized with 3 generalizable categories that people can fall into (Figure 1). Figure 1 is a simplified version of tuberculosis lacking vaccination, treatment intervention, and drug susceptibility.

The susceptible compartment is the population that can be infected with Mtb. The only vaccine available is the bacille Calmette-Guérin (BCG) vaccine which only protects against

Figure 1. Stages of tuberculosis in humans



severe forms of TB in childhood.[2] While the entire population can be susceptible, not everyone will be exposed to Mtb. This is dependent on environmental risk factors such as population, occupation, and ventilation.

Exposure is defined as the population that is exposed to someone with transmissible disease. Likelihood of exposure is dependent on population, occupation, and ventilation.[10] In the vast majority of cases, this is caused by the inhalation of Mtb that travels into the lower respiratory tract and enter the alveolar macrophages and dendritic cells. The innate immune system then attempts to eliminate the bacteria through phagocytosis that leads to inflammatory cytokine and chemokine production.

If the immune system is unable to eliminate Mtb, primary infection occurs. Mtb inhibits the maturation and acidification of phagosomes and lysosomes into macrophages that would cull the Mtb. TNF-α stimulates endothelial cells to express proteins that trigger blood clotting in local small vessels, cutting off blood flow. This prevents Mtb from entering the bloodstream from the alveoli, leading to extrapulmonary TB. [Janeway's Immunology 2012] This cell death signals neutrophils that do not fully cull the multiplying bacteria, and in 6-12 weeks post infection, the adaptive immune system (T lymphocytes) creates antigens and begins to kill the Mtb that resides in the formerly immature macrophages. An attempt is made to encapsulate any remaining Mtb in a mixture of fibroblasts, lymphocytes, Langhans cells, and dendritic cells in a granuloma/tuberculoma. The outcome of this determines if Mtb latency or primary progressive TB occurs, depicted as the two arrows branching from exposure (figure 1).

Latent infection occurs when the tuberculoma is successfully formed and it is not anticipated that progression to disease will occur in the next two years. The patient is left in a chronic state of asymptomatic infection with the bacilli in a state of dormancy or deactivation.[11] These people often have no risk factors associated with TB disease at the time of infection. Secondary TB occurs when the immune system is unable to maintain a tuberculoma and reactivation occurs as seen in figure 1.

Incipient tuberculosis is defined as progression to TB within the next two years found through the metabolic activity of Mtb. This stage is met either through the initial failure to create a tuberculoma by the adaptive immune system, primary progressive TB, or the reactivation of latent Mtb, secondary TB.

Subclinical disease differs from active TB due to its lack of symptoms but evidence of radiographic abnormalities and/or evidence of viable Mtb. Lung cavitation is the common

radiological finding. Subclinical disease, due to the low bacilli count is often not included in transmission sources.[11]

Active TB is symptomatic with radiographic abnormalities and the presence of Mtb. At this stage, transmissibility is variable, determined by degree and duration of infectiousness, location of the Mtb, and the susceptibility of any contacts. This state technically can be reached multiple times through the continuous reactivation and inactivation of Mtb, called cyclic disease.

Extrapulmonary TB

Despite TB being associated with respiratory illness, it is not limited to the respiratory system. Extrapulmonary TB (EPTB) is any TB that is not located in the lungs and consists of 16% of TB cases globally.[12] It is categorized as either primary EPTB or secondary/disseminated EPTB. Primary EPTB is when the initial infection is in a location outside the lungs and is rare. Secondary/disseminated EPTB is caused by the dissemination of TB bacilli from the lungs through hematogenous spread. Table 1 is a non-exhaustive list of EPTB.

TB meningitis, oral TB, cutaneous TB, musculoskeletal TB, and miliary TB are more common in children than other groups.[12] Childhood EPTB are often deadly and confer permanent disability for children who survive. TB meningitis is of particular concern to the very young (<2 years) because of its high rates of morbidity and mortality compared to others (<1 year 10-20% risk; 1-2 years 2-5%).[1] Three stages of TB meningitis occur. Stage I the child lacks any non-constitutional symptoms; stage II the child is conscious but lethargy, confusion, and mild focal neurological symptoms occur; and stage III the child may be in a stupor, coma, and with seizures, palsies, or hemiplegia. Seizures are reported in 50%[9] of children and those >2 years having higher rates of vomiting, gastrointestinal symptoms, and neurological symptoms

compared to those with pulmonary TB.[13,14] Neurological sequelae occurs in anywhere from 30-50% of cases, and permanent impairment occurs in half of those who survive.[9,13,14]

In India, 15-20% of all HIV-negative TB cases are EPTB, while among HIV-positive TB cases 40-50% of new cases are EPTB.[15] This is expected since those who are HIV-positive are less likely to have an immune system that could mount a robust response to the initial infection leading to dissemination and those who have LTBI and later are infected with HIV will not have the immune system capable of maintaining the granuloma.

Risk Factors

Risk factors for exposure to Mtb include crowding, ventilation, alcohol, and smoking, occupation, duration of exposure, and proximity of exposure. Compared to the general public, healthcare workers are more likely to be exposed to TB because those with TB will be seeking care. With crowding, those with TB will be in close proximity to others and without proper ventilation, exposure to Mtb in the air will be greater. Firsthand smoking is a known risk factor for a variety of respiratory related infections and diseases, which includes TB and Mtb infection. Research into secondhand smoking and its association with both Mtb infection and TB is conflicting, with the quality of studies of particular concern.[16]

TB is an opportunistic infection leading to it being the most common cause of death among those who are HIV positive.[2,4,5] The likelihood of progression to TB is 7-10% per year among those with HIV. Compare this to the 30% lifetime risk among those with diabetes and the 10% lifetime risk among those with no risk factors.[4] If these risk factors are present before the primary infection, the immune system will be less likely to successfully cut off blood supply to the site and form the tuberculoma. If these risk factors were acquired during the latency period, the immune system will be less likely to sustain a tuberculoma.

There is a debate on if sex is a true risk factor for Mtb infection and TB or if it is a disparity in access to care.[2] This is noted by the divergence in TB notifications and mortalities beginning with the 35–44-year age group.[2] The prevalence of adult male smokers compared to females may contribute to the TB disparity.[2,10]

According to the WHO's 2022 Global TB Report, undernourishment, alcoholism, smoking, diabetes, and HIV are the greatest risk factors for TB in descending order. Undernourishment accounts for over half of all risk factor associated TB cases with 738,000 incidences, with the next largest being alcoholism at 258,000 in India.[2] In infants, weight-for-age z-score is used to measure if the child's weight is relative to age and is associated with nourishment.

Children are the most likely to have primary progressive TB after being infected with Mtb with infants at the highest risk because of their less robust immune system[1,8,9,17,18] For children <1 year old, the risk of disease from primary tuberculosis is highest at 30-40%, with disseminated TB and TB meningitis at 10-20% if untreated.[17] At that age, the source of infection is most likely another person in the household but they themselves being less infectious. [10,17,18]

Table 1. Forms of TB, adapted from Gopalaswamy et al.[12]

Category	Organs	Host Risk Factors	Defining signs/symptoms
Pulmonary TB			
Pulmonary	- Lungs	HIV, sex, age, immunosuppressive treatments, weight, malnutrition, DM, end-stage renal disease	Fever, weight loss, night sweats, loss of appetite, malaise, fatigue
Miliary	Lungs and ≥2 other sites		Cutaneous lesions, choroidal tubercles, TB meningitis
Extrapulmonary TB			
Head and Neck	- Brain - Neck lymph nodes - Eye - Mouth/tongue	Prior history of TB, trauma to the area, pulmonary and Skeletal TB	Meningitis: seizure, coma, palsies Lymphadenitis: painless lump(s) Ocular: Lack of classical TB symptoms Oral: Painless ulcers in primary infections and painful ulcers in secondary infections
Thorax	- Pleura - Pericardium	Miliary TB	Pleura: pleural effusion, breathlessness Pericarditis: pericardial effusion, constriction of the heart, pericardial inflammation and edema
Skin, Bone, and Muscle	- Skin - Spinal TB - Prosthetic joint	Skin: direct TB inoculation or chancre, abrasions and broken skin, prexisting TB, measles, scarlet fever	Skin: ulcer, hyperkeratotic or verrucous papule, nodule, lesions Spinal: back pain, spinal deformity, neurological deficits
Abdominal	- GI tract - Peritoneum - Solid viscera		GI: abdominal pain, diarrhea Male genitalia: Scrotal swelling, irregular/nodular prostate, genital ulcer, perineal sinus/ fistual, male infertility
Genitourinary	- Kidneys - Male and female genital tract		Kidneys: Renal dysfunction and failure, flank pain, pyuria, hematuria Male genitalia: Scrotal swelling, irregular/nodular prostate, genital ulcer, perineal sinus/ fistual, male infertility Female genitalia: menstrual dysfunction and infertility

Standard Diagnostics

While the gold standard for TB diagnosis continues to be culturing sputum samples, rapid molecular tests, some with the ability to detect drug resistance, have been recommended by the WHO.[2] The GeneXpert stands out for its cartridge set up that produces a clear answer. Though in low- and middle-income countries sputum smear microscopy is often used. This is the same technology used in the discovery of Mtb by Robert Koch over 100 years ago. Clinical symptoms and chest x-rays are non-laboratory ways to detect TB. Symptom-based diagnosis, while not entirely reliable, are vital in low-resource settings where access to x-ray and GeneXpert is unavailable. There is no gold standard for Mtb infection detection.[1,2,18–20] Mtb diagnostic tests are reliant on the adaptive immune response to produce Mtb antigens since the infected are asymptomatic the bacille are trapped in tuberculomas.[18] Those with compromised immune systems due to a variety of risk factors will be less likely to respond to tests, that treatment will not be administered in most cases to a population that are at the greatest risk for progressing to TB.[18] Children, because of their less robust immune system, experience similar diagnostic issues. Additionally, those tested before 6-12 weeks after infection do not produce antigens created by the adaptive immune system, causing false negatives. Only two methods are available to detect Mtb infection: the tuberculin skin test (TST) and IFNγ release assays (IGRAs).[2,18]

The TST was pioneered by Robert Koch with the diagnostic criteria refined by Charles Mantoux.[18] It is an intradermal injection of tuberculin purified protein derivate (PPD) that creates an induration that is measured by a trained healthcare worker 48-72 hours afterwards to determine infection. The PPD has low specificity due to its cross reactivity with non-tuberculosis mycobacterium infection and the BCG vaccine, which is widely used outside of western Europe,

Canada, Australia, and the United States. Still, it is widely used because of its cost effectiveness and logistic simplicity.

The two IGRAs available for commercial use are QuantiFERON-TB (Cellestis) tests and T-SPOT.TB (Oxford Immunotec). While QuantiFERON (QFT) uses whole blood to measure IFNγ concentrations, T-SPOT.TB uses peripheral blood mononuclear cells to count the number of IFNγ spots. Both use the Mtb specific proteins ESAT-6 and CFP10 to test infection through antigen levels.

QFT-Plus gives both qualitative and quantitative results, with IFNγ levels IU/mL for the Nil, TB1-Nil, TB2-Nil, and Mitogen-Nil and the result of either positive, negative or indeterminate. The interpretation do not give a definitive answer to infection, rather stating infection as likely. If the Nil is >8.0 IU/mL of the Mitogen – Nil is <0.50 the results is automatically, indeterminate and the likelihood of infection cannot be determined.

Novel Diagnostics

The lack of sensitivity in one of the highest risk populations has led to research into the use of cytokines outside or in addition to IFNγ to help definitively diagnosis Mtb infection in infants. Of the studies, a handful of cytokines have emerged as contenders for continued evaluation. This includes interleukin (IL) 2[21–25], induced protein (IP) 10[21–25], IL-13 [21,23–25], IL-1α [21,25], tumor necrosis factor alpha (TNF-α) [21–25], granulocyte-macrophage colony-stimulated factor (CM-CSF) [21,23,25], IL-1RA[23–25], IL-3[23,25], and macrophage inflammatory protein (MIP) 1b[23–25]. In a systematic analysis of cytokine and chemokines' ability to differentiate active TB and LTBI in 2020, 14 studies involving children were evaluated.[25] IP-10 was evaluated in 10 studies, with a single study finding a significant difference between the two in children. Likewise, IL-2 was

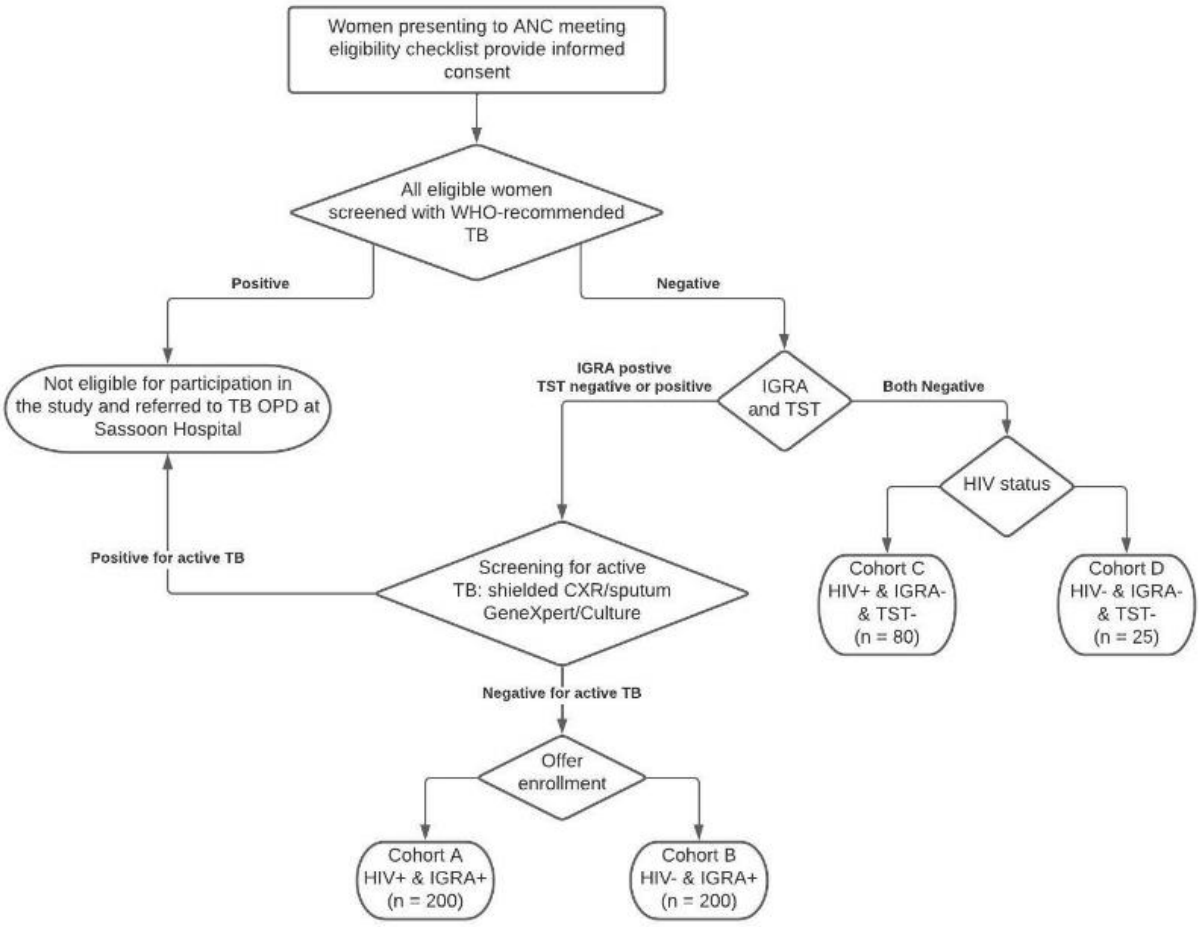
another commonly studied cytokine but with contradictory results. IL-15 and MIP-3α were yet to be evaluated in children, with few studies being conducted in adults.

Methods

Participants

Mothers in their second trimester of pregnancy were enrolled into the Impact of Immune Changes of HIV and Stages of Pregnancy on Tuberculosis (PRACHITi) prospective longitudinal cohort study. They were to be enrolled and followed throughout the duration of their pregnancy and two years postpartum. Infants born to the mothers of the PRACHITi study were then enrolled without any exclusion criteria to be followed for 24 months.

Figure 1. Screening of mothers



Screening and Enrollment of Mothers

Table 2. Inclusion and Exclusion Criteria for Pregnant Women

Cohort	Inclusion	Exclusion
A	• Pregnant women age ≥ 18 years with gestational age > 13 weeks and up to 34 weeks • Documentation of pregnancy • Documentation of HIV+ status during the current pregnancy • Positive IGRA result, irrespective of TST result • Ability and willingness of participant or legal guardian/representative to provide written informed consent	• Current TB suspect or taking treatment for active TB • Taken TB treatment in the last 2 years • CXR consistent with active TB • Currently taking any antibiotic > days, excluding preventive therapies (e.g., Bactrim of PCP prophylaxis, acyclovir for HSV prevention) • Plan to deliver and receive postpartum care somewhere other than Sassoon Hospital • Hemoglobin /le 7.5g/dL (within 30 days) • Current use of immunosuppressive medications (e.g., prednisone, betamethasone, dexamethasone) within the last 14 days • History of autoimmune disease or immunosuppressive condition excluding HIV or diabetes (e.g., lupus, rheumatoid arthritis) • Plan to move outside the study area during the 24 months following delivery • Any condition that, in the opinion of the study staff, would make participation in the study unsafe, complicate interpretation of study outcome data, or otherwise interfere with achieving the aims of the study
B	• Pregnant women age ≥ 18 years with gestational age ≥ 13 weeks and up to 34 weeks • Documentation of pregnancy • Documentation of HIV- status in the last 30 days • Positive IGRA result, irrespective of TST result • Ability and willingness of participant or legal guardian/representative to provide written informed consent	
C	• Criteria are the same as Cohort A except participants will require both a negative IGRA and TST	
D	• Criteria are the same as Cohort B except participants will require both a negative IGRA and TST	

Approximately 1000 pregnant women were pre-screened and referred based on the eligibility criteria that included age, gestational age, HIV status, hemoglobin, and medical/medication history by medical staff. After providing informed consent they were screened with the WHO-recommended TB symptom screening, with those positive being excluded and referred to the Sassoon Hospital's Outpatient Department for treatment of active TB. Women who were negative for active TB were then tested for Mtb infection using either a QFT Gold Test-in-tube (GIT) or QFT Gold Plus (3^{rd}/4^{th} generation QFT kits) and a TST.

After screening, 400 IGRA-positive active-TB negative women entering the index group and 105 IGRA and TST-negative women (HIV+ = 80 women, HIV- = 25) entering the control group. Women were separated into four cohorts based on a variety of exclusion and inclusion criteria both depicted in Figure 1 and table 2. During the enrollment process, further information was collected on a variety of topics that included the woman's HIV status, socioeconomic status, household smoking, and current ART use.

HIV status was categorized using the WHO Clinical Staging System, which lists out four separate stages of HIV/AIDS in adults (Box 1).[26] Mothers who were put into cohort A and cohort C were asked about current ART use at baseline.

Box 1. WHO Clinical Staging System for HIV/AIDS, adapted from Weinberg J et al

- **Stage 1.** Asymptomatic or persistent generalized lymphadenopathy
- **Stage 2.** Unexplained weight loss < 10% of total body weight, recurrent respiratory infections, dermatological conditions
- **Stage 3.** Weight loss > 10% of total body weight, prolonged (> 1 month) unexplained diarrhea, pulmonary TB, severe systemic bacterial infections, mucocutaneous conditions
- **Stage 4.** AIDS-defining illnesses. This includes HIV wasting syndrome, Pneumocystis pneumonia (PCP), recurrent severe or radiological bacterial pneumonia, EPTB, HIV encephalopathy, CNS toxoplasmosis, chronic (>1 month) or orolabial herpes simplex infection, esophageal candidiasis, and Kaposi's sarcoma.

BMI was categorized into four classifications that were adapted from WHO's adult BMI classification and ranked from 1 to 4. The following categories were underweight (>18.5), normal weight (18.5 – 24.9), pre-obesity (25-29.9), and obese class I, II, and III (30+).[27]

Socioeconomic status was scored using the modified Kuppuswamy Socioeconomic scale. The score is a composite score of the head of household's highest obtained level of education and occupation and monthly income of the family. With a range of 3 to 29, a higher score is associated with a higher socioeconomic class that is divided into lower (V), upper lower (IV), lower middle (III), upper middle (II), and upper class (I).[28]

Household smoking was considered anyone in the house that smoked at the time of enrollment, it was assumed that this would be constant throughout the study.

Infant Follow-Up

Infants were clinically evaluated 5 times during their time in the study, two of which blood samples would be drawn for QuantiFERON. Infants were brought to the Sassoon Hospital at birth (+6 days), 6 weeks (±14 days), 3 months (±21 days), 6 months (±21 days), and 12 months (±21 days) of life for clinical evaluations.

Birth outcome, vital signs (temperature, respiration rate, pulse), anthropometrics (weight, length), and HIV status were noted at birth evaluation. HIV status would only be conducted for infants born to documented HIV+ mothers and if the test were not already conducted as part of standard medical care. If the DNA/TNA PCR test results returned indeterminate or failed, then an RNA PCR test would be done. For those with a positive HIV test, 2ml of blood would be collected for HIV confirmation.

At all subsequent evaluations vital signs, anthropometrics, infant feeding practices, development, immunizations, co-morbidities, and TB outcomes were evaluated. At the 6-week

visit an additional BCG scar evaluation was conducted. At 6- and 12-months blood samples were collected to evaluate the infant for a Mtb infection using QFT GIT/Plus. At 6-weeks and 6-months, blood samples were collected for a sample repository that would be used for future research. Additional samples collected included saliva and stool.

Blood Collection

Whole blood from infants was drawn 2 times during the 12-month cohort study at 6-months and 12-months. The total volume of blood that could be collected was capped at 3mL/kg of body weight in an 8-week period.

IGRA testing was conducted at 6-months and 12-months, drawing 4mL of blood each, with any supernatants remaining being stored for future studies. A QFT GIT or QFT Plus test was performed per the package insert. The 4ml of blood could be collected in either a single lithium heparin tube and then separated into the assigned QFT tubes or directly into the QFT tubes. Both the qualitative (positive, negative, indeterminate) and quantitative (concentration of IFN-γ in mitogen, nil, and TB antigen tubes) results were recorded. The remaining supernatant from the 4ml blood sample was stored in 4 aliquots of 50uL at -80*C. Any indeterminate test would result in a request for the patient to return within the week for an additional test to be conducted. If the second test remained indeterminate, it was recorded as such.

A variety of non-whole blood specimens were collected at 6-weeks and 6-months. This included 4mL of PBMC in lithium heparin tubes, plasma harvested from the PBMC and 2mL of serum in noncoated tubes that were cryopreserved in liquid nitrogen for future studies.

Cytokine Analysis

Cytokine concentrations were measured using the Human Th9/Th17/Th22 Fixed Panel Luminex® assay after the completion of the study. The PBMC samples that were cryopreserved for storage in liquid nitrogen were used for flow cytometry. The plasma would be separated and used for noncellular measures of immune response (i.e., cytokines, chemokines). The multiplex assay would produce the concentration of cytokines for IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-15, IL-17A, IL-17E, IL-33, MIP-3α, TNF-α, CD40L, and GM-CSF.

Analysis

The first outcome of this analysis was to evaluate the prevalence and consistency of Mtb immune responses among Indian infants over the first year of life as detected by standard QFT IGRA and a Luminex assay to capture non-IFNγ cytokines. Second to identify sociodemographic and clinical correlates of Mtb infection as detected by QFT and Luminex. Finally, to evaluate the agreement between QFT and Luminex.

Maternal and infant sample size per time step was determined by follow-up visits attended and samples collected at the marked time step. Descriptive analysis was performed with frequency counts for each risk factor separated on cohort and participation in sample collection. If a mother or infant died during the study, the decision of what to do with the information collected so far would be decided on a case-by-case basis. If an infant died during delivery, the infant would be removed from the dataset and not counted towards any analysis on data at birth. If the infant died after delivery but before 6 months, the data collected on the infant at birth would be retained. In the case the infant died after the 6-month follow-up visit, the clinical and diagnostic information would be used in any statistical analysis.

In this study, a cytokine analysis using QFT supernatant was conducted to measure cytokines and chemokine concentrations. Concentrations (IU/mL) of cytokines and chemokines was outputted, and a set of criteria converted them into categorical outcomes of positive, negative, and indeterminate for Mtb infection. A set of three criteria was created: (1) if the difference between the maximum antigen concentration and nil would be above the antigen 90^{th} percentile and greater than or equal to the nil 25^{th} percentile; (2) if the nil was less than two times the average Nil concentration; and (3) if the difference between the maximum mitogen concentration and nil is greater than or equal to the lower limit. If all three criteria were met, this would be considered a positive result. If criterion 1 was not met, it would be a negative. If any of the criteria were missing an output, this would be considered an indeterminate result.

A final Luminex panel result was created from the 17 single analyte results with the possible outputs being likely to have a *M. tuberculosis* infection, positive, and not likely to have a *M. tuberculosis* infection, negative. A positive Luminex panel was defined as at least positive 2 single analyte results with a negative result being 0 or 1 positive single analytes.

A combined results were created from a combination of the QFT and Luminex panel results. The two possible outcomes for the combined Mtb infection result were positive and negative, with the same interpretations as the Luminex panel results of likely and not likely having *a M. tuberculosis* infection. A positive result was having a positive result from Luminex and/or QFT test. This converted any indeterminates from QFT into either a positive or negative, given that a sample was collected from the Luminex assay or the assay could be completed. If a participant did not have either a QFT result or Luminex assay result, the combined result would be the same as the available result to maximize combined results.

Cohen's kappa statistic, κ, was used to measure the agreement between QFT and Luminex panel at 6 and 12 months. The two-sided alternate hypothesis with a 95% confidence interval was used to produce the standard error.

Univariate analysis was conducted using known variables associated with Mtb infection. The continuous weight-for-age z-score variable would be modified into a dichotomous variable with a cut-off point of -2. This would separate the severely and moderately underweight for age from slightly underweight, normal weight, and overweight for age categories. Risk ratios were estimated using the small-sampled adjusted method and normal approximate (Wald) confidence intervals.

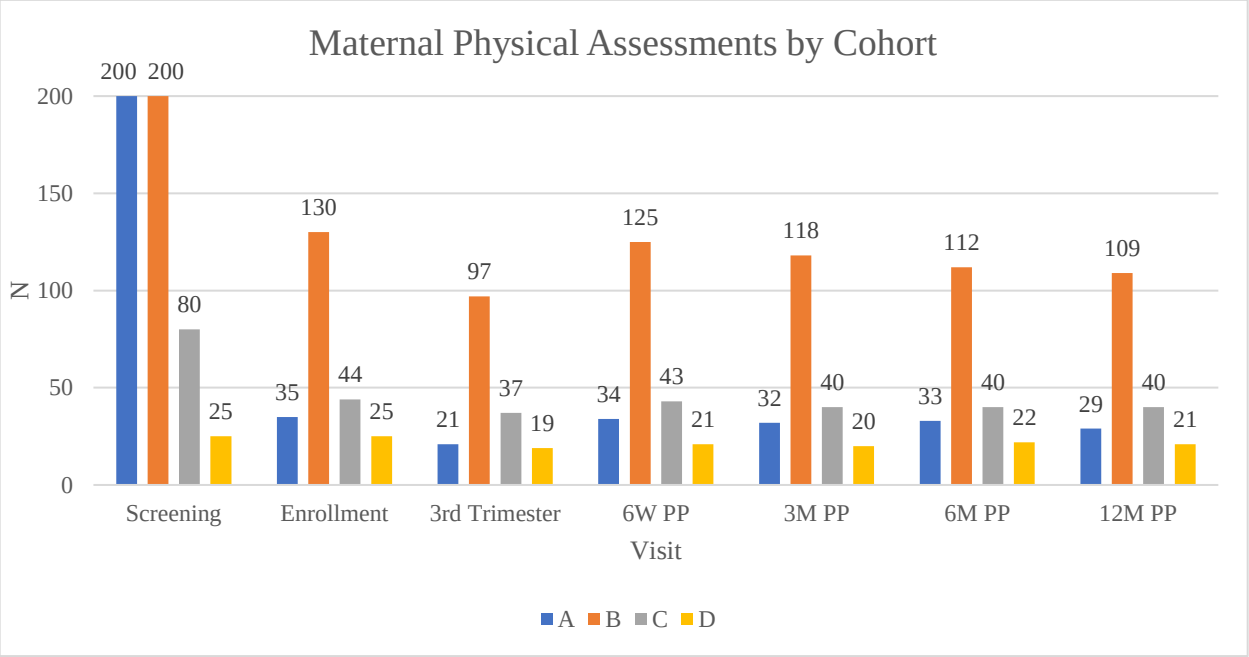
Data was entered into Microsoft Excel (Redmond, WA, USA) and analyzed using R software 4.2.2.

Ethical Approval

The study was conducted to the standards of the International Conference on Harmonisation (ICH E6) or the Institutional Review Board (IRB) of B.J. Medical College, National Institute of Research in Tuberculosis, Weill Cornell Medical College, and the Johns Hopkins School of Medicine. All eligible participants were given an IRB-approved written consent form stating the purpose of the study, risks and benefits of participation, alternatives, and contact information in Marathi or Hindi. Those unable to read or write had the consent form read aloud in the presence of a literate adult witness not affiliated with the study team, and a signature or thumb print was taken.

Results

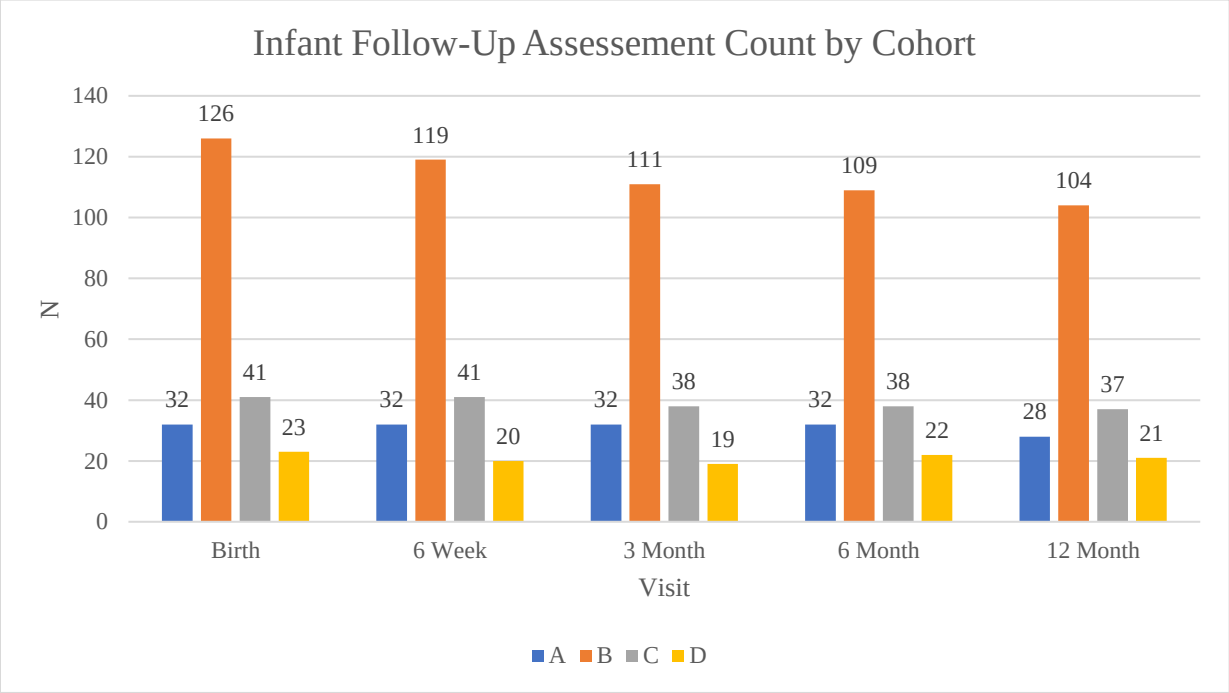
Figure 2. Mothers participating in physical assessments up to 12 months postpartum



Overall, 234 pregnant women were enrolled in the study. Of the screened 200 mothers in cohort A, 200 mothers in cohort B, 80 mothers in cohort C, and 25 mothers in cohort D, only cohort D achieved the anticipated enrollment. At enrollment, cohort A had 35 mothers, cohort B had 130 mothers, cohort C had 44 mothers, and cohort D had 25 mothers (figure 2). Post-enrollment, there were minimal declines in mothers returning for their own physical assessments. There was no physical assessment recorded for labor and delivery. Five mothers continued the maternal physical assessments until the 12 months post-partum visit after their infant died.

In accordance with the WHO system for categorizing clinical HIV/AIDS, 69% were asymptomatic stage I while 28% were mildly symptomatic stage II. One woman was clinically classified as having AIDS and one woman had not previous taken or was currently taking ART at the time of enrollment. The median SES score was 10 which is classified as upper lower class. Thirty-six (15%) of women lived in a house where someone besides them smoked.

Figure 3. Infant participation in follow-up assessments from birth to 12 months



There were 229 infants that were born. Seven infants were removed from the dataset as 5 died during delivery and 2 before their 6-month follow-up visit. Among the 222 infants, 105 (52%) were male. Overall, BCG BCG vaccine uptake was 96%. All HIV exposed infants began antiretroviral (ARV) prophylaxis, in accordance with National PPTCT Guidelines. They would be given Nevirapine with dosage dependent on weight class for up to 6 weeks.[29] A final diagnosis of HIV in infants only occurred in 3 infants.

Weight for age (W/A) at all age groups was on the lower side of normal with the average z-scores at birth, 6 months, and 12 months being -1.3 (±1.0), -1.4 (±1.3), and -1.3 (±1.1). The minimum score at any visit was -7.22, severely underweight, at 6 months and the maximum score was 1.76, normal weight, at 12 months. At all time points, no infant was categorized as overweight or obese.

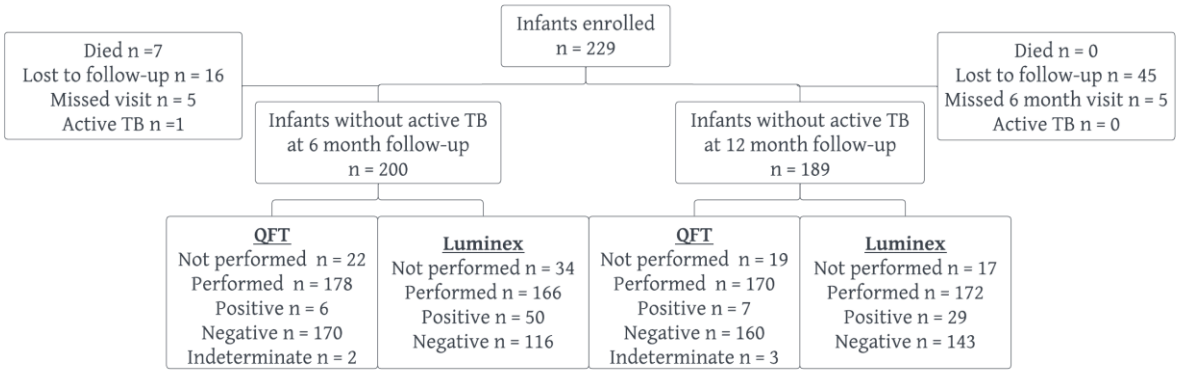
Table 3. Demographics and Clinical Characteristics of Mother-Infant Pairs

Variable	Overall[1] N = 222	Cohort A[1] N = 32	Cohort B[1] N = 126	Cohort C[1] N = 41	Cohort D[1] N = 23
Socio-demographic characteristics					
SE Class/Score	10.2 (3.9)	9.0 (2.6)	10.2 (3.6)	10.3 (4.1)	12 (5.2)
I (26-29)	2 (1)	0 (0)	1 (1)	0 (0)	1 (4)
II (16-25)	20 (9)	1 (3)	9 (7)	5 (11)	5 (20)
III (11-15)	72 (31)	8 (23)	46 (35)	12 (27)	6 (24)
IV (5-10)	139 (59)	26 (74)	73 (56)	27 (61)	13 (52)
V (1-4)	1 (0.4)	0 (0)	1 (1)	0 (0)	0 (0)
Household smoke	36/205 (15)	3 (9.4)	23/112 (18)	4/39 (9.8)	6/22 (26)
Maternal characteristics					
Maternal TBI	158 (71)	32 (100)	126 (100)	-	-
Maternal HIV	73 (33)	32 (100)	-	41 (100)	-
WHO Stage I	44/71 (60)	18 (56)	-	26/39 (63)	-
WHO Stage II	18/71 (25)	8 (25)	-	10/39 (24)	-
WHO Stage III	8/71 (11)	6 (19)	-	2/39 (4.9)	-
WHO Stage IV	1/71 (1.4)	0 (0)	-	1/39 (2.4)	-
Maternal ARV	72/73 (99)	31 (97)	-	41 (100)	-
Infant characteristics					
Male	105 (47)	13 (41)	58 (46)	20 (49)	14 (61)
BCG	214 (96)	32 (100)	119 (94)	41 (100)	22 (96)
ART Initiate[2]	73 (100)	32 (100)	-	41 (100)	-
HIV	3 (1)	0 (0)	0 (0)	3 (8)	0 (0)
WAZ (birth)	-1.3 (1.0)	-1.6 (1.0)	-1.2 (1.0)	-1.3 (1.0)	-1.2 (1.0)
WAZ (6 month)	-1.4 (1.3)	-1.9 (1.8)	-1.2 (1.1)	-1.5 (1.2)	-1.1 (0.8)
WAZ (12 month)	-1.2 (1.1)	-1.2 (1.3)	-1.3 (1.0)	-1.1 (1.3)	-1.2 (0.8)

[1]Mean (SD) or N (%) TBI: Mtb infection. ARV: Antiretroviral therapy. WAZ: Weight-for-age z score. Percentage might not sum to 100% due to rounding.

Mtb Infection Prevalence

Figure 4. Workflow of infant Mtb infection results



At 6 months and 12 months, 200 and 190 infants were available for QFT testing (Figure 4). At 6 months, 6 infants did not have a recorded clinical follow-up that was supposed to accompany the QFT test. This occurred in 10 infants at 12 months. Insufficient blood volume was cited as the most common cause of an infant not having QFT results at 6 months (Table 4). At 12 months, a missing record of QFT encounter, but available record of a clinical follow-up, was the most common cause of missing QFT data (Table 4). There were 16 infants that were absent for their 6-month clinical follow-up and QFT testing but returned for their 12-month visit.

Table 4. QuantiFERON and Luminex Test Performed

Test Outcome	**6 Months (N = 200)**	**12 Months (N = 190)**
QFT Performed	178 (89%)	170 (90%)
QFT Plus	172	169
QFT GIT	6	1
QFT Not Performed	22 (11%)	20 (11%)
Participant refused	3	6
Insufficient blood volume	16	4
Sample could not be collected	2	2
Blood clotted in tube	1	0
Missing Records	0	8
Luminex Performed	166 (83%)	172 (90.9%)
Luminex Not Performed	34 (17%)	18 (9.1%)

Of the 178 QFT tests at 6 months and 170 QFT tests at 12 months, 6 (3.4%) and 7 (4.1%) were positive (Table 5). Of the 166 Luminex tests at 6 months and 172 Luminex tests at 12 months, 50 (30%) and 29 (16%) were positive (Table 5). At both 6 months and 12 months, one of the infants with indeterminate results returned to rerun the QFT test to confirm the indeterminate finding.

There were 147 infants who were tested for a Mtb infection at 6 and 12 months using QFT and 146 supernatant samples available for the Luminex assay test. QFT found no persistent positives or indeterminates while Luminex found 8 (5.5%) incidences (Figure 5). Using QFT, 5 (3.4%) infants converted to positive compared to 12 (8.2%) using Luminex (Figure 5). Reversion occurred in 6 (4.1%) infants using QFT while this occurred in 38 (26%) infants using Luminex.

Figure 5. Sankey diagram of infant test results between 6 and 12 months

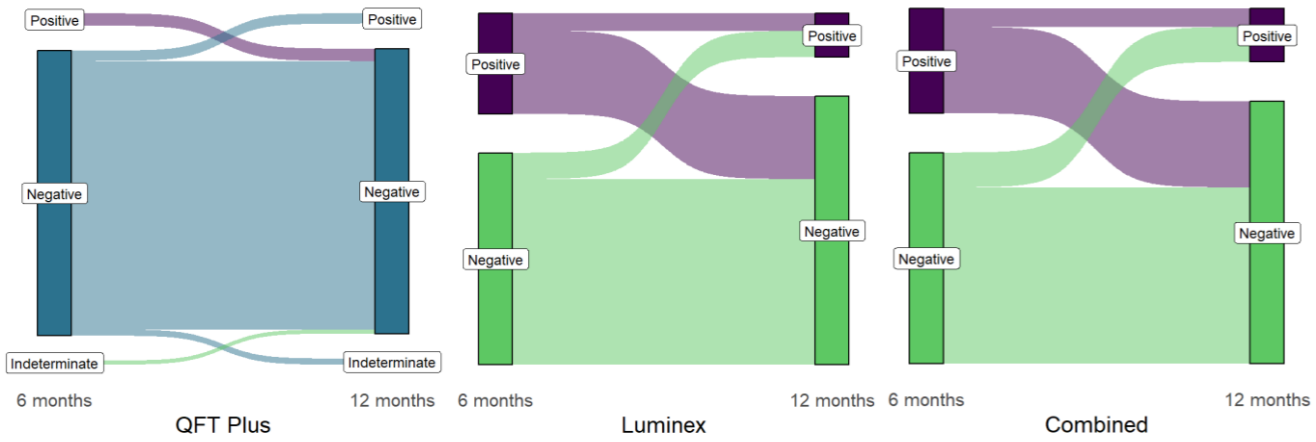


Table 5 depicts the individual cytokine results that made up the Luminex test. The prevalence of positives between 6 and 12 months decreased. At 6 months, IFNγ, IL1β, IL2, IL4, IL10, IL12, IL15, IL17A, TNFα, CD40, and GMCSF reported positives in ≥10% of infants. At 12 months, no single cytokine reached 10%. IL2 and IL4 are the closest to 10% prevalence at 8.7% and 8.1%, respectively.

Table 5. QuantiFERON Gold Plus and Luminex Results

Test	N	Negative (%)	Positive (%)	Indeterminate (%)	N	Negative (%)	Positive (%)	Indeterminate (%)
	6 Months				**12 Months**			
IFN-γ	164	142 (87)	20 (12)	2 (1.2)	172	160 (93)	11 (6.4)	1 (0.6)
IL-1β	160	138 (86)	16 (10)	6 (3.8)	172	162 (94)	6 (3.5)	4 (2.3)
IL-2	163	144 (88)	17 (10)	2 (1.2)	172	157 (91)	15 (8.7)	0 (0)
IL-4	161	145 (89)	17 (10)	0 (0)	171	155 (91)	14 (8.1)	2 (1.2)
IL-5	162	148 (91)	11 (7.5)	3 (1.9)	172	157 (91)	11 (6.4)	4 (2.3)
IL-6	163	141 (87)	10 (6.1)	12 (7.4)	172	160 (93)	5 (2.9)	7 (4.1)
IL-10	161	139 (86)	20 (12)	2 (1.2)	172	160 (93)	10 (5.8)	2 (1.2)
IL-12	163	141 (87)	20 (12)	2 (1.2)	172	160 (93)	11 (6.4)	1 (0.6)
IL-13	162	139 (86)	15 (9.3)	8 (4.9)	172	162 (94)	10 (5.8)	0 (0)
IL-15	161	138 (86)	21 (13)	2 (1.3)	172	163 (95)	8 (4.7)	1 (0.6)
IL-17A	161	139 (86)	20 (12)	2 (1.2)	172	162 (94)	8 (5.2)	1 (0.6)
IL-17E	161	158 (98)	0 (0)	3 (1.9)	172	172 (100)	0 (0)	0 (0)
IL-33	163	141 (87)	19 (12)	3 (1.8)	172	160 (93)	10 (5.8)	2 (1.2)
MIP-3α	161	148 (92)	5 (5.0)	5 (3.1)	172	160 (93)	5 (2.9)	7 (4.1)
TNF-α	161	143 (89)	17 (11)	1 (0.6)	172	157 (91)	12 (6.9)	3 (1.7)
CD40L	163	141 (86)	17 (10)	5 (3.1)	172	160 (93)	11 (6.4)	1 (0.6)
GM-CSF	162	139 (86)	21 (13)	2 (1.2)	172	161 (94)	9 (5.2)	2 (1.2)
Luminex >1	166	116 (70)	50 (30)	-	172	143 (84)	29 (16)	-
QFT Plus	178	170 (95)	6 (3.4)	2 (1.1)	170	160 (94)	7 (4.1)	3 (1.8)
Combined	178	123 (69)	55 (31)	-	177	144 (82)	33 (18)	-

Table 6. Comparative Results of QFT and Luminex

QFT	Luminex, 6 Months				κ statistic
	+	-	N/A	Total	-0.009
+	1	3	2	6	
-	49	111	10	170	
±	0	2	0	2	
Total	50	116	12	178	

QFT	Luminex, 12 Months				κ statistic
	+	-	N/A	Total	0.110
+	3	4	0	7	
-	25	130	5	160	
±	1	2	0	3	
NA	0	7	13	20	
Total	29	143	18	190	
+ Positive test result; - Negative test result, ± Indeterminate test result					

Table 6 shows there was no agreement (κ = -0.009) between QFT and Luminex at 6 months while there was slight agreement (κ= 0.110) at 12. At 6 months, agreement on negatives (111/170, 65%) was more common than positives (1/6, 17%). This trend continued at 12 months with 130/160 (81%) negatives agreeing versus 3/7 (43%) positives.

Of infants that had positive Luminex test, the majority just met the criteria with 2 cytokines being positive (Figure 6). The average amount of cytokines that were positive at both 6 month and 12 months was 4. One infant at 6 months had 15/17 cytokines measured being positive with only IL17E and MIP3α being negative.

Figure 6. Prevalence of positive cytokines in positive Luminex panel

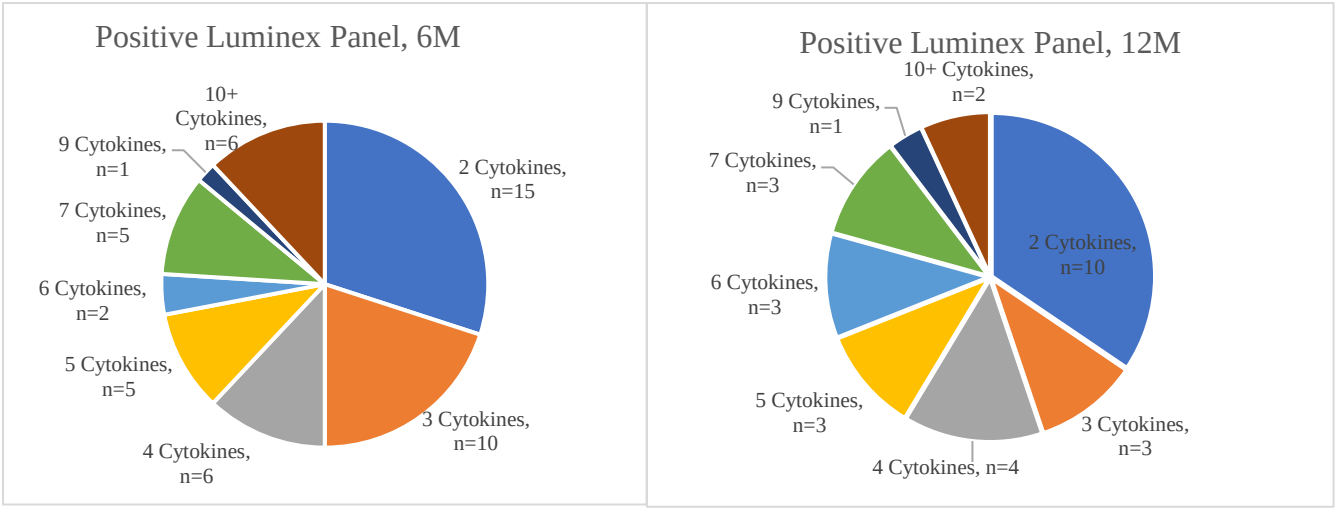


Table 7 breaks down cytokine results among those with Luminex positive results. IFNγ, IL10, IL12, IL15, and GMCSF were positive in ≥ 40% of infants at 6 months. This contrasts with the distribution at 12 months only having IL2 and IL4 being ≥ 40% positive. IL17E did not contribute to positive Luminex results at either 6 months or 12 months. MIP3α had the second lowest prevalence of positives across both tests with 10%.

Table 7. Cytokine results among those with positive Luminex results

Cytokine	**N**	**+ (%)**	**- (%)**	**± (%)**	**N**	**+ (%)**	**- (%)**	**± (%)**
	6 Months				**12 Months**			
IFNγ	50	20 (40)	29 (58)	1 (2)	29	11 (38)	17 (59)	1 (3.5)
IL1β	48	14 (29)	31 (65)	3 (6.3)	29	5 (17)	22 (76)	2 (6.9)
IL2	49	14 (29)	34 (69)	1 (2.0)	29	12 (41)	17 (59)	0
IL4	49	13 (27)	36 (73)	0	29	13 (45)	14 (48)	2 (6.9)
IL5	49	7 (14)	42 (86)	0	29	4 (14)	23 (79)	2 (6.9)
IL6	50	9 (18)	35 (70)	6 (12)	29	4 (14)	21 (72)	2 (14)
IL10	49	20 (41)	28 (57)	1 (2.0)	29	10 (34)	17 (59)	2 (6.9)
IL12	50	20 (40)	29 (58)	1 (2.0)	29	9 (31)	19 (66)	1 (3.5)
IL13	49	12 (25)	30 (61)	7 (14)	29	7 (24)	22 (76)	0
IL15	49	20 (41)	28 (57)	1 (2.0)	29	8 (28)	20 (69)	1 (3.5)
IL17A	48	18 (38)	29 (60)	1 (2.0)	29	4 (14)	24 (83)	1 (3.5)
IL17E	49	0	46 (94)	3 (6.1)	29	0	29 (100)	0
IL33	49	18 (37)	29 (59)	2 (4.1)	29	10 (35)	17 (59)	2 (6.9)
MIP3α	49	5 (10)	44 (90)	0	29	3 (10)	26 (90)	0
TNFα	48	15 (31)	32 (67)	1 (2.1)	29	11 (38)	16 (55)	2 (6.9)
CD40L	49	16 (33)	29 (59)	4 (8.2)	29	11 (38)	17 (59)	1 (3.5)
GMCSF	49	20 (41)	29 (59)	0	29	8 (28)	19 (66)	2 (6.9)

Table 8 evaluated the association between clinical variables and possible Mtb infection using QFT and Luminex. Excluding WAZ at 6 months, there were no clinical or socio-demographic variables that were associated with infant Mtb infection. A WAZ > -2, being moderately or severely underweight at 6 months, was inversely related to a positive Luminex test. This was a statistically significant finding. In all QFT relative risk calculations, at least 1 group had a frequency less than 5.

Table 8. Univariate analysis

Variable	N	QFT RR[1]	p-val	N	Luminex RR[1]	p-val	N	Combined RR[1]	p-val
6 Months									
Maternal HIV	176	0.31* (0.04, 2.56)	0.38	166	1.19 (0.74, 1.89)	0.44	178	1.08 (0.69, 1.69)	0.68
Maternal TBI	176	Undefined	0.11	166	0.88 (0.54, 1.44)	0.73	178	0.99 (0.61, 1.61)	0.90
Household Smoke	175	Undefined	0.30	165	0.69 (0.33, 1.45)	0.33	177	0.57 (0.27, 1.21)	0.12
Sex	172	0.47* (0.09, 2.47)	0.55	163	1.06 (0.67, 1.68)	0.72	174	0.98 (0.63, 1.52)	0.98
BCG	172	Undefined	0.97	163	Undefined	0.69	174	Undefined	0.68
WAZ at Birth <-2	172	0.57* (0.07, 4.76)	0.80	163	0.69 (0.36, 1.32)	0.27	174	0.67 (0.36, 1.24)	0.20
WAZ at 6M <-2	171	1.13* (0.21, 5.95)	0.69	161	0.29* (0.12, 0.69)	0.0006	172	0.41 (0.20, 0.83)	0.005
12 Months									
Maternal HIV	167	0.62* (0.12, 3.08)	0.75	172	0.56 (0.26, 1.24)	0.17	177	0.66 (0.33, 1.33)	0.28
Maternal TBI	167	0.45* (0.10, 1.93)	0.49	172	1.49 (0.64, 3.45)	0.20	177	1.24 (0.60, 2.57)	0.40
Household Smoke	167	2.89* (0.68, 12.2)	0.12	171	1.14 (0.51, 2.56)	0.68	176	1.22 (0.58, 2.56)	0.55
Sex	163	1.88* (0.38, 9.43)	0.22	169	1.05 (0.54, 2.03)	0.76	173	1.21 (0.65, 2.23)	0.45
BCG	163	Undefined	1	169	Undefined	0.83	173	Undefined	0.81
WAZ at Birth, <-2	163	1.19* (0.24, 5.86)	0.66	169	1.09 (0.51, 2.36)	0.74	173	0.90 (0.42, 1.91)	0.86
WAZ at 12M <-2	153	1.07* (0.20, 5.61)	0.73	158	1.18 (0.58, 2.40)	0.58	161	1.09 (0.54, 2.18)	0.80

[1]95% confidence interval
* Single cell ≤ 5
TBI: Mtb infection. WAZ: Weight-for-age z score. Those listed as undefined have cells that contain 0

Table 9 shows the results of infants with mothers who were diagnosed with TB disease. There were only three incidences of a positive Luminex test, all occurring at 6 months. At 6 months, the 3 infants that were tested using QFT did not have Luminex results. Infant ID 10046 declined having a blood sample drawn on both occasions.

Table 9. QuantiFERON and Luminex results for infants with mothers with active TB

ID	Cohort	QFT 6M	Luminex 6M	Combined 6M	QFT 12M	Luminex 12M	Combined 12M
10046	B	Declined	Missing	N/A	Declined	Missing	N/A
10076	A	Negative	Missing	Negative	Missing	Missing	N/A
10152	A	Negative	Missing	Negative	Missing	Missing	N/A
10278	C	Negative	Negative	Negative	Negative	Negative	Negative
10666	B	Negative	**Positive**	**Positive**	Negative	Negative	Negative
10893	B	Negative	Negative	Negative	Negative	Negative	Negative
10989	B	Missing	Missing	N/A	Negative	Negative	Negative
11092	B	Negative	**Positive**	**Positive**	Negative	Negative	Negative
11710	A	Negative	Missing	Negative	Missing	Missing	Missing
11720	A	Negative	**Positive**	**Positive**	Negative	Negative	Negative

Discussion

This study was the first to assess longitudinal non-IFNγ cytokine responses to Mtb specific antigens in both HEU and unexposed infants. Our primary findings indicated an overall additional 58 infants with possible TBI beyond the 13 who tested positive by QFT-Plus. There was poor agreement of the assays at both 6 and 12 months.. Mtb infections detected by the Luminex panel was associated with a low categorical weight-for-age z-score. These findings suggest that combining IFNγ with non-IFNγ cytokines could increase sensitivity to detect infant Mtb infection and weight-for-age affects Luminex panel's ability to detect possible Mtb infection in infants at 6-months.

Similar to other pediatric studies, we demonstrated that non-IFNγ cytokines could improve detection of Mtb infection. [22,30] IL10, IL12, IL15, IL17E, IL33, CD40L, and TNFα contributed to ≥ 30% of positive Luminex tests. TNFα has been evaluated multiple times both for its ability to detect Mtb infection and active TB using QFT supernatant. [22,30] Similar to Tebruegge, we found that IL10 was able to detect Mtb infection. An infant's limited cell mediated immunity may lead to false negative IGRA results, making the addition of other non-IFNγ cytokines valuable.

While there were no infants with sustained QFT positives, there were 8 infants with sustained Luminex positives and 17 infants converted from negative to positive by Luminex at 12 months. Nemes found that adolescents, those who converted to QFT positive (IFNγ < 0.2 IU/mL to IFNγ > 0.7 IU/mL) 360 days after a baseline negative were at a 10-fold higher risk of developing TB disease compared to those with sustained QFT positive (IFNγ > 0.7 IU/mL).[31] Additionally, the likelihood of conversion over a 6 month period in adolescents with TBI by QFT was 15.8% and sustained positives was 11.6%.[32] Nemes' finding of conversion were higher compared our findings, which may represent distinct epidemiology of TBI by geographic region and age.

Persistent positives in our cohort were driven by positive concentrations of IL2, CD40L, IL10, and IL33 (each with 2 occurrences) with IFNγ to a lesser extent.

There was an inverse correlation between weight-for-age z-score and a positive Luminex panel at 6 months. Infants categorized as either moderately or severely underweight were less likely to have a positive Luminex result. The impact of undernourishment and low BMI on IGRA and TST tests has mainly focused on adults with TB. [33] It is theorized that IGRA-false-negative results may be caused by inefficient activation of antigen-specific CD4 T-cells.[34] . In small studies, expression of IFNγ and IL12 was reduced in undernourished children compared to

well-nourished and was inverted for IL4 and IL10.[33] Additionally, there was a finding of impaired T-cell activation in undernourished children compared to well-nourished children without illness.[33] This is in line with our Luminex findings.

HIV exposure was found not to be associated with Mtb infection by QFT and Luminex detection. This is in contrast with Marquez et al, who found the odds of TB infection among HEU children was 21.2 times higher than HUU children when adjusting for age, BCG vaccination, and positive maternal TST or QFT. The prevalence of positive QFT at 1-11 months though was 2.3% and 12-23 months 5.3% with TST reporting much higher prevalence. Unlike with our study with reported maternal usage at enrollment of 99%, only 71% of mothers reported taking ART.[20]

Limitations of this study included the sample size and lack of a gold standard to define Mtb infection. The lack of a gold standard for the detecting Mtb infection means we cannot determine the accuracy of either QFT or Luminex. This study highlights the uncertainty when determining the prevalence of Mtb infections in a population. In prior studies, IGRA accuracy for TBI has been correlated with performance among known cases of TB disease, and/or evaluating prognostic value for TB disease progression.

Conclusion

The use of Th9/Th17/Th22 cytokines and chemokines to detect the presence of TB infection in infants in India yielded greater prevalence than with QuantiFERON alone. Luminex and QuantiFERON results had no association and slight association depending on time. A low weight for age z-score was associated with negative Luminex panel and combined result. Future studies should determine if the Luminex panel can predict progression to TB disease.

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