# Immunological aspects of latent tuberculosis infection during pregnancy

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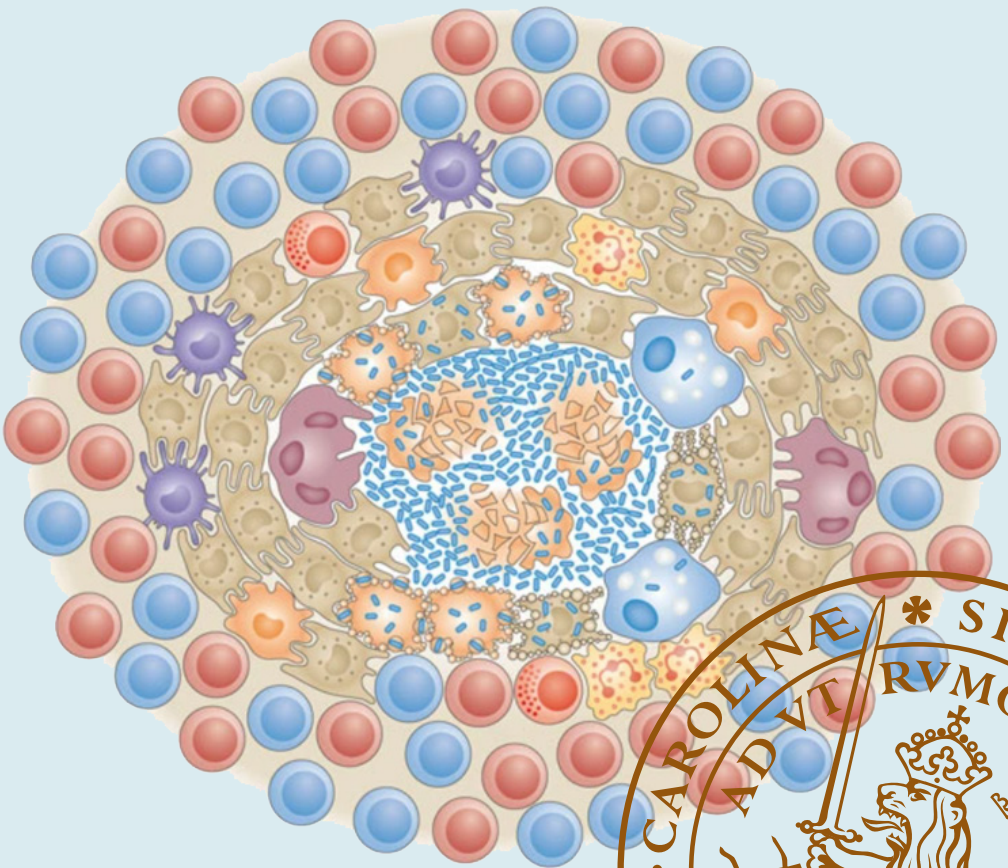

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# Immunological aspects of latent tuberculosis infection during pregnancy

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**Abstract**

Pregnancy-induced immune modulation might lead to reactivation of latent tuberculosis infection (LTBI). This thesis explores aspects of immune-based LTBI diagnostics and how pregnancy affects the immune control of Mycobacterium tuberculosis (Mtb) infection. We studied a prospective cohort of women recruited during pregnancy in Ethiopia. LTBI testing was performed by quantification of interferon-γ (IFN-γ) in Mtb-antigen-stimulated whole blood supernatants using the QuantiFERON-TB Gold Plus (QFT) assay.

In paper I, we found that 277/829 (33%) of pregnant women had LTBI using the conventional IFN-γ cut-off level (0.35 IU/ml). However, borderline results (0.20-0.70 IU/ml) were common, especially in HIV-positive women. In paper II, we characterized Mtb-antigen cytokine responses for LTBI classification in women with borderline IFN-γ results. A combination of MCP-2, IP-10 and IL-1ra classified 42% of women with borderline IFN-γ as having high likelihood of LTBI. In paper III, we studied longitudinal patterns of Mtb-triggered IFN-γ secretion during pregnancy and post-partum. We observed that Mtb-stimulated IFN-γ response was elevated during the 3rd trimester compared to early pregnancy and post-partum. In paper IV, we investigated longitudinal kinetics of Mtb-specific and -non-specific cytokine responses in women with LTBI. We found elevated expression of Mtb-specific IL-2 and IP-10, and reduced TGF-β1, secretion at the 3rd trimester. Non-specific levels of IL-2, IP-10 and MCP-2 were elevated post-partum in women with LTBI. In conclusion, these findings suggest that cytokines other than IFN-γ, could be used as biomarkers to assess LTBI status of pregnant women. The dynamic immune responses in women with LTBI indicate increased exposure to Mtb at later stages of pregnancy, which in turn suggests that LTBI is transiently reactivated during pregnancy.



**Key words** Interferon-γ; immune mediators; latent tuberculosis infection; Mtb-antigen reactivity; pregnancy; QuantiFERON-TB Gold Plus

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Cover photo: Structure and cellular constituents of the tuberculous granuloma. Adapted from Nature Reviews Immunology, Ramakrishnan L. et al., with permission from Springer Nature, Copyright (2012).



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*It always seems impossible until it's done.*
Nelson Mandela

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# Original papers

I. Walles JK, Tesfaye F*, Jansson M, Balcha TT, Winqvist N, Kefeni M, Garoma S, Belachew F, Sturegård E, Björkman P. Performance of QuantiFERON-TB gold plus for detection of latent tuberculosis infection in pregnant women living in a tuberculosis- and HIV-endemic setting. PloS One 2018;13: e0193589.

II. Tesfaye F, Sturegård E, Walles J, Winqvist N, Balcha TT, Karlson S, Mulleta D, Isberg PE, Jansson M, Björkman P. Alternative biomarkers for classification of latent tuberculosis infection status in pregnant women with borderline Quantiferon plus results. Tuberculosis 2020; 124: 101984.

III. Tesfaye F, Walles J, Sturegård E, Winqvist N, Balcha TT, Kefeni M, Jansson M, Björkman P. Longitudinal Mycobacterium tuberculosis-specific interferon gamma responses in Ethiopian HIV-negative women during pregnancy and postpartum. J Clin Microbiol 2021; 59: e00868-21.

IV. Tesfaye F, Sturegård E, Walles J, Bekele B, Bobosha K, Björkman P, Jansson M. Dynamics of pregnancy-shaped immune responses in women with latent tuberculosis infection [manuscript].

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# Related Papers

Walles J, Otero LG, Tesfaye F, Jansson M, Balcha TT, Sturegård E, Winqvist N, Hansson SR, Björkman P. Tuberculosis infection and stillbirth in women in Ethiopia – a prospective cohort study. PLoS One (accepted for publication; 2021)

Walles J, Tesfaye F, Jansson M, Balcha TT, Sturegård E, Kefeni M, Merga G, Hansson SR, Winqvist N, Björkman P. Tuberculosis infection in women of reproductive age – a cross-sectional study at antenatal care clinics in an Ethiopian city. Clin Infect Dis. 2020; 73:203-210.

Olsson O, Tesfaye F, Sökilde R, Mazurek J, Abebe M, Aseffa A, Skogmar S, Balcha TT, Rovira C, Björkman P, Jansson M. Expression of microRNAs is dysregulated by HIV while Mycobacterium tuberculosis drives alterations of small nucleolar RNAs in HIV-positive adults with active tuberculosis. Front Microbiol (accepted for publication; 2021)

# Popular science summary

Tuberculosis (TB) caused by Mycobacterium tuberculosis bacteria is considered to be the single pathogen responsible for the greatest number of deaths due to infectious diseases in the world during the recent decades. In 2019, 10 million people fell ill and about one and a half million people died in the disease. Ethiopia is among the 30 countries most affected by TB worldwide, with 140 cases per 100 000 inhabitants in 2019. Thus, TB remains one of the major public health problems in the country, causing more than 30 000 deaths per year. The WHO's "End TB Strategy" goal is a 90% reduction in TB incidence by 2035 and major efforts are currently being taken to achieve this goal.

A quarter of the world's population is estimated to have latent TB, a condition with small numbers of dormant bacteria in the lungs. Persons with latent TB have no symptoms of TB disease and cannot transmit TB. However, latent TB can be reactivated, mainly as a consequence of weakened immunity, and it is therefore an important cause of TB disease. Without treatment, about 10% of infected persons will develop TB disease within the first two years of infection. For persons whose immunity is weak, especially those with HIV infection, the risk of developing TB disease is 20% higher than healthy persons with normal immunity.

Even though pregnancy is a natural phenomenon that women experience during their reproductive age, pregnancy has also been suggested to confer an increased risk of active TB disease, probably due to reactivation of latent TB infection. The reasons for this are linked to the physiological immune changes that occur during pregnancy, to prevent rejection of the fetus. However, these pregnancy-related immune modifications could also make the pregnant woman more susceptible to infections. It has been estimated that globally more than half a million maternal deaths are due to TB. Women suffering from TB disease during pregnancy are faced with a number of risks, such as spontaneous abortion, giving birth before term and having a baby with a low birth weight – even worse - TB is even associated with death of both mother and child. The risk of TB is especially high in HIV-positive pregnant women, meaning that the combination of HIV and pregnancy further increase the risk for reactivation of latent TB.

A major challenge for clinicians is to accurately diagnose latent TB. None of the currently available latent TB diagnostic techniques, such as tuberculin skin test (TST) and interferon-gamma release assays (IGRAs), can directly detect the

dormant bacteria. Instead, these tests measure immune reactivities, reflecting contact with TB bacteria. IGRA is a TB-specific test, which is based on the stimulation of blood with TB bacteria proteins (antigen), and immune response to the TB antigen is detected by measuring a signaling molecule (cytokine) called interferon-gamma (IFN-γ). However, performance of IGRA can be affected by immune changes, for example those in people with HIV, but perhaps also those that occur during pregnancy. Thus, measuring a single cytokine, such as IFN-γ, might not be adequate for use in persons with immune suppression, and accordingly other alternative diagnostic methods are needed. Moreover, both the process of how latent TB is reactivated during pregnancy, and how immune cells act to control latent TB infection in pregnant women, are poorly understood. Thus, the aim of this thesis has been to explore different aspects of immune-based latent TB diagnostics in pregnant women and to investigate the impact of pregnancy on immune responses in women with latent TB. For this purpose, we designed a cohort study and collected blood samples from 2093 Ethiopian pregnant women at different timepoints during pregnancy and after delivery. Latent TB testing was performed by measuring IFN-γ cytokine in TB-antigen stimulated blood samples.

In the first study, the performance of a new version of IGRA test, called QuantiFERON-TB Gold Plus assay (including two TB antigens; TB1 and TB2), was evaluated for detection of latent TB in pregnant women. Eight hundred twenty-nine pregnant women (49 HIV-positive) were included; of these 277 (33%) had latent TB using the recommended IFN-γ cut-off level (0.35 IU/ml). The newly added TB2 antigen detected TB infection similarly to the original TB1 antigen in both HIV-positive and negative women. We also observed that many women had test results that were near the cut-off level. Such results are considered to be uncertain, and are commonly labeled as borderline results (defined as 0.20-0-70 IU/ml). Having results within this borderline range was more common in HIV-positive women.

In the second study we selected 96 study participants from the same cohort, to explore improved diagnosis of TB in women with QuantiFERON-TB Gold Plus borderline results. For this purpose, we measured eight cytokines, other than IFN-γ, in blood samples stimulated with TB1 and TB2 antigens. Here, three of the cytokines, interferon-gamma inducible protein 10 (IP-10), monocyte chemoattractant protein-2 (MCP-2) and interleukin-1 receptor antagonist (IL-1ra) could distinguish the borderline group from the TB-negative women. A combination of these markers added robustness to the test and classified 42% (13) of women with borderline IFN-γ to have high likelihood of TB infection.

In the third study we investigated the impact of pregnancy on TB-antigen specific IFN-γ secretion in HIV-negative women followed longitudinally during pregnancy and after delivery. We found that TB antigen specific IFN-γ levels were increased late in pregnancy, as compared to at earlier time-points and after delivery, in women with latent TB infection, whereas the mitogen-stimulated IFN-γ response (which

reflects the overall ability of immune cells to produce IFN-γ) was decreased during the late pregnancy phase.

In the fourth study, we investigated follow-up samples from the same women, as in study three, to measure additional cytokines released by different immune cells after TB-antigen stimulation. We found that proinflammatory immune responses, such as interleukin-2 (IL-2) and IP-10 cytokine levels, were elevated, while the expression of the regulatory cytokine transforming growth factor beta 1 (TGF-β1) decreased, in late phase pregnancy. Moreover, non-specific levels of the proinflammatory cytokines were elevated after delivery in women with latent TB.

These results highlight examples of biomarkers that could be used to improve diagnosis of latent TB during pregnancy, particularly in women with HIV. The increased TB-antigen specific cytokine release during late pregnancy indicate that the immune cells of these women are exposed to TB bacteria, which indicates latent TB reactivation. Of note, none of these women developed active TB disease during follow-up, suggesting that the TB reactivation is transient and does not lead to TB disease in these HIV-negative women. Taken together, this thesis adds knowledge to the field of immune responses in pregnant women with latent TB, which could help to improve diagnostics and understanding of TB reactivation during pregnancy.

# Populärvetenskaplig sammanfattning

Tuberkulos (TB), orsakad av Mycobacterium tuberculosis-bakterier, är en av de mest dödliga infektionssjukdomarna i världen. Under 2019 insjuknade 10 miljoner människor och cirka en och en halv miljon människor dog i sjukdomen. Etiopien är bland de 30 mest TB-drabbade länderna i världen, med 140 TB-fall per 100 000 invånare 2019. Således är TB fortfarande ett av de största folkhälsoproblemen i Etiopien, och orsakar mer än 30000 dödsfall per år. WHO:s "End TB Strategy", har som målsättning att TB-fallen per år ska minska med 90% fram till 2035, och stora ansträngningar görs för närvarande för att uppnå detta mål.

En fjärdedel av världens befolkning uppskattas bära på latent tuberkulos, en vilande form av infektionen. Personer med latent TB uppvisar inga kliniska symtom på aktiv TB-sjukdom, men är en viktig källa till smittspridning vid TB-reaktivering, främst vid försvagad immunitet. Utan behandling kommer cirka 10% av de smittade personerna att utveckla tuberkulos under de första två åren efter infektion. För personer vars immunitet är svag, särskilt hos de med HIV-infektion, är risken att utveckla TB-sjukdom 20% högre än hos friska personer med normal immunitet. Anledningen till detta är att HIV leder till immunbrist.

Även om graviditet är en naturlig företeelse som kvinnor upplever under sin reproduktiva ålder, har det visat sig att graviditet också är en riskfaktor för latent TB-reaktivering. Orsakerna till detta är kopplade till fysiologiska förändringar i immunförsvaret, som sker under graviditeten för att förhindra avstötning av fostret. Men dessa graviditetsrelaterade förändringar i immunförsvaret kan också göra den gravida kvinnan mer mottaglig för infektioner. Det har uppskattats, globalt, att mer än en halv miljon mödrar dör på grund av tuberkulos, främst under fertil ålder. Kvinnor som lider av tuberkulos under graviditeten utstår ett antal risker, såsom spontan abort, att föda barn för tidigt och att få ett barn med låg födelsevikt – ännu värre – tuberkulos är även förknippat med dödsfall för både mor och barn. Dessutom är risken för tuberkulos förhöjd hos HIV-positiva gravida kvinnor, vilket innebär att kombinationen av HIV och graviditet ytterligare ökar risken för reaktivering av latent tuberkulos.

En stor klinisk utmaning är att korrekt diagnostisera latent tuberkulos. Detta, eftersom de tillgängliga diagnostiska teknikerna för latent tuberkulos, såsom tuberkulin-hudtest (TST) och interferon-gamma-frisättningsanalyser (IGRA), är immunbaserade tester, vilket innebär att de inte direkt upptäcker bakterier, snarare

upptäcker immunreaktivitet, vilket reflekterar tidigare kontakt med bakterierna. IGRA är ett TB-specifikt test, som är baserat på stimulering av blod med TB-bakterieprotein (antigen), och att immunsvaret mot TB-antigen detekteras genom att mäta en signalmolekyl (cytokin) som kallas interferon-gamma (IFN-g). Prestandan hos IGRA kan dock påverkas av immunförändringarna, till exempel de som inträffar under graviditet. Att mäta en enskild cytokin, såsom IFN-g, kanske inte är tillräcklig för användning hos personer där immunsvaret är påverkat, och följaktligen behövs andra alternativa diagnostiska metoder. Dessutom är förståelse av hur gravida kvinnors immunceller svarar för att kontrollera latent TB, och hur graviditet ökar risken för latent TB-reaktivering hos gravida kvinnor, inte klarlagd. Därför har syftet med denna avhandling varit att utforska olika aspekter av immunbaserad latent TB-diagnostik hos gravida kvinnor och att studera graviditetens inverkan på immunsvar hos kvinnor med latent TB. För detta ändamål utformade vi en kohortstudie och samlade in blodprover från 2093 etiopiska gravida kvinnor under graviditeten och efter förlossning. Latent TB-testning utfördes genom att mäta IFN-g-cytokin i TB-antigenstimulerade blodprover.

I den första studien utvärderades en ny version av IGRA-testet som heter QuantiFERON-TB Gold Plus, inkluderande två TB-antigener (TB1 och TB2), för upptäckt av latent TB hos gravida kvinnor. Åttahundratjugonio gravida kvinnor (49 HIV-positiva) inkluderades; av dessa hade 277 (33%) latent TB utifrån rekommenderad IFN-g-gränsvärde. Det nyligen adderade TB-antigenet (TB2) upptäckte TB-infektion på liknande sätt som den ursprungliga antigenet (TB1) hos både HIV-positiva och negativa gravida kvinnor. Vi observerade också att många kvinnor hade resultat inom det diagnostiska osäkerhetsintervallet som kallas ”borderline”-intervallet. Detta var vanligare hos HIV-positiva kvinnor.

I den andra studien valde vi ut 96 studiedeltagare från samma kohort, för att utforska förbättrad diagnos av tuberkulos hos kvinnor med QuantiFERON-TB Gold Plus-resultat inom ”borderline”-intervallet. För detta ändamål mätte vi åtta cytokiner, istället för enbart IFN-γ, i blodprover stimulerade med TB1- och TB2-antigener. Här kunde tre av cytokinerna, interferon-gamma-inducerbart protein 10 (IP-10), monocyt kemoattraktant protein-2 (MCP-2) och interleukin-1 receptorantagonist (IL-1ra), skilja borderline-gruppen från gruppen av TB-negativa kvinnor. En kombination av dessa markörer gjorde testet ytterligare robust och klassificerade 42 % (13) av kvinnorna med borderline IFN-γ att med hög sannolikhet bära TB.

I den tredje studien undersökte vi graviditetens inverkan på TB-antigenspecifik IFN-γ-sekretion hos HIV-negativa kvinnor som följdes longitudinellt under graviditeten och efter förlossningen. Vi fann att TB-antigenspecifika IFN-γ-nivåer ökade sent under graviditeten, jämfört med en tidigare tidpunkt och efter förlossningen, hos kvinnor med latent TB. Medan det mitogenstimulerade IFN-γ-svaret (vilket återspeglar den övergripande förmågan hos immunceller att producera IFN-γ) minskade under den sena graviditetsfasen.

I den fjärde studien undersökte vi prover från samma kvinnor, som i studie tre, för att mäta ytterligare cytokiner som frisätts av olika immunceller efter TB-antigenstimulering. Vi fann att proinflammatoriska immunsvar, såsom interleukin-2 (IL-2) och IP-10 cytokin-nivåer, var förhöjda, medan uttrycket av det reglerande cytokinet, transformerande tillväxtfaktor beta 1 (TGF-β1) minskade, i den senare graviditetsfasen. Dessutom var ospecifika nivåer av proinflammatoriska cytokiner förhöjda efter förlossning hos kvinnor med latent TB.

Dessa resultat belyser exempel på biomarkörer som skulle kunna användas för att identifiera potentiell TB-risk, och erbjuda förbättrad diagnos av latent TB, under graviditet, särskilt hos kvinnor med HIV. Den förhöjda TB-antigenspecifika cytokinfrisättningen under senare delen av graviditeten tyder på att immunceller hos dessa kvinnor exponerats för TB-bakterier, vilket indikerar latent TB-reaktivering. Det kan noteras att ingen av dessa kvinnor utvecklade aktiv TB-sjukdom under uppföljningen, vilket tyder på att TB-reaktiveringen hos HIV-negativa kvinnor är övergående och utan kliniska symtom. Sammantaget tillför denna avhandling kunskap om immunsvar hos gravida kvinnor med latent tuberkulos, vilket kan bidra till att förbättra diagnostik och förståelse runt reaktivering av tuberkulos.

# Abbreviations

| | |
|---|---|
| ANC | antenatal care |
| APCs | Antigen presenting cells |
| BCG | Bacillus Calmette-Guerin |
| CD4 | cluster of differentiation 4 |
| CD8 | cluster of differentiation 8 |
| CFP-10 | culture filtrate protein |
| DCs | dendritic cells |
| DosR | dormancy of survival regulon |
| ELISA | enzyme-linked immune-assay |
| ELISPOT | enzyme-linked immunospot |
| ESAT-6 | early secreted antigenic target-6 |
| GM-CSF | granulocyte-macrophage-colony-stimulating factor |
| HIV | human immunodeficiency virus |
| IDO | indoleamine 2,3-dioxygenase |
| IFN-γ | interferon-gamma |
| IGRAs | interferon-γ release assays |
| IL | interleukin |
| IL-1ra | interleukin-1 receptor antagonist |
| IP-10 | interferon-γ-induced protein 10 |
| JAK/STAT | Janus kinase/signal transducers and activators of transcription |
| KMC | k-mean clustering |
| KNN | k-nearest neighbor |
| LTBI | latent TB infection |
| M1 | classical macrophages |
| M2 | alternative macrophages |
| MCP | monocyte chemoattractant protein |
| MHC | major histocompatibility complex |
| MIG-γ | monocyte induced interferon-gamma |

| | |
|---|---|
| MIP-1α | macrophage inflammatory protein-1 alpha |
| MIP-1β | macrophage inflammatory protein-1 beta |
| Mtb | Mycobacterium tuberculosis |
| MTBC | Mycobacterium tuberculosis complex |
| MTCT | mother-to-child transmission |
| MUAC | upper arm circumference |
| NKs | natural killer cells |
| PCR | polymerase chain reaction |
| PDGF- BB | platelet-derived growth factor-BB |
| PMTCT | prevention of mother-to-child transmission of HIV |
| PPD | purified protein derivative |
| QFT | QuantiFERON |
| QFT-GIT | QuantiFERON-TB Gold In-Tube |
| QFT-Plus | QuantiFERON-TB Gold Plus |
| RANTES | regulated on activation, normal T cell expressed and secreted |
| RD | region of difference |
| ROC | receiver operating characteristic |
| rRNA | ribosome ribonucleic acid |
| TB | Tuberculosis |
| TGF-β1 | transforming growth factor-β-1 |
| Th | T helper |
| TNFα | tumor-necrosis factor alpha |
| TPT | tuberculosis preventive therapy |
| Tregs | regulatory T cells |
| TST | tuberculin skin test |
| VEGF | vascular endothelial growth factor |
| WHO | World Health Organization |
| VL | viral load |

# INTRODUCTION

## Epidemiology of Tuberculosis

Tuberculosis (TB), one of the oldest human diseases known, remains a global health emergency in the 21st century (1). According to the Global TB report 2020, 10 million new active TB cases and 1.4 million deaths occurred in 2019, making TB one of the greatest causes of death from a single infectious agent (2). TB affects all age groups, including 8.8 million adults and 1.2 million children (aged <15 years), as reported in 2019. Of these, 0.8 million TB cases and 208 000 deaths were reported among people living with HIV (2). In total, 30 high TB burden countries accounted for 87% of all estimated new cases of TB worldwide (Figure 1). Most of the cases, 44%, were reported in South-East Asia, followed by 25 % in Africa, where the highest TB incidence is observed, due to high HIV prevalence, and various socio-economic problems (2).

Since 1993, when the World Health Organization (WHO) declared TB as a global emergency (1). several strategies for prevention and control of TB have been launched, such as the STOP TB strategy in 2000 and the End TB strategy in 2015, particularly in endemic settings. One of the goals of the End TB Strategy is a 90% reduction in TB incidence by 2035. However, the global annual TB incidence decline rate is hitherto about 2%, and the 2020 End TB Strategy milestone of 20% reduction was not reached (achieved only 9%) (2).

In order to reach this goal, multisectoral TB diagnosis, treatment and prevention services need to be improved to address the broader determinants that influence TB epidemics and its socioeconomic impact. Even though the direct observed therapy endorsed by WHO improves adherence to anti-TB treatment, there are major problems associated with poor treatment outcomes, especially in high TB burden low-income countries. Examples of such factors are poor socioeconomic conditions and lack of knowledge about TB (3). As a result of inadequate TB treatment programs, multi- and extensively drug resistant TB strains have emerged. In 2019, an estimated 3.3% of new TB cases and 17.7% of previously treated cases had multidrug or rifampicin-resistant TB, reflecting a 10% increase from 2018 worldwide (2). Recently, the COVID-19 pandemic has also had a negative impact on access to TB diagnosis and treatment (4).

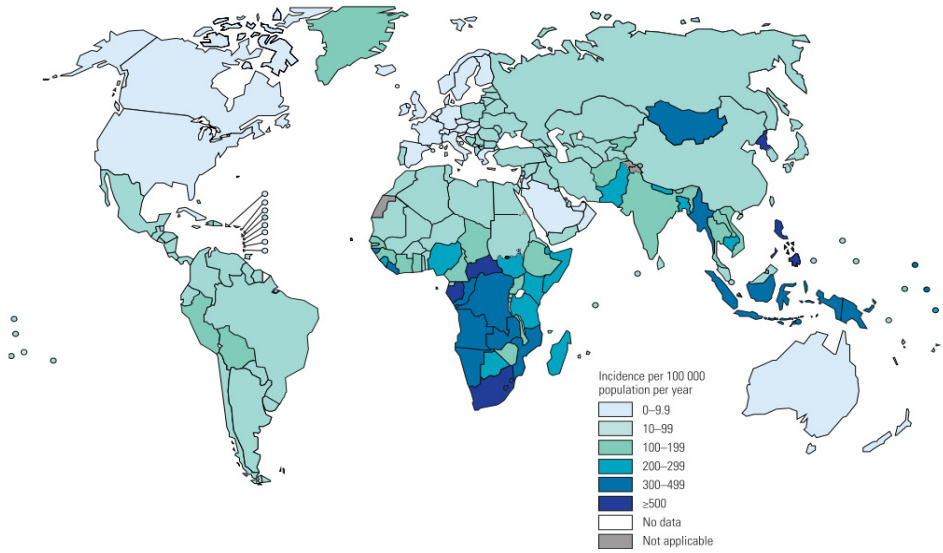


**Figure 1.** Estimated TB incidence rates, 2019. Adapted from WHO global tuberculosis report 2020 (2)

Ethiopia, the second most populous country in Africa with a population of more than 110 million, is one of the 30 high TB incidence countries in the world, with an incidence of 140 cases per 100 000 inhabitants in 2019 (2). TB remains one of the major public health problems in the country, resulting in more than 30 000 deaths per year. Although Ethiopia reached one of the milestones of the End TB strategy, with TB treatment success rate of 88% (17), an estimated one-third of active TB cases are not diagnosed, indicating high TB exposure and transmission in the community (2). HIV coinfection is a major contributing factor for developing active TB in Ethiopia, where an estimated 613, 000 adults were living with HIV (5). In order to reach effective and durable TB control, specific and sensitive point-of-care diagnostic methods, integrated TB-HIV care and TB case detection followed by provision of treatment are needed.

Approximately 25% of the world population have latent TB infection (LTBI), with 5% to 15% lifetime risk of developing active TB, mainly within the first 2 to 5 years following initial infection (6,7). Although only a minority of people with LTBI develop active TB, latently infected persons constitute a reservoir of TB infection in the community. Reactivation of LTBI is usually due to disruption of host immune control of dormant live bacilli. Major risks for LTBI reactivation are various immunosuppressive conditions, such as HIV co-infection, malnutrition, immunosuppressive therapy and a range of chronic diseases (including renal failure, diabetes mellitus, silicosis and liver cirrhosis).

Identification of individuals at risk (for example, people living with HIV and household contacts of people with TB) for progression of LTBI to active disease and providing tuberculosis preventive therapy (TPT) is recommended by WHO for the management of LTBI. Testing for LTBI for provision of TPT is not recommended in most low-income countries, including Ethiopia. However, TPT based on isoniazid for six-months is recommended for HIV-positive individuals and household contacts of cases with contagious TB.

# Tuberculosis in women

Overall, the prevalence of active TB is markedly higher among men than women globally, with 56% of notified cases in men (2). The reasons for this difference in sex distribution are not fully understood. It has been suggested that socioeconomic and cultural factors lead to worse access to health care among women (8). On the other hand, biological factors such as sexual hormones, and sex-related genetic characteristics may contribute to reduced susceptibility to TB in women (8). However, an increase in the proportion of women notified with active TB has been observed during the last decades, especially in sub-Saharan Africa (9). This phenomenon may partly be explained by female predominance of HIV infection in this world region (9).

In addition, TB is considered to be one of the leading non-obstetric causes of death in women during reproductive age (15–45 years), with an estimated 3.2 million new cases and half-million deaths annually in this category, and with the highest case burden in low-income countries (2,10–12). Active TB has also been associated with increased incidence of various adverse pregnancy outcomes, including preterm delivery, low birth weight, perinatal and maternal death (13).

Several register-based studies performed in high-income countries have reported an increased incidence of active TB in connection to pregnancy (15,20). There is limited data on this relationship from low-income countries. However, women with HIV have high risk of active TB during pregnancy, including those receiving ART (16,18,28). Pregnant women are more susceptible to several infectious diseases, such as influenza virus, hepatitis E virus and malaria (19). It is possible that physiological pregnancy-related immune modifications also affect the immune control of Mtb replication and lead to LTBI reactivation in latently infected women. The incidence of active TB in relation to pregnancy has been reported to be highest post-partum (20,21). It is possible that pregnancy can mask symptoms of TB which results delay in diagnosis and leads to increased case findings postpartum (typically 3-6 months after delivery). In women living in low-endemic areas, most cases of active TB during pregnancy are probably due to reactivation of LTBI. In areas with ongoing TB transmission, newly acquired TB infection could also be responsible.

The relative proportions of reactivated LTBI and new TB infection in this context are usually not possible to determine.

## Pregnancy, tuberculosis and HIV co-infection

Both TB and HIV infection have harmful reciprocal consequences for maternal and child health (22). Apart from high risk of active TB in women with HIV during pregnancy (16), the risk of mother-to-child transmission (MTCT) of HIV as well as TB is increased in co-infected women, particularly in the absence of antiretroviral treatment (18,23,24).

HIV infection leads to depletion of CD4+ T cells (which play an important role for containment of Mtb infection (25). Pregnancy changes the immunological balance towards anti-inflammatory responses in order to prevent reject of the fetus (26), which also could lead to reduced immune control of LTBI.

TB/HIV co-infection is estimated to be responsible for considerable maternal and infant morbidity and mortality, especially in Asian and African countries (12,27). The risk of developing active TB has been reported to be increased in HIV infected pregnant women as compared to non-pregnant HIV-positive, with a two-fold increased incidence in HIV seropositive compared with HIV negative pregnant women in South Africa (16). In another study, the risk of LTBI progression to active TB was reported to be >4 times higher in HIV positive pregnant women (28). Furthermore, among severely immunosuppressed pregnant women with HIV, positive QFT results were associated with postpartum active TB and increased maternal and infant mortality (28). The effect of LTBI treatment among HIV-positive on the incidence of active TB is uncertain (29). Currently, WHO recommends isoniazid preventive therapy for all people with HIV regardless of LTBI status after excluding active TB. However, the latter issue may be especially challenging in HIV-positive pregnant women, and it is possible that active TB may not be recognized (which in turn could result in isoniazid monotherapy in such cases).

## *Mycobacterium tuberculosis*: the bacterium

TB is caused by the Mycobacterium tuberculosis complex (MTBC) species that comprises M. tuberculosis, M. africanum, M. bovis, M. canettii, M. caprae, M. pinnipedii and others which are 99% genetically homogeneous and have identical 16S rRNA sequences (30). Among others, Mycobacterium tuberculosis (Mtb) is the predominant and well-known cause of TB in humans. It was first discovered by Robert Koch in 1882 as the etiological agent of TB disease (31).

Mtb is a slow growing facultative intracellular aerobic rod-shaped bacterium.

Similar to other mycobacteria, Mtb has a unique cell wall, which resists acid decoloration due to high-molecular-weight glycolipids, such as lipomannan, lipoarabinomannan, mycolic acids, peptidoglycan and other lipids. The characteristic features of the Mtb cell wall are accountable for acid-fastness and confers resistance to harmful compounds which contribute to its virulence (31,32). Furthermore, these cell wall components have immunomodulatory properties that interfere with the host immune response and contribute to its survival within the phagosomes.

# Transmission and pathogenesis

Mtb is transmitted from person-to-person through inhalation of small (<5 μm) aerosolized droplet nuclei containing live bacteria which are expelled in exhaled air from individuals with pulmonary TB (33). Following inhalation, Mtb is rapidly taken up and phagocytized by alveolar macrophages that manage to kill and eliminate the bacteria in most exposed persons through innate immune responses (34). However, in some individuals this first-line defense is not complete, leading to establishment of Mtb infection in pulmonary foci (35). During this early stage of infection, bacilli spread to regional lymph nodes, with ensuing adaptive immune responses that occur 6-8 weeks after infection (35). In the majority of infected persons, Mtb infection is contained by these immune responses in granulomas in the lung parenchyma, a condition referred to as LTBI.

Since the presence of viable bacilli cannot be determined, LTBI is defined as cellular immune response to stimulation by Mtb antigens, with exclusion of active TB (36). The term LTBI was first introduced by Clemens von Pirquet, an Austrian pediatrician who coined the terms allergy and allergen (37). Following Koch's discovery of the tubercle bacilli in 1890, von Pirquet noted skin reactions to intracutaneously injected tuberculin (an extract from Mtb cultures) in a high proportion of children without clinical active TB. Furthermore, he observed that the rates of positive skin reactions (defined as >5 mm) increased with age (from ~5% in infants to ~70% at the age of 10 years), reflecting high exposure to contagious TB in Europe at the time of these studies.

Thus, exposure to Mtb can lead to different outcomes; bacterial clearance through host innate immune responses, establishment of LTBI through adaptive immune responses, or failure of initial immune containment, leading to direct development of active TB (Figure 2). The outcome of TB infection mainly depends on the individual immune status, but can also be influenced by different degrees of virulence among Mtb strains (38,39). In total, approximately 5-10% of persons with LTBI develop active TB disease, which occurs during the first two years after

exposure in the majority of cases. However, in some persons with LTBI, reactivation can occur several years after exposure, usually as a result of immune suppression. The natural course of LTBI remains incompletely understood; whereas viable bacilli can persist for decades in some individuals, it is likely that spontaneous resolution of LTBI occurs in many people over time (40).

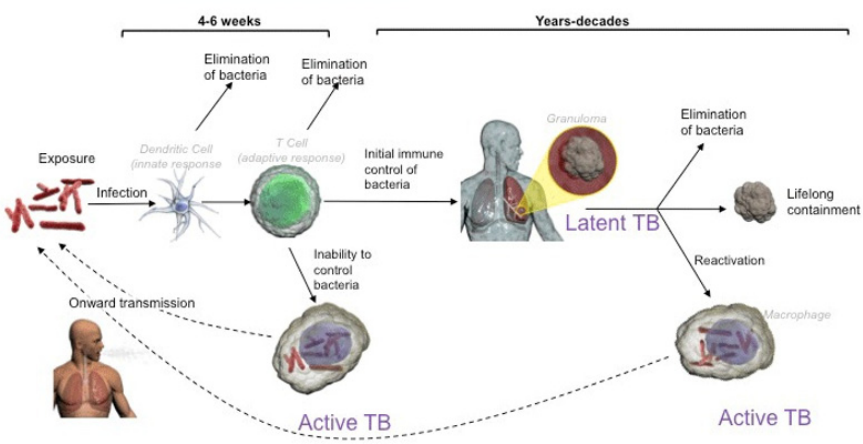


**Figure 2.** Natural history of TB infection (41). Adapted from online source: infectionlandscapes.org/2013/04/tuberculosis.html.

# Spectrum of TB infection

TB infection has traditionally been classified into two entities: latent TB infection and active TB disease. However, this classification is likely to be over-simplified, and TB infection may instead be considered as a continuous spectrum of host-pathogen interactions depending on the balance between bacterial activity and host immunological responses. This spectrum can range from the presence of small numbers of dormant bacilli contained in pulmonary granulomas to active bacterial replication and clinical disease (42). The gradual and dynamic changes that can occur are reflected in further terms that are used, such as incipient TB infection, sub-clinical active TB, and symptomatic TB disease (Figure 3) (43).

LTBI reactivation can be seen as the process of weakening Mtb-specific immune control, which can result in development of active TB disease as an end result. Various risk factors for the reactivation of LTBI and development of TB disease have been identified; among these, untreated HIV infection is currently considered to confer the greatest risk of reactivation (44).

Incipient TB refers to an asymptomatic phase of infection, with higher Mtb replicative activity in granulomas, but without detectable bacilli in routine clinical samples. If this heightened bacterial activity is not arrested by immune responses, infection can progress to subclinical TB. This is considered as an asymptomatic stage of TB infection in which TB bacilli can be detected in clinical samples (43,45). Asymptomatic TB disease has been reported to be relatively common, and persons with subclinical pulmonary TB may also be contagious (43,45). The gradual progression from LTBI to active TB disease may be rapid or slow, and can also be cyclic in nature, with episodes of elevated bacterial activity which may be partly controlled by Mtb-triggered immune responses (43) and as depicted in Figure 3.

Persons with active TB disease frequently have systemic symptoms, such as fever, night sweating, fatigue and weight loss. In addition, organ-specific symptoms and signs are often present, and depend on the organs involved. Histopathologically, active TB is often characterized by granulomas with central necrosis (46). These granulomas can expand and invade tissue structures, which can lead to pulmonary cavitation and bronchial spread of infection. However, in persons with cellular immunosuppression (such as people with advanced HIV infection), this granulomatous inflammatory reaction is frequently dampened, and the histopathological features resemble non-specific inflammatory conditions. Apart from bronchial spread of infection within the lungs, TB bacilli can also disseminate by hematogeneous or lymphatic routes to other parts of the body, which can lead to development of different types of extrapulmonary and disseminated active TB (47).

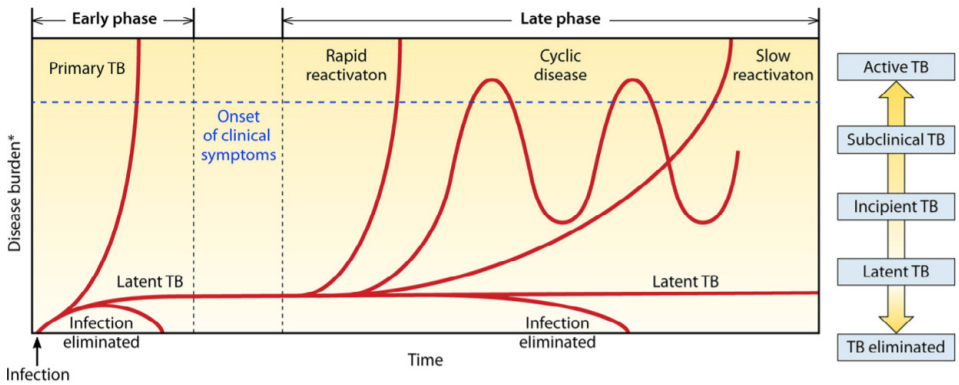


**Figure 3.** The spectrum of TB infection. From Clinical Microbiology Reviews, Drain P, (43),Copyright (2018). Reprinted with permission from American Society for Microbiology.

# Immune response to Mtb

## Innate immune response

The early protective immunity against Mtb is induced by innate immune cells such as macrophages, dendritic cells (DCs), natural killer cells (NKs) and neutrophils (48).

Alveolar macrophages mediate the first line of defense against Mtb infection, when they encounter and ingest the bacilli in the lung. Macrophages can eliminate Mtb through production of reactive oxygen and nitrogen species, and phagosome-lysosome fusion, where the engulfed pathogen is degraded (34). On the other hand, Mtb manipulates the infected macrophage by blocking phagosome maturation and fusion with the lysosome to promote its intracellular survival, and use macrophages as a niche for replication and reservoir, which results in persistent antigen accumulation within the granulomas during chronic infection (49,50). The activation of the innate immune responses is through pathogen pattern recognition receptors, such as Toll-like receptors, which involves ligand recognition, for example Mtb components such as lipoarabinomannan, and intracellular signal transduction up on Mtb infection (51). These host pathogen interactions trigger intracellular signaling cascades, including the activation of the NF-κB transcription factor. This in turn results in the induction and regulation of a large set of genes promoting innate immunity, inflammation, proliferation, and apoptosis control.

This activation also leads to secretion of pro-inflammatory cytokines such as tumor-necrosis factor alpha (TNFα) and interferon gamma (IFN-γ), as well as interleukins (IL-1 β, IL-6, IL-12 and IL-18), chemokines, antimicrobial molecules and triggers a cascade of cellular functions including phagocytosis, inflammasome activation, autophagy, apoptosis and control or elimination of the invading pathogens (48,52). For example, IL-12 and IL-18 promote IFN-γ production of macrophages in response to Mtb, which increases phagocytosis (53,54). As a result, IFN-signaling in these cells increase activation and promotion of host defense to control bacterial growth. The influx of neutrophils into the lungs is also involved in the process of killing Mtb early during infection, but are actively manipulated by the bacteria through necrosis (55). Overall, influx of leukocytes, including activated DCs and macrophages, as well as fibroblasts into the site of infection results in granuloma formation, crucial to the containment of Mtb (56).

NK cells facilitate directed lysis of Mtb-infected cells through the production of perforin, granulysin, and granzymes (57). Besides, these cytolytic activities, NK cells also facilitate IFN-γ production in either antigen-independent manner in response to the costimulatory cytokines or after direct exposure to Mtb components, such as oligodeoxynucleotides (57).

A critical role of activated classical macrophages (M1) is also to present Mtb antigens to T cells through major histocompatibility complex (MHC) and expression of costimulatory molecules, and thereby facilitate control of the bacteria (58). DCs also play an important role in the phagocytic process and antigen presentation to T cells by migrating to lymph nodes (59). Thus, DCs are especially critical at the interface of the innate and adaptive immune responses.

## Adaptive immune response

The immune control of Mtb relies on effective interactions between antigen-presenting cells (APCs) and different T cell subsets (Figure 4), in the local lung-draining lymph node, where circulating naïve T cells recognize Mtb antigens in the context of MHC on the APCs surface (60). Activated T cells migrate to site of infection and contribute to control of the bacterial replication by secreting proinflammatory cytokines IFN-γ, IL-2 and TNF-α, which induce anti-mycobacterial activity in macrophages. The containment of Mtb, comprising both CD4+ and CD8+ T cells (60).

*CD4 T cells*

CD4 T cells recognize Mtb peptide antigens presented on MHC-class II molecules. Upon activation, naïve CD4 T cells differentiate into major T cell subsets, including T helper (Th)1, Th2, Th17, and regulatory T cells (Tregs) depending on expression of specific transcription factors and specific cytokine secretions (61,62). The differentiation of these cells is from precursor Th0 cells and is under the control of the cytokine milieu, such as IL-12 or IFN-γ, IL-4, and transforming growth factor beta (TGF-β), respectively. The outcome of Mtb infection is determined by the type of CD4+ T cells subset, resulting either in proinflammatory, anti-inflammatory or regulatory responses.

Immune control of Mtb is predominantly facilitated by CD4+ Th1 cells. Th1 cells secret cytokines, such as IFN-γ, IL-2 and TNF-α, which results in activation of macrophages and CD8+ T cells, and the control of Mtb infection. IFN-γ, which is a typical pro-inflammatory cytokine secreted by activated CD4+ Th1cells, as well as by other lymphocytes, has an important role in control of Mtb (60).

Instead, the dominance of CD4+ Th2 cells has been described to be linked to TB disease progression by the secretion of cytokines such as, IL-4, IL-5 and IL-10 (63). These Th2 cytokines may inhibit Th1 cell immune responses, which in turn results in loss of Mtb immune control and clinical TB progression (63–65). This suggests that the Th1-Th2 cell responses against Mtb need to be balanced for effective control of the infection (66). Furthermore, IL-4 has been shown to modulate the macrophage phenotype and induce differentiation of the alternative macrophages (M2), that display reduced microbicidal activities (58,67). Importantly, IL-4 promotes Th2

differentiation which induces humoral immunity through the production of antibodies to protect against extracellular pathogens and parasites (68). Humoral immunity has also been shown to be involved in the control of Mtb infection (69).

The CD4+ Th17 cells, secreting IL-17, mainly contributes to protection and clearance of extracellular pathogens, but are also involved in Mtb responses and early bacterial control (70), although the specific role to Mtb control remains uncertain. IL-17 mediates induction of other pro-inflammatory cytokines and chemokines to promote inflammation and neutrophil recruitment to the site of infection which leads to granuloma formation (71,72). However, excess expression of IL-17 is associated with TB pathology and autoimmune diseases (73).

Tregs constitute a functionally distinct subset of CD4+ T cells, and are involved in self-tolerance and suppression of certain cell-mediated immunity, but are also important in minimizing excessive inflammation and host tissues damage (74,75). During Mtb infection, Tregs contribute to the suppression of the Th1 immune responses by inhibiting the effector activity of CD4+ and CD8+ T-cells (76–78). It has also been shown that rising levels of Mtb antigen result in the expansion and accumulation of Tregs at the site of infection, and thereby downregulate antigen-specific T cells and impeding macrophage activation (79–81). However, the mechanisms behind Treg mediated immune suppression are not fully understood, but involves secretion of inhibitory cytokines such as TGF-β and IL-10 (82). Elevated frequency of Tregs have been observed in the peripheral blood of TB patients, compared with healthy controls, and subsequently decline during TB treatment (83,84).

*CD8 T cells*

CD8+ T cells are also involved in the immune defense against Mtb (85,86). By and large, CD8+ T cells recognize Mtb antigens that have been processed in the cytosol compartment and loaded onto MHC-class I molecules. Upon specific antigen recognition, stimulated CD8+ T cells can produce cytotoxic and antimicrobial molecules such as granzymes, granulysin and perforin to eliminate infected cells or even direct killing of the Mtb bacilli (87,88). Furthermore, CD8+ T cells secrete cytokines such as IFN-γ, TNF-α, and IL-2, which increase activation of immune cells and induce apoptosis of infected target cells (89).

Studies performed in mice and non-human primate models have demonstrated the role of Mtb-specific CD8+T cells in the control of Mtb infection (90,91). In humans, higher percentage of Mtb-specific CD8+ T cell responses have been reported in TB patients, which were associated with the presence of higher Mtb antigen load and granulomas as compared with those in LTBI and health control subjects (92,93). Moreover, difference in phenotypic and functional characteristics of Mtb-specific CD8+ T cells has also been observed in active TB vs LTBI; thus Mtb-specific CD8+ T-cell responses vary with the activity and the clinical presentation of Mtb infection (92).

*Cytokines and Chemokines*

Host immune responses to infectious disease include signaling mediated by a group of proteins collectively known as cytokines. Cytokines are soluble, low-molecular weight protein molecules mainly secreted by leukocytes and various other cells in response to a stimuli, and they play an important role in cell-to-cell communication (94). Cytokine is a general term that represents signaling molecules, for example interleukins, molecules within the interferons and tumor necrosis factor families, and chemokines that regulate the recruitment of immune effector cells to different sites (94,95).

The recognition of the Mtb antigens by macrophage and DCs leads to activation and secretion of cytokines which induce more of innate immune response and initiation of adaptive immunity against Mtb. Cytokines produced during Mtb infection could be proinflammatory, which are beneficial for Mtb control, but could also cause tissue damage if the response is excessive, or anti-inflammatory, which may blunt Mtb controlling responses in the infected host (96). The proinflammatory cytokines, such as IFN-γ, IL-1β, IL-6, IL-12, IL-17 and TNF-α promote inflammation and induce the immune response, including activation of macrophage and T cells, at the site of infection, which enhance host immune defense and facilitate clearance of the pathogen (95,97).

The IFN-γ cytokine is principally expressed by specific lymphocyte subsets and one function is macrophage activation, that enhance microbicidal function and phagocyte activity. IFN-γ is a type II interferons that binds to the IFN-γ receptor and mediator of transcriptional regulation of more than 200 genes in macrophage through the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway (98,99). The importance of IFN-γ for control against Mtb has been demonstrated in knockout mice and in TB patients with IFNγ deficiency (100–104). It is also an important marker for the detection of Mtb-specific CD4+ Th1-cell responses in humans and in animal models (100). On the other hand, several studies have reported lack of association between levels of Mtb-specific IFN-γ producing CD4+ T cells and degree of immune control to Mtb; instead elevated Mtb-specific IFN-γ levels have been correlated with high bacterial load and severity of the disease (105–107). Furthermore, some studies have suggested CD4+ Th1 mediated Mtb control, which would be IFN-$\gamma$ independent (106,108). These findings taken together indicate that IFN-γ is fundamental, but not sufficient for Mtb control. Thus, protection may be mediated by multifunctional T cells or CD4 + Th1 cells by mechanisms not limited to IFN-$\gamma$ secretion.

By their functional roles and cytokine secretion patterns, T cells can largely be divided into effector T cells (secreting IFN-γ), effector-memory T cells (secreting both IFN-γ and IL-2) and central memory T cells (secreting IL-2) (109,110). Furthermore, the cytokine secretion patterns of T cells have been shown to be distinct at different clinical stages of TB infection (active, latent, and cured TB)

(111). For example, IL-2 has been shown to be associated with low bacterial burden and LTBI, while IFN-γ has been correlated with high antigen load and the progression from LTBI to active disease (112–116). However, the capacity of Th1 cells to produce IFN-γ after Mtb stimulation is reduced in persons with different types of immunosuppression, who have increased risk of progression to active TB.

IL-2 is potent T cell growth factor and promotes clonal expansion and differentiate of T cells into effector T cells upon antigen activation. IL-2 driven responses have shown to be more important at the site of infection (117). IL-2 has also been shown to facilitate the development of activated CD4+ or CD8+ T cells into memory cells through IL-2 receptor signaling in mice (118).

TNF-α is a key proinflammatory cytokine, at activation of macrophages and during granuloma formation, for control of Mtb (119). It is also involved in different inflammatory diseases, such as rheumatoid arthritis (RA) and other autoimmune diseases. For example, the increased risk of LTBI reactivation in RA patients taking ant-TNF-α immunotherapy indicates the importance of this cytokine (120).

Cytokines with anti-inflammatory or regulatory functions such as IL-10 and TGF-β are involved in suppression of the proinflammatory Mtb controlling response, which may facilitate TB development (95). IL-10 suppresses macrophage activation which in turn leads to down-regulation of IL-12 and subsequently Th1 cell development and IFN-γ secretion (121). TGF-β also inhibits T cell proliferation and proinflammatory cytokine production, and is also an inhibitor of cytotoxic T-cell effector function in granuloma (122,123).

Chemokines, a subgroup of cytokines, exhibit potent chemotactic activity for various cell populations and contribute to the recruitment and trafficking of specific immune cells to the site of Mtb infection. Thus, Mtb induces elevated levels of a variety of chemokines which includes CCL2 (MCP-1), CCL8 (MCP-2), CCL7 (MCP-3) CXCL8 (IL-8) and CXCL10 (IP-10) secreted from antigen presenting cells after stimulation by proinflammatory cytokines such as IFN-γ and TNF-α (124,128). The involvement of chemokines in host responses to Mtb infection includes the recruitment of myeloid cells into the lung for activation of adaptive immunity, and formation of protective granulomas as it has been observed in animal models (126). Among others, IP-10 is a highly expressed chemokine in response to antigen stimulation (127). It is secreted by monocytes and expressed 100-fold higher levels than IFN-γ, and it is mainly triggered by IFN-γ, but also by other cytokines (128). Its main function is to attract activated Th1 cells to sites of infection. Similarly, MCP-2 is also chemotactic for T-cell, as well as monocytes, and can also be induced at high levels (about 10-fold higher levels than IFN-γ) (128).

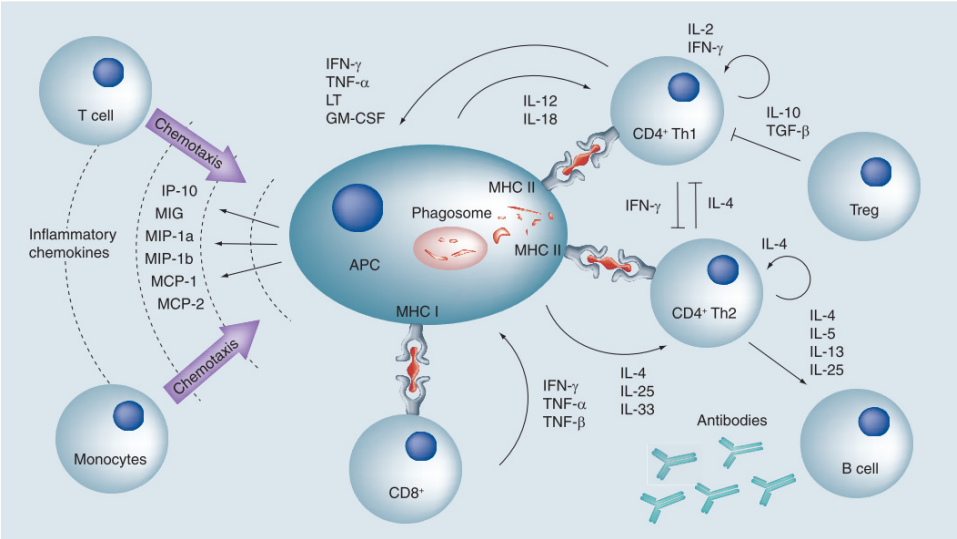


**Figure 4.** Overview on interactions between antigen-presenting cells and different T cell subsets which results cytokines and chemokines release from the respective cells in response to Mycobacterium tuberculosis infection. Reproduced with permission from Expert Review of Respiratory Medicine, Ruhwald M. and Ravn P. (129), Copyright Taylor and Fransic.

## Granuloma formation

TB granuloma is a hallmark structure, mostly located in the lung and consist of aggregate of Mtb infected and uninfected immune cells that contain the growth of Mtb (130). The granuloma is predominantly made up of macrophage and T cells, including CD4+ and CD8+ T cells and other cells such as DCs, neutrophils, B cells, giant and plasma cells as well as central necrotic macrophages (Figure 5). It is an important immune microenvironment for coordinating the interaction of these cells in restricting the dissemination and replication of the bacteria to uninfected tissues (131). Granuloma formation is initiated when inhaled Mtb bacteria are phagocytosed by macrophages, who fail to kill bacteria, which results in viable bacteria remaining inside the infected macrophages (132). This leads to the induction of apoptosis and consequently recruitment of additional innate immune cells such as neutrophils, DCs and monocyte derived macrophages, which further promote inflammation and initial establishment of Mtb infection in the granuloma. Adaptive immunity, mainly mediated by Th1 producing proinflammatory cytokines such as IFN-γ and TNF-α, and chemokines that attract T cells to the site of infection, also greatly contribute to granuloma formation and persistent containment of the Mtb infection (133).

Although granuloma could limit the bacteria expansion at the infection site, Mtb inside the granuloma constantly activate the host immune cells, and if this process is hampered in conditions with immune suppression, this may lead to dissemination

of the infection, especially in individuals with HIV infection and other risk factors (134). In such condition, the granuloma becomes a site of reactivation of bacteria from dormancy, or failure to develop latency after recent infection, which causes development of primary TB disease. However, in about 90% of infected and immune competent individuals the latent infection remains dormant for life without progressing to clinical TB.

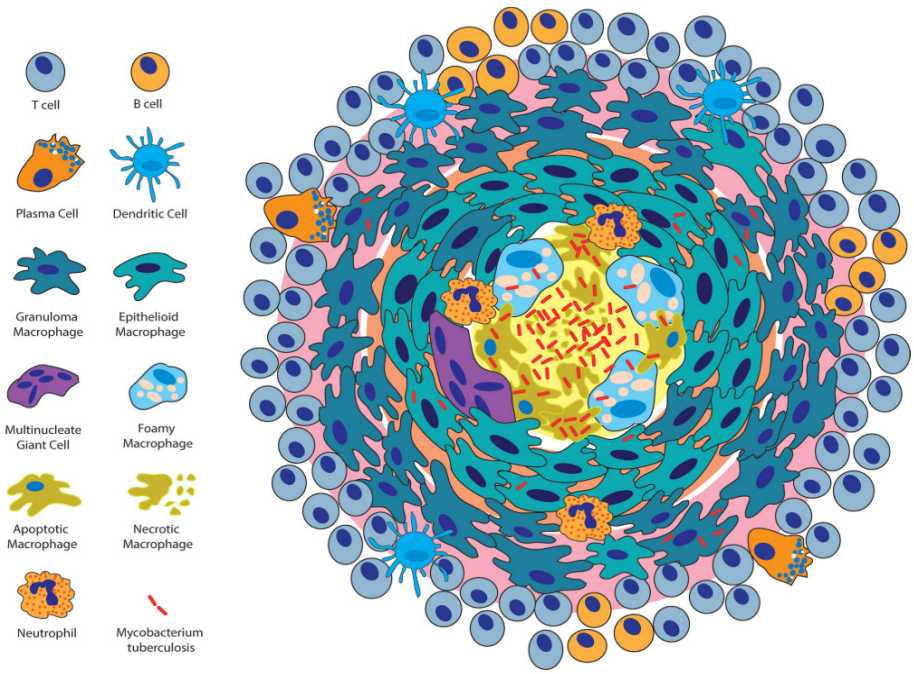


**Figure 5.** Structure of the tuberculosis granuloma. From Pathogens and Disease, McClean CM. 2016 (49). Reprinted with permission from Oxford University Press.

# Diagnosis

## Diagnostic methods for active TB

Bacteriological methods for active TB diagnosis include microscopy, mycobacterial culture and nucleic acid amplification techniques or polymerase chain reaction (PCR) (135). Sputum smear microscopy is a rapid, and simple tool for diagnosing pulmonary TB using Ziehl–Neelsen staining technique. Despite its low sensitivity, it serves as a primary diagnostic test and remains the cheapest and the most accessible TB diagnostic tool in developing countries with high TB prevalence (135). Culture is the gold standard test for TB diagnosis. Liquid culture techniques

have higher sensitivity for detection of Mtb than solid culture (135). Culture-based techniques are required for drug susceptibility testing and detection of drug resistant TB (135,136). However, TB culture can take up to six weeks for a definite result to be released, and requires high biosafety level laboratories.

PCR is a sensitive and specific tool for rapid detection of Mtb DNA in clinical samples such as sputum and blood (137). Among several PCR systems that have been used for detection of Mtb, Xpert MTB/RIF assay (Cepheid Inc., CA, USA) has been widely implemented in low-income countries since 2010 (135). It is an automated real-time sputum processing, cartridge based assay that simultaneously detects TB and rifampicin resistance on sputum and selected extrapulmonary specimens (138). The sensitivity is greater than that of smear microscopy, but lower than culture (139).

Due to difficulties in detection of Mtb in patients with suspected TB disease, TB can also be diagnosed based on clinical, radiographic and/or histo-pathological criteria (140).

## Diagnostic methods for latent TB infection

Per definition, Mtb bacilli cannot be detected in persons with LTBI, the diagnosis of which is based on immunological tests that measure reactivity to Mtb antigens. The diagnosis of LTBI is challenging for several reasons: (i) lack of a diagnostic gold standard (36) (ii) the commercially available tests rely on detection of cell-mediated immune response to Mtb, which have lower performance in immunosuppressed individuals and have low predictive value for progression to active TB (141,142) (iii) current diagnostic tests cannot distinguish active TB disease and LTBI (and also not individuals with true latent infection, i.e. persistent viable bacterial replication, that could pose a risk of reactivation, from individuals with cleared infection and remaining immune reactivity). In the absence of a gold-standard test, the accuracy and sensitivity of LTBI tests are sometimes evaluated using culture-confirmed active TB cases as surrogate reference standards (143). However, the response of these tests may be affected by active TB, and false negative reactions are rather common, especially in immunosuppressed individuals (143). On the other hand, specificity is assessed by testing low-risk individuals with no known TB exposure in low-TB burden setting (143).

Tuberculin skin test (TST) and T-cell interferon-γ release assays (IGRAs) are the currently used LTBI diagnostic tools. Both tests measure past or present Mtb exposure by detection of cellular immune reactivity to Mtb.

Historically, TST has been the most common method for LTBI testing. Nowadays, IGRAs are used as a routine method for LTBI diagnosis and have largely replaced TST in many high-income countries. However, both TST and IGRAs have lower diagnostic performance in immunosuppressed patients, but IGRAs offer higher

specificity than TST, especially in bacillus Calmette-Guérin (BCG)-vaccinated individuals (142,144,145). The WHO recommends either TST or IGRAs tests such as Quantiferon for diagnosis of LTBI (36).

*Tuberculin skin test*

This test has been used for detection of LTBI for over a century (37,146). TST is an in vivo test performed by intradermal injection of purified protein derivative (PPD), an extract from Mtb cultures containing >200 mycobacterial antigens (147). The skin reaction that causes induration at the site of injection reflects a delayed-type hypersensitivity response to the tuberculin antigens. After tuberculin injection, most infiltrating cells are neutrophils (after 4–6 h), activated macrophages (12 h) and CD4+ and CD8 T-cells that migrate out into the skin by 48 h. The underlying mechanism of this cellular infiltration is not completely clear, but the response is mediated by pro-inflammatory cytokines, such as IFN-γ, and TNF-α stimulation.

The size of induration is measured within 48 to 72 h and the criteria for TST positivity depend on the risk of TB and presence of immunosuppression in different groups. Three cut-off levels are recommended for categorizing a positive tuberculin reaction: >5 mm (person with HIV, recent contact with active TB and patients with organ transplantation or other severe immunosuppressive conditions), >10 mm (people at high risk of TB exposure, such as recent immigrants from high TB burden countries, and children less than 4 years), and >15 mm (person with no risk factor for TB) (148).

However, cross-reactivity (to antigens in PPD which are shared by Mycobacterium bovis (present in the bacillus Calmette-Guerin (BCG) vaccine) and other non-tuberculosis mycobacteria, limit its specificity (149–151) Furthermore, TST sensitivity is also reduced in individuals with immunosuppressive conditions such as malnutrition, HIV infection, immune suppressive therapy, and other chronic diseases (152–155). Variability in measurement of induration, interpretation of repeat testing (which may be difficult due to immunological boosting), test conversion and reversion are among several factors that affect TST reproducibility (156).

*Interferon-γ release assays*

IGRAs are in vitro tests which measure the response of T cells to mycobacterial antigens that are highly specific for Mtb: 6 kDa early secreted antigenic target (ESAT-6) and 10 kDa culture filtrate protein (CFP-10), which are located in the region of difference 1 (RD-1) and additional single peptide tuberculosis 7.7 (TB7.7) -encoded in RD11 of the Mtb genome (157). These antigens are absent in all BCG strains, as well as in most other environmental mycobacteria (148,158); thus, in contrast to TST, the specificity of IGRAs is less affected by infection with non-tuberculous mycobacteria (152).

Two IGRAs are commercially available, QuantiFERON (QFT) and T-SPOT.TB (TSPOT). Both tests are based on the same principle, with stimulation of whole blood with Mtb specific antigens leading to release of IFN-γ from Mtb-specific Th1 cells. In QFT assays, the concentration of IFN-γ is quantified in supernatants by enzyme-linked immune-assay (ELISA), whereas TSPOT measures the number of IFN-γ producing peripheral blood mononuclear cells using an enzyme-linked immunospot (ELISPOT) assay (159–161). The QFT test evaluation is performed by stimulation reactions with negative control (nil, for background signal correction) and positive control (mitogen, for general T-cell responsiveness). A test is considered positive if the Mtb-specific IFN-γ levels are above the recommended cut-off value ≥0.35 IU/ml (after nil value is subtracted), and negative when IFN-γ is <0.35 IU/ml. Test results with nil values >8.0 IU/ml and/or mitogen <0.5 IU/ml are considered to be indeterminate.

Variability in QFT results with serial testing is a common phenomenon (162,163). QFT test variability can be due to preanalytical, technical, or patient related factors (163). One problem in relation to the use of QFT assay is the interpretation of results around the dichotomous cut-off (≥0.35 IU/ml) (164,165). Using a single cutoff point may be inadequate for differentiation of persons with and without TB infection. Several studies have also reported high variability of QFT results around this cut-off level (166,167). For this reason, the use of a borderline range (or uncertainty zone), has been suggested (168,169). Currently, there is no established definition of the QFT borderline zone, although the range 0.20–0.70 IU/ml is commonly used (168,170).

Although borderline QFT results may be due to false positive reactions in many cases (164,171), such results could also represent true TB infection, particularly in immune suppressed individuals (172).

Previous versions of QFT (QFT-GIT) assays were exclusively based on the effector function of CD4+ T-cells, mainly mediating the production of IFN-γ in response to Mtb antigen (173). Apart from CD4+ T cells, CD8+ T-cells have been found to have a role in response to Mtb antigens, with association to recently acquired TB infection (174,175). For this purpose, a new generation of the QFT assay, QuantiFERON-TB Gold Plus (QFT-Plus), has been developed with the aim to detect cell-mediated immune responses to Mtb antigens from both CD4+ and CD8+ T-lymphocytes.

*QuantiFERON-TB Gold Plus*

The 4th generation of the QFT assay, QFT-Plus, was introduced by Qiagen in 2015 (176). QFT-plus is a novel version of the QFT-GIT assay with similar basic principles, but designed to improve the sensitivity for detection of LTBI in immune suppressed subjects (177,178). This assay includes two TB-specific antigen tubes: TB antigen 1 (TB1) and TB antigen 2 (TB2). TB1 contains long peptides derived from ESAT-6 and CFP-10 (TB-7.7 present in QFT-GIT has been removed) which

specifically stimulates CD4+ T-cells, whereas TB2 includes an additional set of short peptides of these antigens which stimulates CD8+ T-cells.

It has been reported that CD8+ T cells response is more frequently detected in active TB patients (60%) compared with LTBI (15%) (92) and in subjects with recent Mtb exposure (179). Mtb-specific CD8+ T cells were also found to be highly expressed in children with active TB disease (180). Thus, it is possible that the QFT-Plus may have better sensitivity for LTBI detection in individuals with recently acquired TB infection. However, high concordance has been reported between results of QFT-GIT and QFT-plus in both immunocompromised (181–183) and immunocompetent subjects eligible for LTBI screening (184,185). Similarly, high agreement between TB1 and TB2 antigens response has been reported in the QFT-Plus assay (186,187).

# Biomarkers for TB infection

A biomarker (biological molecule found in blood or other body fluids) is defined as a ‘‘characteristic that is objectively measured and evaluated as an indicator of normal biological process, pathological process or pharmacological response to therapeutic intervention’’ (188). In infectious disease, biomarkers can either be host or pathogen derived and in TB infection these markers can be used for diagnostic and prognostic tools, monitoring of anti-TB treatment succuss and evaluation of vaccines response (189,190). In particularly, biomarkers are used to predict durability of treatment success in active TB patients, to indicate reactivation risk as well as treatment success in latently infected persons or to indicate new TB vaccines efficacy in other group of people (191). Among others, cytokines and chemokines expressed as a part of the host immune response against Mtb, have been investigated in both antigen stimulated and unstimulated samples at different stages of TB infection (97,128).

In the IGRAs, IFN-γ has been used as a readout marker for Th1 immune-based TB diagnostic. In a wide-range of studies, in human and animal models, more comprehensive profiling of T cells, APCs and other adjacent immune-competent cells (secreting cytokines in the T-cell–APC interaction) have been shown to produce several panels of cytokine, chemokine and inflammatory markers in response to Mtb antigen (128). Thus, the secretion of multiple markers by different functional cells in response to Mtb specific antigen stimulation have been proposed as an alternative potential immunodiagnostic biomarker that could improve the diagnostic sensitivity of IGRAs (128,161).

Chemokines have been reported to be alternative TB biomarkers. These includes chemokines such as IP-10, MCP-1, MCP-2, MCP-3, macrophage inflammatory protein (MIP-1 α and β), and monokine inducible by interferon-γ (MIG), and their secretion is primarily triggered by IFN-γ and other proinflammatory cytokines.

Indeed, IP-10 is the most extensively studied alternative biomarker in both active TB and latently TB infected individuals (192,193). Furthermore, the high, amplified, expression of IP-10, as compared with the IFN-γ expression levels, makes this chemokine an attractive diagnostic marker for TB detection, particularly in those individuals living with HIV (194–196) and in children (197–199). The high sensitivity of IP-10 in detecting TB in immunocompromised subjects could be that its initial secretion is not CD4 T cell dependent and not as much affected by low CD4+ T cell count. Moreover, IP-10 secretion, which mainly is stimulated by IFN-γ and TNF-α, can also be activated by multiple other cytokines, such as IFNa/b, IL-1β, IL-2, IL-17, and IL-27 (128). Other chemokines such as MCP-2 have also been reported to be promising markers for TB (111).

# Immunological Changes during Pregnancy

The central role of the immune system is to protect the host from infectious pathogens. During pregnancy the maternal immune system undergoes transient modification in order to promote tolerance to fetal antigens (Figure 6) (200). Pregnancy is characterized by, not only the local changes that occur at the feto-maternal interface, but systemic alterations in maternal immunity which consecutively can affect the course of infectious diseases (201). Thus, pregnancy-related immune adaptation could make the pregnant woman more susceptible to infections (19). In general, a healthy pregnancy is characterized by a shift in the proinflammatory and anti-inflammatory responses (202). This change is more pronounced during the third trimester of pregnancy in which the activity of Th2, Treg cells and M2 macrophages is elevated, whereas the responses of Th1, Th17, M1 macrophages and NK cells are down regulated (201). Furthermore, pregnancy-induced hormones such as estradiol, estriol and progesterone are substantially increased during pregnancy, regulating the proportion and function of T cells (201). For example, the elevated concentrations of progesterone induced blocking factor stimulate the CD4+ T cell differentiation to Th2 cells, which increase the production of regulatory and anti-inflammatory cytokines and decreased the Th1 secreted pro-inflammatory cytokines over the course of pregnancy (201).

Apart from a Th1/Th2 shift, to maintain maternal-fetal tolerance, pregnancy is also accompanied by changes in the Th17/Treg cell balance, resulting in a reduction of Th17 and expansion of Treg responses (203). Reduced proportions of Treg cells and increased in Th17/Treg ratio has been observed in women with the history of recurrent spontaneous abortion (204). Thus, the balance between Th17 and Treg cells is crucial for successful pregnancy outcome.

Hence, Tregs play an important role in regulating the maternal immune system to tolerate the fetus, which expresses paternal antigens. Studies have demonstrated

high numbers of Tregs in decidua and in the periphery, in mice and in humans, during pregnancy (205,206). It has also been shown that fetus specific Treg cells migrate from the periphery to the decidua, with suppression of antigen-specific immune responses over the course of pregnancy (207–209). Pregnancy-related complications such as miscarriage and preeclampsia has also been associated with reduced expression of Tregs in the placental decidua (210,211), further supporting that Treg cells are central for the maintenance of pregnancy.

Expansion of Tregs in decidua and periphery during pregnancy suppresses maternal Th1/Th17 activity (203). The mechanism behind the suppressive effect of Tregs is not completely understood. It has been proposed that the Treg responses are mediated in a cell-contact dependent manner through the recognition of co-stimulatory molecules that suppress the activity of effector T cells (212). Feto-maternal tolerance is generated after the Treg cells recognize fetal-antigens by maternal APCs and tolerance is established in antigen-specific manner (212).

Another Treg suppressive mechanism is through a cell-contact independent manner, by production of regulatory and anti-inflammatory cytokines, such as TGF-β and IL-10 (203). Pregnancy enhances expression of these cytokines which can stimulate the expansion and suppressive activity of Tregs and prevent immunological rejection of the fetus. TGF- β is an important factor in establishing early embryo development, trophoblastic differentiation, and proliferation in human fetal growth (213). It has also been shown to induce Treg cells to synthesize IL-10. These cytokines seem to be very important early during pregnancy and their increased levels have been associated with successful pregnancy outcome (214,215).

In addition, trophoblasts, which develop in the placenta, induce feto-maternal tolerance by expressing an inhibitory enzyme called indoleamine 2,3-dioxygenase (IDO). IDO suppresses the secretion and activity of maternal immune cells (216). Furthermore, trophoblasts induce immune suppression through the secretion of regulatory cytokines, such as TGF-β and IL-10 (217).

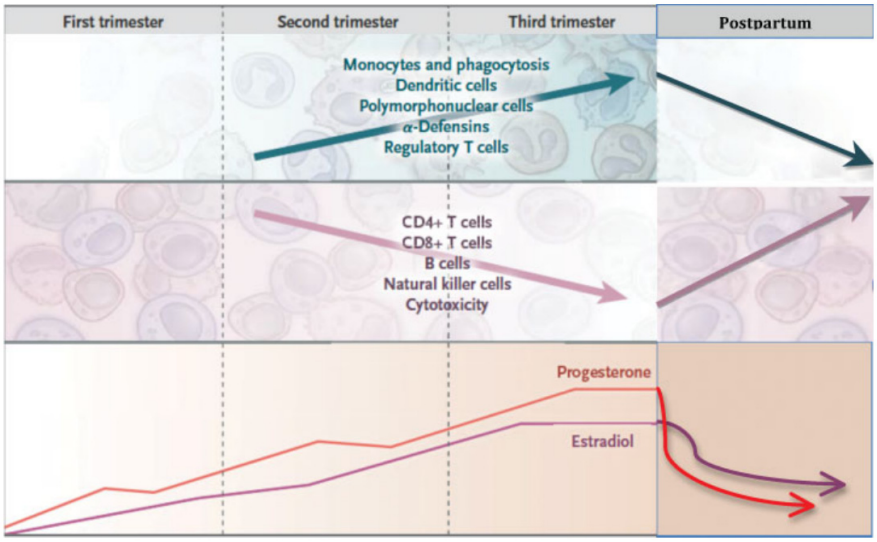


**Figure 6.** Immune and hormone levels change during pregnancy. Reproduced with permission from New England Journal of Medicine, Kourtis A. (19), Copyright Massachusetts Medical Society.

# Effect of pregnancy on LTBI immune control and performance of diagnostic methods

Pregnancy associated immune modification might affect immune control of Mtb in pulmonary granulomas, which in turn could confer increased risk of reactivation of LTBI. IFN-γ, a central marker of Th1 immunity, has a key role in immune protection against Mtb infection, and it is possible that the capacity for Mtb-specific IFN-γ secretion is reduced during pregnancy.

Previous studies on the impact of pregnancy on Mtb-specific IFN-γ secretion have mainly been performed in women with HIV. In longitudinal studies from TB endemic countries, lower levels of IFN-γ, both in response to Mtb antigens and mitogen, have been observed during pregnancy, with increasing levels postpartum, in HIV-positive women (218,219). A similar pattern was found in HIV-negative women in a small study from India (220), whereas no significant changes in Mtb-triggered IFN-γ secretion were observed in 25 HIV-negative women in the US followed longitudinally during pregnancy (221). Furthermore, in a study from India, the stage of pregnancy was found to influence the performance of immune based LTBI diagnostic assays, also in HIV-negative women (220). Taken together, these findings indicate that pregnancy influences the cellular response to Mtb antigen in both HIV positive and negative women, although with more profound effect in women with HIV.

Timely detection of LTBI offers the possibility of preventive therapy, with reduced risk of subsequent reactivation. The indication for LTBI screening and TPT in pregnant women remains controversial (222). TPT for women of reproductive age could reduce TB related maternal and infant mortality; however, exclusion of active TB may be difficult during pregnancy, and the risk of isoniazid-induced hepatotoxicity is higher in pregnant women (223). In addition, the physiological immune modifications that occur during pregnancy might influence the performance of current immune based LTBI diagnostic assays. The diagnostic performance of QFT-GIT has been compared with TST in pregnant women (218,220,224). In these studies, QFT-GIT identified more women with LTBI than TST, and decreased IFN-γ concentrations were reported during pregnancy compared to postpartum (218,225). Poor performance of the QFT-GIT assay has been reported in pregnant women with HIV from Kenya, with 15% indeterminate test results (28); furthermore, positive QFT results during pregnancy were associated with postpartum active TB as well as with maternal mortality (28).

There is limited data on the proportions of QFT borderline results in pregnant women, but it is plausible that this phenomenon is more common during pregnancy. Whereas QFT borderline results may be due to false-positive reactivity in persons at low risk of TB exposure (164), a borderline QFT result in a pregnant woman living in a TB-endemic setting could instead represent true infection (226). Thus, in persons with different forms of immune suppression, a lower threshold for defining QFT reactivity could be considered. Another strategy could be the use of immune mediators other than IFN-γ as readout markers in the QFT assay (227); however, this approach has not been investigated in pregnant women.

# AIMS

## General aim

The overall aim of this thesis was to explore how pregnancy influences immune responses to latent TB infection.

## Specific aims

I. To evaluate the performance of the QuantiFERON-TB Gold Plus assay for detection of latent tuberculosis infection during pregnancy (paper I)

II. To explore alternative Mtb-antigen stimulated cytokine responses, separately or in combinations, for LTBI status determination of pregnant women with QFT IFN-γ results in the borderline range (paper II)

III. To investigate the impact of pregnancy on Mtb-triggered IFN-γ levels in whole blood of HIV-negative women sampled longitudinally during pregnancy and postpartum (paper III)

IV. To investigate the dynamics of cytokine responses, Mtb-specific and non-specific, in women with LTBI followed longitudinally, during pregnancy and post-partum, in order to study how pregnancy impacts immune control of LTBI (paper IV)

# MATERIALS AND METHODS

## Study setting

All studies were conducted in the city of Adama, Ethiopia. Ethiopia is a landlocked country located in the Horn of Africa. With over 110 million inhabitants, Ethiopia is the second most populous nation and the tenth largest in Africa with a total area of 1.104 million $km^2$. Adama is located 100 km east of the capital city of Ethiopia, Addis Ababa along the Addis Ababa-Djibouti highway, the main transport corridor in Ethiopia, and is the main commercial centre, in East-Shewa zone of the Oromia region.

In the city the health services are provided in primary (health centers) and secondary (hospitals) levels of cares in public and private health sectors. Our studies were conducted in two selected public health centres: Adama health center and Geda health center; and one referral hospital, Adama hospital medical college, all located in the Adama city, with an uptake area with over 300 000 inhabitants. These health facilities provide health care for TB, HIV, maternal and child health including prevention of mother-to-child transmission of HIV (PMTCT) and other health services to the community. TB, HIV and maternal follow up care is provided for free in these public sectors. For pregnant women, the antenatal care (ANC) visits occur at 1st, 2nd, 3rd trimesters during pregnancy and at labor, and after delivery for infant vaccination. In connection to ANC visit, screening for TB and HIV is performed. At these health facilities, around 8 000 women register annually for ANC and majority of deliveries occur at the facilities. Pregnant women with high-risk pregnancies are referred to the hospital ANC clinic for follow up and delivery.

The Adama-Lund University research station was established in 2010 in collaboration with Oromia Regional Health Bureau. This research station has previously been used as a platform for studies on HIV/TB co-infection in public health centers. This infrastructure has also been used for the TB-pregnancy cohort study and currently the project is working in close collaboration with Armauer Hansen Research institute (AHRI). The project staffs are well experienced for data monitoring and entry. We have used REDCap software for data management.

# The Adama TB-pregnancy cohort: Study procedures

All studies included in this thesis were based on samples collected from an ongoing prospective cohort of pregnant women. The overall purpose of this cohort study is to explore different aspects of TB infection in relation to pregnancy. The cohort was initiated in October 2015 and recruitment of pregnant women into the cohort was completed in February 2018 after having reached the target of 2200 women. Pregnant women presented for their first visit to ANC for the current pregnancy with residence in the study catchment area were eligible for inclusion. Women who had attended ANC prior to screening during the current pregnancy and those referred to the hospital ANC clinic from health centers outside the study uptake area were excluded. At enrolment, written informed consent was received from all participants. Sociodemographic, medical and obstetric history was collected using standardized questionnaires. Physical examination was performed by trained health facility staff in the respective clinics. Sputum samples for bacteriological investigation of active TB were collected from participants with symptoms or signs suggestive of active TB at any time during the study period, and for all HIV-positive participants at enrolment (irrespective of clinical manifestations). Questionnaire-based interview and clinical examination regarding symptoms suggestive of TB were repeated at subsequent visits during pregnancy and at all follow up visits after delivery (until 4 years after enrolment). In addition, participants were urged to contact the study clinics in the event of symptoms suggestive of active TB. Participants who did not come for their scheduled visits were actively traced by dedicated staff.

TB bacteriological testing was performed using smear microscopy, liquid culture and GeneXpert MTB/RIF PCR on two consecutive spontaneously expectorated morning sputum samples. Mid-upper arm circumference (MUAC) was measured to assess nutritional status. HIV testing was done using rapid tests for all participants according to national guidelines (14). For HIV-positive women, blood was obtained for analysis of CD4 cell count (FACSCalibur, Becton Dickinson) and HIV RNA quantification (viral load, VL, using Abbott m2000rtTM, Abbott Molecular Inc.). Blood QFT-Plus testing was collected in heparin tubes for LTBI investigation. All study samples were transported to the Adama Public Health Research and Referral Laboratory Center, where processing, laboratory testing, and storage was performed.

# Study design and participants

The studies of paper I and II include cross-sectional data, and paper III and IV comprise longitudinal data in participants with LTBI and controls selected from the cohort. A summary of the study design with included study groups and investigated study variables are presented in Table 2.

Paper I and II included both HIV-positive and negative women, whereas in paper III and IV all study participants were HIV-negative. In paper II, the study participants were selected from the cohort and categorized in to three groups based on the QFT IFN-γ response as indicated in Table 2. Participants with current or previous active TB (except in paper I) were excluded from these studies.

**Table 2.** Summary of study design and study participants

| Paper | Study design | Study participants | Sample size | Variables investigated |
|---|---|---|---|---|
| **I** | Cross-sectional | HIV-negative | 780 | QFT-Plus assay performance |
| | | HIV-positive | 49 | TB2 additional yield<br>IFN-γ borderline distribution |
| **II** | Cross-sectional | HIV-negative and HIV-positive* | | Patterns of alternative biomarkers in different QFT categories |
| | | QFT-low<0.20 IU/ml | 33 | Alternative biomarkers for QFT-borderline classification |
| | | QFT-borderline 0.20–0.70 IU/ml | 31 | |
| | | QFT high ≥ 0.70 IU/ ml | 32 | |
| | | Controls | 20 | |
| **III** | Longitudinal | HIV-negative women | 363 | Levels of Mtb-stimulated IFN-γ in QFT supernatants at i) 1st/2nd trimester vs. ii) 3rd trimester vs. iii) 9 months postpartum |
| **IV** | Longitudinal | HIV-negative women | | Dynamics of Mtb-specific and non-specific immune mediators at i) 1st/2nd trimester vs. ii) 3rd trimester vs. iii) 9 months postpartum |
| | | LTBI+ (QFT ≥0.35 IU/ml) | 22 | |
| | | LTBI- (QFT <0.20 IU/ml | 10 | |

*12 HIV-positive participants were included in each QFT category (except controls).

# Study definitions

## Latent TB infection

Nil-subtracted QFT-IFN-γ results ≥0.35 IU/ml in either TB1 or TB2 were defined as LTBI positive while IFN-γ results <0.35 IU/ml defined as LTBI negative.

In paper III, LTBI was defined as nil-subtracted QFT-IFN-γ response >0.20 IU/ml, while absence of LTBI was defined as IFN-γ <0.20 IU/ml (also in paper IV) after excluding previous or current active TB. In this study, the reason that the lower cut-off was chosen (than the standard threshold 0.35 IU/ml) to consider the reduced capacity of IFN-γ secretion during pregnancy.

### QFT IFN-γ borderline results

QFT IFN-γ borderline results were defined as IFN-γ results in the range between 0.20 IU/ml-0.70 IU/ml.

### Lower borderline QFT IFN-γ results

QFT IFN-γ results between 0.20-0.34 IU/ml.

### Higher borderline QFT IFN-γ results

QFT IFN-γ results between 0.35-0.70 IU/ml.

### Active tuberculosis

Current active TB was defined as a positive bacteriological test result (either smear microscopy, GeneXpert PCR or culture), and/or clinical manifestations meeting Ethiopian national TB guideline criteria for active TB. Previous active TB was defined as self-reported history of previous treatment for active TB.

## Laboratory procedures

### QuantiFERON-TB Gold Plus

LTBI testing was performed using the QFT-Plus assay (Qiagen, Carnegie, Australia). Four ml of venous blood was collected into lithium heparin tubes and transported to the study laboratory. Within 8 h of venipuncture, 1 ml of heparinized whole blood was dispensed into each of four QFT incubation tubes; a negative control (nil), a positive control (mitogen) and two Mtb-specific antigen coated tubes (TB1 and TB2). The tubes were mixed by inversion and incubated at 37° C for 18 h. After incubation, the tubes were centrifuged and QFT supernatants were harvested and dispensed into three aliquots; one aliquot was stored at -20 °C (for IFN-γ ELISA), and the remaining two aliquots were stored at -80 °C for

quantification of other cytokines. The concentration of IFN-γ was measured using the QFT ELISA according to the QFT-Plus protocol. The ELISA readout was calculated using the QFT-Plus software. IFN-γ nil result >0.80 IU/ml or IFN-γ mitogen <0.50 IU/ml interpreted as indeterminate.

### Multiplex immune mediator analysis

For papers II and IV, we measured the concentration of 32 immune mediators in duplicate in QFT supernatants stimulated with Mtb antigen (TB1 and TB2) and unstimulated (nil) using Magnetic Luminex assay (R&D Systems Inc., Minneapolis, MN) on the Bio-Plex 200 platform (Bio-Rad Laboratories Inc., Hercules, CA). The 32 immune mediators included in the assay were eotaxin, granzyme A, granzyme B, GM-CSF, IFN-γ, IP-10, interleukins (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, IL-13, IL-15, IL-17a, IL-21), IL-1ra, MIG, MIP-1α, MIP-1β, MCP-1, 2 and 3, osteopontin, PDGF- BB, RANTES, resistin, TGF-β1, TNF-α and VEGF. Immune mediators that have been associated with TB infection and pregnancy in literature search were considered for these studies.

For both studies, pilot experiments were performed to determine optimal dilutions. In the pilot studies, immune mediators with concentrations below the assay detection limits and markers without detectable Mtb-specific responses were omitted from further analysis. Analyses of GM-CSF, IL-10, TNF-α, and IL-2 were below the lower limit of detection in the initial assays used, and were repeated with high sensitive assays (HS). For paper II, the concentrations of eight markers were analyzed with different dilutions in samples obtained during pregnancy: MCP-1, MCP-2, IL-6, resistin and osteopontin in 1:2; and IP-10, IL-8 and IL-1ra in 1:30 dilution. For paper IV, six cytokines (IL-1ra, IL-2, IP-10, MCP-2, MCP-3 and TGF-β1) were selected for longitudinal analysis. The lower or upper standard concentrations were used for supernatants with cytokine concentrations below or above the multiplex assay detection limits, respectively. The TB-specific cytokine responses of TB1 and TB2 were determined by subtracting the concentration detected in the nil supernatant.

## Statistical analyses

Nonparametric tests were performed for all studies. Cross-sectional comparisons between two groups were performed using Mann-Whitney U-test, whereas Kruskal-Wallis followed by Dunn's multiple comparisons test was used for the three group comparisons. Pairwise longitudinal comparisons were performed in related samples using the Wilcoxon signed-rank test. Friedman test, followed by Dunn's post-test,

were used for longitudinal comparisons at three time points. Associations were determined using Spearman Rank correlations.

In paper I, Kappa-statistic was performed to determine the agreement between TB1 and TB2. In paper II, Receiver operating characteristic (ROC) analysis was performed to evaluate the discriminatory potential of the markers to identify different QFT categories. Furthermore, we used k-Nearest Neighbor (KNN) and k-mean clustering (KMC) supervised machine learning algorithms to classify women with QFT-borderline results with regard to their likelihood of having LTBI. In the absence of a diagnostic gold standard for LTBI, we selected two reference groups for these analyses: non-pregnant HIV-negative women with IFN-$\gamma$ <0.20 IU/ml considered to be at low likelihood of LTBI; and pregnant HIV-negative women with IFN-$\gamma \geq 0.70$ IU/ml considered to be at high likelihood of LTBI. In the KNN algorithm, participants with low and high likelihood LTBI reference groups were assigned as the training dataset while the borderline and remaining study groups were assigned as the test dataset. Then the individuals assigned in the test group were classified to the nearest neighbors to low or high likelihood of LTBI in the n-dimensional space of the normalized cytokine levels. For this, several number of neighbors (K = 1, 3, 5, 7, 9) were repeatedly applied. Besides, in the KMC classification algorithm the reference groups were used as pre-determined cluster centroids and the test cases were classified based on the lowest Euclidian distance to either low or high centroid.

All data analysis was performed using Graph pad prism software, version 8 and IBM SPSS statistics version 25.

# Ethical considerations

The studies obtained ethical approval from National Research Ethics Review Committee, Addis Ababa, Ethiopia and the Regional Ethical Review Board at Lund University, Sweden. The studies were also supported by the institutional review board (IRB) at Armauer Hansen Research Institute (AHRI) and Oromia Regional Health Bureau, Addis Ababa, Ethiopia. Written informed consent was obtained from all participants. All records were kept under confidential condition and anonymous study codes were used throughout the study for each participant.

# RESULTS

## Paper I: Performance of QuantiFERON-TB Gold Plus for detection of latent tuberculosis infection in pregnant women living in a tuberculosis- and HIV-endemic setting

Cross-sectional analysis was performed to evaluate the performance of QFT-Plus assay; including two preparations of Mtb-specific antigens [TB1 and TB2] for detection of LTBI from 829 Ethiopian pregnant women (49 HIV-positive) enrolled during antenatal care.

Using the recommended cut-off level 0.35 IU/ml, 277 (33%) of women were QFT-positive, with similar proportions among HIV–positive and HIV–negative individuals (16/49 [33%] vs. 261/780 [33%], respectively). We found high agreement between TB1 and TB2 antigens (κ = 0.92) with strong correlation between IFN-γ concentrations triggered by these antigens (Spearman's Rho 0.89). Discordant results were identified in 29 (3.5%) subjects, with similar distribution between TB1(1.9%) and TB2 (1.6%) antigen formulations. Furthermore, lower IFN-γ levels were observed in women with discordant results compared to women with concordant positive results. In addition, borderline range IFN-γ concentrations (defined as 0.20-0.70 IU/ml), triggered by either TB1 or TB2 antigens, were noted for a high proportion (14/49, 29% TB1; 12/49, 24% TB2) of HIV-positive women, compared to (59/780, 7.6% TB1 and TB2) among HIV-negative women. HIV-positive women also had significantly lower median IFN-□ release compared to HIV-negative participants (TB1: 0.47 vs. 2.16 IU/ml; $p<0.01$, TB2: 0.49 vs. 2.24 IU/ ml; $p<0.001$). In women tested at late stages of pregnancy, we observed slightly higher levels of Mtb antigen stimulated IFN-γ (TB1: rho 0.13, $p = 0.031$; TB2: rs= 0.11, $p = 0.061$), while levels of mitogen induced IFN-γ were lower compared to those in women tested early in pregnancy (rs =-0.32, $p<0.0001$).

## Paper II: Alternative biomarkers for classification of latent tuberculosis infection status in pregnant women with borderline Quantiferon Plus results

We explored alternative cytokine markers in supernatants of whole blood stimulated with Mtb antigens for classification of women with borderline QFT results. For this, 96 participants were selected and categorized into three QFT groups: QFT-low (IFN-γ<0.20 IU/ml, n=33); QFT-borderline (IFN-γ 0.20–0.70 IU/ml, n=31); QFT-high (IFN-γ>0.70 IU/ml, n=32), with inclusion of 12 HIV-positive women in each group. For comparison, HIV negative non-pregnant women (n=20) with no current or previous history of active TB, and with negative QFT results (IFN-γ <0.20 IU/ml) were included as controls. The concentration of eight markers (IL-1ra, IL-6, IL-8, IP-10, MCP-1, MCP-2, osteopontin and resistin) were measured in QFT supernatants and evaluated separately and in combination. Concentrations of MCP-2, IP-10 and IL-1ra were higher in QFT-borderline compared to QFT-low participants in both antigen stimulations ($p < 0.001$).

We found that the concentration of three cytokines, MCP-2, IP-10 and IL-1ra were higher in the QFT-high and borderline groups compared to QFT-low and controls in both TB1 and TB2 antigen stimulations ($p<0.001$), regardless of HIV serostatus. In HIV-positive women, IP-10 and MCP-2 also differentiated the QFT-borderline from the QFT-low group ($p<0.05$). In Receiver operating characteristic (ROC) analysis, the concentrations of MCP-2, IP-10 and IL-1ra in Mtb-antigen stimulated supernatants could distinguish women with IFN-γ borderline results. Using KNN classification analysis, a combination of these three markers classified 42% (13/31) of women with borderline results as having high likelihood of LTBI, whereas 17/31 women (55%) were classified as having low likelihood of LTBI. Among the 13 women with borderline results classified as having high likelihood of LTBI, all had IFN-γ levels between 0.35 and 0.70 IU/ml.

## Paper III: Longitudinal Mycobacterium tuberculosis-Specific Interferon γ Responses in Ethiopian HIV-Negative Women during Pregnancy and Postpartum

We compared IFN-γ levels in whole blood stimulated with Mtb-specific antigens (TB1 and TB2) and mitogen in women followed longitudinally during pregnancy (1st/2nd and 3rd trimesters) and post-partum to explore the impact of pregnancy on the dynamics of Mtb- and mitogen-triggered IFN-γ secretion. Women with samples available from two and/or three occasions (n=363) showing Mtb-specific IFN-γ responses ≥0.20 IU/ml were included. The lower cut-off of IFN-γ (IFN-γ≥0.20

IU/ml) was chosen to define QFT positive results considering the reduced capacity for IFN-γ secretion during pregnancy, and to improve detection of longitudinal fluctuations. Women with HIV infection were excluded from analysis.

We found that Mtb-triggered IFN-γ levels were elevated at 3rd compared to 1st/2nd trimester (n=38; TB1: 2.8 vs 1.6 IU/ml, p=0.005; TB2: 3.3 vs 2.8 IU/ml, p=0.03) and post-partum (n=49; TB1: 3.1 vs 2.2 IU/ml, p=0.01; TB2: 3.1 vs 2.3 IU/ml, p=0.03), p=0.02), whereas no significant differences were observed between post-partum and 1st/2nd trimester (n=276, TB1: 2.3 vs 1.9 IU/ml, p=0.60; TB2: 2.5 vs 1.9 IU/ml, p=0.20). Similar results were observed in women sampled on all three occasions. In contrast, mitogen-triggered IFN-γ responses were lower at 3rd compared with 1st/2nd trimester (21.0 vs 34.9 IU/ml, p=0.02). In this study, we observed QFT conversion in 71/610 (11.6%) and reversion in 52 (18.4%) women, using lower and upper limits of the borderline range (0.20 to 0.70 IU/ml), during pregnancy and postpartum.

# Paper IV: Dynamics of pregnancy-shaped immune responses in women with latent tuberculosis infection

In this study we further investigated how pregnancy affects the immune control of LTBI in HIV-negative women. For this, we studied longitudinal expression patterns of cytokines secreted by different immune cells in response to Mtb-antigen. In addition, we analyzed the levels of these cytokines in Mtb unstimulated samples to explore the dynamics of systemic inflammation in pregnant women with or without LTBI.

In response to stimulation with Mtb-specific antigens, the expression of IL-2 and IP-10 was elevated in women with LTBI at the 3rd trimester compared to at 1st/2nd trimester (median 139 pg/mL vs. 62 pg/mL, p=0.006; 4,999 pg/mL vs. 2,310 pg/mL, p=0.03, respectively), whereas the concentration of Mtb-antigen stimulated TGF-β1 was decreased at the 3rd trimester compared to 1st/2nd trimester (-6.8 ng/mL vs. 2.3 ng/mL, p=0.02).

The unstimulated levels of IL-2, IP-10 and MCP-2 were significantly increased postpartum compared to 1st/2nd trimester in women with LTBI (IL-2: 5 vs 2 pg/mL, p=0.002; IP-10: 331 vs 76 pg/mL, p=0.0001 and MCP-2: 80 vs 45 pg/mL, p=0.0001), and to 3rd trimester levels (IL-2: 5 vs 2 pg/mL, p=0.03; IP-10: 331 vs 138 pg/mL, p=0.007 and MCP-2: 80 vs 47 pg/mL, p=0.0001). Compared with women without LTBI, the non-specific responses of IL-2 and IP-10 were also higher postpartum in women with LTBI (IL-2: 2 vs 5 pg/ml, p=0.002 and IP-10: 87 vs 331 pg/ml; p=0.0001, respectively). Furthermore, Mtb-specific and non-specific cytokine responses with a pro-inflammatory profile showed positive correlation at all three time points, with stronger correlation at 3rd trimester.

# DISCUSSION

This thesis explores the impact of pregnancy on the immune control of LTBI, and the findings support our hypothesis that pregnancy associated immune modification impacts immune responses to TB infection. This could have implications for immune-based diagnostic tests for TB, but also for the natural course of TB in latently infected women. The outcome of LTBI depends on the balance between the bacilli and host immunity, which changes during the course of pregnancy. This phenomenon cannot be directly studied in humans at the sites LTBI, but may be reflected by patterns of mediators secreted by different immune cells.

## Performance of QFT-Plus assay for LTBI detection during pregnancy

All diagnostic methods for LTBI are based on detection of immune responses to Mtb and cannot determine whether viable bacilli are present or not. Furthermore, these methods cannot discriminate between LTBI and active TB disease. IGRAs have better specificity than TST, but the sensitivity of both TST and IGRAs depend on the capacity of infected individuals to mount immune response upon Mtb antigen stimulation. The QFT-Plus assay was developed with the aim to improve the sensitivity of IGRAs in immunosuppressed subjects. This assay comprises two Mtb-specific antigen preparations; the TB1 antigen mainly stimulates CD4 +T-cells whilst the shorter peptide formulations included in the TB2 antigen tube stimulate both CD4 and CD8+ T-cells. The addition of TB2 short peptide antigens could therefore improve LTBI detection rates in persons with cellular immunodeficiency and reduced capacity for CD4+ T cell IFN-γ secretion (for example, people with HIV), which can lead to false negative IGRA results. This has also been reported in other conditions, for example in person receiving immunosuppressive therapy (228), but it is uncertain how the immune modifications that occur during pregnancy affect these responses.

In paper I, we therefore evaluated the performance of QFT-Plus for detection of LTBI in pregnant women. Our results showed a strong correlation between TB1 and TB2 ($\kappa$=0.92) IFN-γ responses in both HIV-negative and positive women. Thus, the addition of TB2 did not increase detection of LTBI among these women (most of

whom were HIV-negative and with no other immunosuppressive conditions). Our study was the first to evaluate the performance of QFT-Plus for the diagnosis of LTBI in pregnancy. A recent study from Uganda also reported strong correlation in IFN-γ responsiveness between TB1 and TB2; however, 18% of women considered to have LTBI only showed TB2 reactivity (229). Furthermore, indeterminate results were found in 9.2% participants in that study, but such results were rare in our studies.

Previous studies have found higher rate of test positivity for QFT-Plus compared to QFT-GIT in persons with active TB (230–233) and in those with recent TB exposure (177). The selective TB2 response appears to be of greater importance for detection of active TB than for LTBI (175,234), in line with studies showing CD8+ T cells responses to be more prominent in active TB than in LTBI (92,234–236), as well as in persons with recently acquired Mtb infection than remotely infected individuals (177). Mtb-specific CD8+ T cell responses have also been shown to be less affected by HIV co-infection (237), and have been observed among young children with active TB disease (180).

However, according to our results (Paper I), the contribution of CD8 T-cell responses appears to be modest for the diagnosis of LTBI in pregnant women. The performance of the assay for detection of LTBI was comparable in women with and without HIV infection. In another study, QFT-Plus showed satisfactory performance among HIV-positive persons with active TB but sensitivity was lower in severe immunosuppression (CD4<100 cells/µl) (233). Most of the HIV-positive women included in our study were on antiretroviral therapy.

One problem related to the use of QFT assays is test variability around the binary threshold level of 0.35 IU/mL at repeated testing. IFN-γ values around this cut-off have been shown to be subject to high rates of conversion and reversion in serial QFT-testing of healthcare workers in low TB-endemic settings (238,239). This phenomenon has presented practical problems in correct interpretation of QFT results. In 2010, Centre of Disease Control (CDC) recommended re-testing of individuals with QFT results near the dichotomous cut-off value (240). The high variability has also led to the proposal of a borderline range (uncertainty zone) for interpretation of QFT results. In low-TB burden countries, different borderline range has been proposed; for example, in Sweden, a borderline range of 0.20–0.99 IU/ml is commonly used, and a high proportion of subjects with borderline results were negative (<0.20 IU/ml) on retesting (164). In our study (Paper I), we explored the distribution of QFT-Plus results within the borderline range of 0.20-0.70 IU/ml. This definition was initially proposed by Nemes et al (168) in a South African cohort, and has since been used by other researchers (238). We noted that Mtb-triggered IFN-γ levels within this borderline range were common in pregnant women (8.8% in TB1 and 8.6% in TB2), especially among HIV-positive women, among whom 29% had IFN-γ levels within the borderline range compared to 7.6% among HIV-negative women.

Although several technical factors could explain test variability, the presence of immune suppression, as observed by lower Mtb-specific IFN-γ production in HIV-positive pregnant women, is a plausible reason for high proportions of QFT borderline results in our study population. For individuals residing in low-endemic regions, the reversion of borderline results to negative on repeated testing probably indicates false positive QFT results (164). While IFN-γ borderline results may be due to false-positive reactions in persons with low pre-test likelihood of TB infection, such results could also represent true LTBI, especially in individuals with different types of immunosuppressive conditions living in TB-endemic settings. This suggests consideration a lower cut-off level to define positive QFT results in such individuals. For this reason, we separately presented data using the lower cut-off (0.20 IU/ml) along with the recommended threshold level (0.35 IU/ml) in our studies. However, further studies are needed to determine whether this lower cut-off level is adequate to improve test sensitivity and define positive QFT results in immunosuppressed individuals.

## Alternative biomarkers for classifying LTBI status among pregnant women

IFN-γ is used as a single readout marker of T cells responsiveness to Mtb in IGRAs, but other mediators secreted by immune cells in response to Mtb stimulation might be considered as biomarkers for determination of LTBI status. In particular, IFN-γ values within the borderline range leads to problems in clinical interpretation. Even with the inclusion of TB2 in the QFT plus assay, in an attempt to improve sensitivity, we found that concordant IFN-γ borderline results in response to both TB1 and TB2 are common, in accordance with other studies (169,241). Thus, alternative strategies to elucidate the LTBI status of subjects with borderline QFT IFN-γ results need to be considered.

In paper II we therefore hypothesized that measurement of other cytokines than IFN-γ in Mtb-antigen stimulated whole blood could lead to better discrimination between women with and without LTBI, with focus on women with IFN-γ values within the borderline range. We found three markers, MCP-2, IP-10 and IL-1ra, that distinguished women with high (≥0.70 IU/ml) and borderline QFT IFN-γ levels from those with low levels (<0.20 IU/ml), in both TB1 and TB2 antigen stimulations, with comparable performance in HIV-positive and HIV-negative pregnant women. Moreover, these three markers showed the highest discriminating performance between persons with QFT-borderline and QFT-low IFN-γ results, suggesting that these markers may improve the sensitivity of the QFT-Plus assay for detecting LTBI in pregnant women with IFN-γ borderline results.

Many researchers have focused on exploring biomarkers with capacity to discriminate between active TB and LTBI (242–245). We mainly used such studies to select candidate biomarkers for the analyses in paper II and IV. Panels of secreted cytokines from QFT supernatants have been suggested as potential biomarkers for this purpose, although none of these markers has been approved for clinical use (128,246,247). Our results are in agreement with previous studies showing elevated levels of IP-10, MCP-2 and IL-1ra in QFT-positive supernatants compared with controls (128,247). In accordance with our finding of elevated IP-10 and IL-1ra concentration in persons with borderline QFT results, Wergeland et al. (227) reported higher TB antigens stimulated levels of these markers in persons with QFT borderline results compared to controls, suggesting that individuals with this cytokine profile have true LTBI. These results also suggests that patterns of these markers could be used to further analyze samples with QFT IFN-γ results within the borderline range. However, the design of our study was not intended to define a threshold for this discrimination.

Several alternative markers have been suggested to increase the sensitivity of LTBI detection in immunosuppressed subjects (128,247). Among others, IP-10 chemokine has been investigated for this purpose (127,249,250). In our study (Paper II), we observed similar secretion patterns of IP-10 and MCP-2 in both HIV positive and negative pregnant women in response to Mtb antigen stimulations. This suggests that these markers could be more robust than IFN-γ for detection of LTBI in persons with HIV. IP-10 is induced by IFN-γ, but is expressed at higher levels than IFN-γ (128). Since IP-10 mainly is secreted by monocytes and also can be induced by various cytokines (128), its response to TB is less dependent on CD4 cell mediated immune response, suggesting that IP-10 may be a promising alternative IGRA readout marker in immunocompromised subjects (195,196,250). Similarly, MCP-2 is also a chemokine and functions as a chemoattractant of T-cells to the site of Mtb infection (251). It has also been explored as a TB biomarker, but has been reported to have insufficient capacity for this purpose in HIV-positive persons (252). IL-1ra is a biological antagonist of IL-1 produced by macrophages as well as a range of other cells (253). IL-1ra has been proposed as a potential marker for TB infection and for monitoring of TB treatment response (247,254).

Next, using a combination of the QFT Mtb-antigen stimulated IP-10, MCP-2 and IL-1ra expression patterns, we set out to classify the LTBI status of women with borderline IFN-γ results with the help of machine learning algorithms. In the absence of a diagnostic gold standard for LTBI, we applied stringent criteria for selecting two reference groups: i) low likelihood of LTBI; which were HIV-negative, non-pregnant women without prior active TB and QFT-IFN-γ <0.20 IU/ml; ii) high likelihood of LTBI; which were pregnant, HIV-negative women with QFT-IFN-γ >0.70 IU/ml. Using KNN algorithms, a combination of the three markers clustered 13 (42%) of the women with borderline results together with the positive control group, and according classified them as having high-likelihood of

LTBI. All of these women had QFT-IFN-γ results above the recommended threshold (>0.35 IU/ml). These results support the accuracy of this IFN-γ cut-off level for LTBI definition in pregnant women.

Thus, in Paper II we explored the concept of using alternative cytokine markers separately or in combination to investigate borderline IFN-γ responses after Mtb antigen stimulation in pregnant women. Apart from measuring alternative cytokines stimulated by the antigens (ESAT-6 and CFP-10) included in the QFT formulation, whole blood stimulation by inclusion of other Mtb antigens with improved immunogenic potential might be considered to determine the LTBI status of subjects with QFT borderline results. For examples, antigens expressed during TB latency, such as dormancy of survival regulon (DosR), the specific region of the Mtb genome containing about 50 genes that are triggered during latency (255). Numerous studies have explored dormancy antigens such as Rv0081, Rv1733c, Rv1737c, Rv2029c, Rv2628 for discriminating active TB from LTBI (256). Long term stimulation of whole blood with these antigens might boost the response of various cytokine biomarkers, which could be considered for further investigation of persons with borderline QFT IFN-γ results.

# Impact of pregnancy on the immune control of latent TB infection

Pregnancy-associated immune modification is necessary to prevent rejection of the fetus, but could also impair the control of Mtb in latently infected individuals. Th1 cellular immunity is of particular importance for control of TB infection (60). Reduced Th1 responses during pregnancy have been linked to increased risk of infectious diseases such as influenza (257), malaria (206), cytomegalovirus and other herpesviruses (19), as well as SARS-CoV-2 (260). The impact of pregnancy on Mtb-specific immune responses in women with LTBI is not well understood, and has mainly been studied in HIV-positive women (218,219,225). In our cross-sectional analysis in paper I, we observed that Mtb antigens stimulated IFN-γ levels were higher, while mitogen induced IFN-γ concentration was reduced, in women tested at later stage of pregnancy, irrespective of HIV co-infection.

In paper III, we aimed to further explore this unexpected finding by investigating the longitudinal dynamics of Mtb-triggered IFN-γ levels during pregnancy and post-partum. We found that Mtb-stimulated IFN-γ levels were elevated and mitogen-induced IFN-γ response was decreased at the 3rd trimester, while no changes in IFN-γ levels were observed comparing early pregnancy and 9 months post-partum. Our findings differ from those in several other cohorts of pregnant women (218,219,225). For example, Mathad, et al. (220) reported lower Mtb-triggered IFN-γ concentrations at delivery in 60 HIV-negative Indian women sampled

longitudinally. In another study performed in the US, Lighter-Fisher, et al. (221) found comparable Mtb-stimulated IFN-γ levels at different trimesters of pregnancy in 25 HIV-negative women. Although Mtb-triggered IFN-γ responses increased in later pregnancy in a majority of our study participants, some women showed declines at this timepoint of pregnancy. This variability in immune profiles between individuals could be due to differences in bacterial activity in women classified as having LTBI; whereas some of these women may harbor viable bacilli, in others the positive QFT reaction could reflect TB-specific memory T cell responses and spontaneously cleared TB infection.

Our results of elevated Mtb-elicited IFN-γ response during later stages of pregnancy are in clear contrast with previous longitudinal studies in Indian and Kenyan women with HIV, showing decreased Mtb-specific IFN-γ responses at this timepoint (218,219). Furthermore, a recent longitudinal multicenter study in women with HIV living in high TB burden countries found lower Mtb-triggered IFN-γ responses at delivery, with subsequent increases postpartum (225). Although we were unable to investigate the underlying mechanisms involved in increasing Mtb-triggered IFN-γ response later during pregnancy, we consider it likely that this reflects higher exposure to Mtb antigens. In turn, this could be due to elevated bacterial activity in LTBI granulomas, caused by physiological immune modifications during pregnancy.

The contrasting longitudinal expression patterns of Mtb-triggered IFN-γ found in our study and in other studies could be due to differences in HIV co-infection status. It is possible that women with HIV and LTBI have reduced capacity to mount adequate Th1 cellular responses to increased Mtb activity during pregnancy (262). This phenomenon could also be involved in the high risk of active TB observed in women with HIV living in TB-endemic settings (226).

IFN-γ plays an important role in the immune control against LTBI reactivation (60), with the containment of Mtb bacteria in the granulomas of latently infected person mainly relying on IFN-γ secreting Th1 immune response. Reduced capacity of Th1 cells to secrete Mtb antigen specific IFN-γ has been reported by several investigators (263,264), and both pregnancy and HIV could impact IFN-γ-producing CD4 T cells in women with LTBI (218,265).

In contrast to the patterns of Mtb-specific IFN-γ secretion, mitogen-stimulated IFN-γ responses were reduced during the 3rd trimester compared to early pregnancy and post-partum. Interestingly, these patterns found in HIV-negative women in our study are similar to those reported in women with HIV in other longitudinal studies (218,219). These results are in accordance with previous findings, showing that pregnancy linked alteration in systemic immunity is most pronounced at later stages of pregnancy (26).

Since the immune response to Mtb infection during pregnancy involves several types of cellular responses, quantification of a single cytokine marker might not

provide sufficiently detailed information on how pregnancy influences the immune control of TB. In paper IV, we therefore studied a range of immune mediators that can be released by different subsets of T cells and other immune cells in response to Mtb-antigen stimulation. In line with the Mtb-specific IFN-γ expression observed in study III, we found that Mtb-specific IL-2 and IP-10 expression was increased at 3rd trimester compared to earlier stages of pregnancy and post-partum in HIV-negative women with LTBI.

Elevated levels of IL-2 and IP-10 at late pregnancy further support the hypothesis that Mtb-induced expression of proinflammatory cytokines indicate higher Mtb antigen exposure, which in turn suggests increased bacterial replication in granulomas of latently infected women. IL-2 is predominantly secreted by central memory T cells, but also by effector memory T cells (110). In our study, the expression pattern of Mtb-stimulated IL-2 release at the 3rd trimester was comparable to that of IFN-γ. These results suggest that cellular Mtb antigen exposure during late pregnancy activates memory T cells. Furthermore, Mtb-specific IL-2 response was increased post-partum. This finding may be compared with those reported by Millington et al. (112), showing an increased response of Mtb-specific T cells secreting IL-2 in persons following TB treatment completion, implying that IL-2 responses reflect expansion of memory T-cells. The Mtb-specific responses of IP-10 and MCP-2, which are largely driven by IFN-γ, also showed longitudinal changes during pregnancy, similar to those observed for IFN-γ. Thus, the elevated secretion of Mtb-specific IP-10 in the 3rd trimester further support the hypothesis of increased Mtb antigen exposure during late-stage pregnancy.

These results are in contrast with another study performed in Ethiopia, which reported suppression of Mtb-specific Th1 cytokines and enhanced secretion of Th2 cytokines in pregnant women with LTBI (266). Another study from India also described lower levels of Mtb-stimulated IFN-γ and IL-2 in samples obtained close to delivery (219). Both these studies included women with HIV (219,266), whereas all women in our studies were HIV-negative. Similar to the findings in Paper III, with regard to Mtb-triggered IFN-γ secretion, we consider it plausible that these differences are explained by failure of HIV-positive persons to mount adequate Mtb-specific immune responses to control increased bacterial replication during pregnancy. Our results suggest that these responses may lead to resumed immune control of LTBI in most HIV-negative women, and that the reactivation of LTBI is of transient character. This is supported by the fact that none of the women with LTBI described in paper III and IV developed active TB during follow-up.

In order to maintain maternal tolerance to fetal antigens, Treg cells expand during pregnancy. These cells secrete anti-inflammatory cytokines, such as TGF-β (267). TGF-β1 is a multifunctional cytokine, which also is involved in the pathogenesis of TB by inhibition of T cells and macrophages (268). In paper IV, we assessed TGF-β1 response as a marker of Treg activity during pregnancy and post-partum, and found lower levels of Mtb-induced TGF-β1 during the 3rd trimester compared to

early pregnancy. It is possible that the decrease of Mtb-specific TGF-β1 during late pregnancy reflects relocation of memory Treg cells to pulmonary sites of TB infection (269), similar to what has been reported for experimental influenza infection (270).

Apart from studying TB-specific immune responses during pregnancy, we also explored patterns of non-specific of cytokine secretion longitudinally. In women with LTBI, the levels of IL-2, IP-10 and MCP-2 were increased post-partum compared to early and late pregnancy. This finding is in accordance with an Indian study showing decreased systemic inflammatory markers in women with LTBI during pregnancy (271). In addition, women with LTBI had higher levels of IL-2 and IP-10 postpartum compared to women without LTBI, and IP-10 levels were also higher at 3rd trimester in women with LTBI compared to those without LTBI. These findings could imply increased systemic inflammation, a phenomenon which might also be induced by transient reactivation of LTBI infection.

In paper III and IV we chose to study the TB-specific immune response in HIV-negative pregnant women with LTBI in order to understand the influence of pregnancy on the immune control of LTBI. These women had no reported other comorbidities known to affect the immune response to TB infection. In these subjects the elevated Mtb-specific proinflammatory cytokine responses could indicate transient TB reactivation that remained asymptomatic during follow-up. Thus, we cannot with certainty exclude that these women experienced active TB, but with an asymptomatic clinical picture, which might have resolved without treatment. In TB-endemic settings, asymptomatic active TB is not uncommon, and studies have reported positive sputum cultures in persons without clinical signs and symptoms (272,273). Therefore, it would have been interesting to obtain sputum samples for TB culture from women with immunological signs of TB infection during pregnancy. We plan to include this in our ongoing studies. However, we have assessed incidence of active TB with bacteriological sputum testing for participants with suggestive clinical manifestations during follow-up, and none of these persons met bacteriological nor clinical criteria for TB. Since only a minority of our participants were HIV-positive, and since longitudinal samples were not available from most of these women, we did not include women with HIV for comparison. Moreover, the extent of immunosuppression of women with HIV is heterogeneous; in our cohort, most women were receiving antiretroviral therapy at enrolment, and the median CD4 cell count was relatively preserved.

# CONCLUSIONS

- The QFT-Plus assay identified Mtb-infection in 277 among 829 pregnant women (33%) using the recommended cut-off level 0.35 IU/ml, with high agreement between TB1 and TB2 antigen formulations included in the assay. The proportions of women classified as having LTBI were similar with regard to HIV infection status.
- QFT IFN-γ borderline results were found among a high proportion (29%) of HIV-positive women (compared to 7.6% among HIV-negative women). Women with HIV also had significantly lower median IFN-γ levels compared to HIV-negative participants
- The concentrations of three cytokines (MCP-2, IP-10 and IL-1ra) in Mtb-antigen stimulated supernatants could differentiate women with IFN-γ borderline results from those with IFN-γ results ≤0.2 IU/ml or >0.70 IU/ml, with similar performance in women with and without HIV infection.
- A combination of MCP-2, IP-10 and IL-1ra classified 42% of women with borderline results as having high likelihood of LTBI.
- In longitudinal analysis we observed elevated Mtb-triggered IFN-γ secretion and lower mitogen stimulation during the 3rd trimester of pregnancy compared to 1st/2nd trimester in HIV-negative women, suggesting increased Mtb antigen exposure at later stages of pregnancy.
- Further investigations revealed dynamics in the Mtb-stimulated and unstimulated cytokine profiles, mainly detected by increased expression of Mtb-triggered IL-2 and IP-10, and decreased Mtb-triggered TGF-β1, secretion at the 3rd trimester of pregnancy compared to 1st/2nd trimester. This provided further support to the hypothesis that Mtb antigen exposure increases at later stages of pregnancy. This phenomenon could be due to reactivation of LTBI; however, since none of the women developed active TB during follow-up, we consider this to be of transient character
- Non-specific cytokine response demonstrated higher levels of IL-2, IP-10 and MCP-2 at post-partum compared to early and late pregnancy in women with LTBI.

# FUTURE PERSPECTIVES

Although register based studies performed in low-incidence settings show an increased risk of active TB during pregnancy and post-partum (15,20), there is lack of data on the burden of TB in connection to pregnancy in TB-endemic regions, posing challenges for optimal management of TB in pregnant women. Reactivation of LTBI is a major source of new TB cases and is an obstacle for accomplishment of the global End TB strategy. For this purpose, WHO recommends testing and treatment in individuals at high risk of LTBI reactivation. However, the diagnosis of LTBI is challenging due to lack of methods that can identify those at highest risk of progressing to active TB. With the available methods for detecting LTBI, prior studies have shown that performance may be reduced during pregnancy, especially in women with HIV.

The studies included in this thesis are based on Mtb antigen-stimulated whole blood obtained during pregnancy and post-partum follow-up from women living in a TB-endemic setting. In this setting, the new QFT-Plus assay showed similar performance in identifying LTBI in pregnant women with and without HIV infection. Although the rate of indeterminate results was low, women with HIV had lower IFN-γ responses and a substantial proportion of these women had QFT IFN-γ results within a borderline range. Re-testing of persons with borderline results is recommended (164,240), but this strategy may not reduce the occurrence of borderline results on retesting. Lowering the QFT threshold level to define LTBI in persons with immunosuppression could instead be considered.

In addition, currently available IGRAs, including the QFT-Plus assay, are expensive and labour-intense to implement for clinical use in resource-limited settings. For this reason, LTBI screening is not performed and IPT is provided blindly for people with HIV in high TB burden countries, such as Ethiopia. Therefore, simpler LTBI diagnostic methods are needed. For this purpose, we plan to evaluate an automated IGRA test, QFT® Access, designed to TB detection in setting with limited infrastructure, in our ongoing cohort study.

Measuring immunodiagnostic biomarkers other than IFN-γ, that are secreted by different immune cells in QFT supernatants, has been suggested as potential strategy to improve performance of the QFT assay (128). We have identified biomarkers that may improve the detection of LTBI separately or in combination. However, a diagnostic gold standard is lacking to evaluate the sensitivity of these markers. Other

studies have included persons with active TB for such evaluation, but we used machine learning classification algorithm and designed our reference groups for low and high likelihood of latent TB infection. The combination of IP-10, MCP-2 and IL-1ra appear to be most promising alternative markers to elucidate the LTBI status of women with QFT-borderline results. Further studies are warranted to define the threshold levels for determination of LTBI using these markers, as well as validation in larger studies before to consider in clinical use. Furthermore, additional studies of promising and potential novel antigens to differentiate viable LTBI from cured and recent LTBI from remote LTBI are needed to validate Mtb-specific immune responses to LTBI.

The impact of pregnancy for control of Mtb in latently infected women is not completely understood. The findings in paper III and IV add knowledge on how pregnancy affects the immune control of LTBI. Our data show higher Mtb-stimulated proinflammatory cytokine patterns at later stage of pregnancy in HIV-negative women. This could indicate restimulation of T cell responses due to increased bacterial replication in LTBI granulomas as an indicator of transient TB reactivation.

The increasing proinflammatory cytokines response during 3rd trimester of pregnancy could also be a sign of TB reactivation. Although the women included in our study did not show clinical signs of active TB, we cannot exclude asymptomatic active TB (which may have resolved spontaneously without TB treatment), and sputum cultures at this time-point could reveal if this is the case.

Beyond measuring Mtb-specific cytokine expression, it would be interesting to perform detailed phenotypic and functional characterization of immune cell subsets in whole blood of pregnant women with LTBI, which could further clarify how pregnancy affects the immune control of Mtb infection.

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