1) TITLE: Impact of maternal HIV on *Mycobacterium tuberculosis* infection among peripartum women and their infants

Short title: Mother infant TB infection Incidence and Prevalence Study (MITIPS)

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5) SUMMARY

The global burden of tuberculosis disease (TB) in pregnancy[1] contributes to substantial morbidity and mortality among HIV-infected women and their children.[2] HIV increases progression from *Mycobacterium tuberculosis* (Mtb) infection to TB,[3] and the risk of TB appears higher in pregnant/postpartum women.[4] However, it is not known whether maternal HIV increases susceptibility to peripartum maternal or infant Mtb *infection*. The prevalence of Mtb infection among HIV-exposed infants is poorly defined and critical to determine due to high rates (30-50%) of progression to TB among young children.[5] Additionally, the performance of latent tuberculosis (LTBI) diagnostic tests, including tuberculin skin tests (TST) and interferon gamma release assays (IGRA), may be affected by both HIV[6] and pregnancy.[7] Defining the risk of Mtb infection in these populations is important to strengthen TB prevention efforts in maternal child health (MCH) settings. **Using an observational prospective parallel longitudinal cohort design, we will compare Mtb infection incidence (measured by IGRA) between HIV-infected and uninfected pregnant women and Mtb infection prevalence between HIV-exposed and unexposed children. We will also evaluate maternal HIV status and peripartum stage effect on LTBI test performance**

Design:	Observational, prospective study of parallel longitudinal cohorts of HIV-infected and HIV-uninfected pregnant women and their infants
Population:	HIV-infected and uninfected pregnant women and their infants
Sample size:	400 pregnant women (200 HIV+/200 HIV-) and 400 infants (200 HIV-exposed, 200 unexposed)
Intervention:	Mothers will be serially tested for *M. tuberculosis* (Mtb) infection with both an interferon gamma-release assay (IGRA) and tuberculin skin test (TST) in pregnancy, 6 weeks and 12 months postpartum. Infants will be serially tested for Mtb infection at 6 weeks and 12 months of age and TST at 12 months of age.
Study duration:	5 years. Mothers will be followed longitudinally from enrollment in pregnancy to 1 year postpartum. Infants will be followed longitudinally from birth to 1 year of age.

Study sites: Kisumu County Hospital, Ahero sub-district and Bondo district Hospital, western Kenya. Additional sites if needed for enrollment goals: Siya Country Referral Hospital, Ranchuonyo sub-County Hospital, Lumumba Health Center, and Jaramogi Oginga Odinga Teaching and Referral Hospital (JOOTRH) in western Kenya.

Objectives:
1) To estimate the risk of Mtb infection in HIV-infected and uninfected peripartum women
2) To estimate the risk of Mtb infection in HIV-exposed and unexposed infants
3) To determine LTBI diagnostic test performance (QFT-Plus and TST) in HIV-infected and uninfected peripartum women

Hypotheses:
1) HIV will be associated with increased risk of IGRA conversion by 12 months postpartum.
2) Mtb infection prevalence will be higher among HIV-exposed infants due to immunologic differences following maternal HIV exposure and/or increased exposure to active TB.
3) IGRA will identify twice as many women with Mtb infection as TST. TST/IGRA discordance will be higher in HIV-infected women, particularly during pregnancy.

Specific Aims:
1) Determine the effect of maternal HIV on risk and timing of maternal peripartum Mtb infection.
2) Determine the effect of maternal HIV on risk and timing of infant Mtb infection.
3) Determine the effect of HIV status and peripartum stage on LTBI test performance.

R01PDTB: *Nested study to measure the effect of effect of HIV exposure and infection on immunity to TB in children using de-identified previously approved samples:*

De-identified specimens from this cohort (MITIPS which includes HIV-exposed uninfected [HEU] and HIV-unexposed uninfected [HUU] infants) will be used to address AIM 1 and 2 below in a new R01 project (*HIV-infected infants mentioned in these aims are from another cohort and do not apply to the MITIPs*). The study procedures remain unchanged and Emory co-investigators (Day, Cranmer) are already co-investigators on this proposal: Consent for immune responses to TB is part of the parent proposal. De-identified specimens from the Urgent Versus Post-Stabilization ART in HIV-1 Infected Children With Severe Co-Infections (completed PUSH cohort) will be used to address AIM 3 below (consent for immune responses to TB are part of the parent proposal and use of stored specimens was already approved). The following aims include more detailed infant immune characterization than in the parent grant.

Aim 1: Determine the effect of HIV exposure and infection on trained immunity in BCG-vaccinated infants. Hypothesis: Maternal HIV exposure and infant HIV infection dampen induction of trained immunity to Mtb in BCG-vaccinated infants. **Aim 1a.** Define and compare the phenotype and functional signatures of monocyte and NK cell responses to mycobacteria in HUU, HEU, and HIV-infected infants. **Aim 1b.** Determine the effect of maternal and infant factors on infant innate anti-mycobacterial immune responses and impact of immune responses on risk of subsequent Mtb infection.

Aim 2: Test the hypothesis that maternal HIV exposure and infant HIV infection modify antimycobacterial immunity via epigenetic reprogramming of innate immune cells. Hypothesis: Maternal HIV exposure and infant HIV infection results in maladaptive epigenetic reprogramming of infant immune responses important for mediating protection against Mtb infection. **Aim 2a.** Define and compare HIV-associated chromatin accessibility and transcriptome profiles of monocyte and NK cells in HEU, HUU, and HIV-infected infants. **Aim 2b.** Determine the association between chromatin accessibility, transcriptome profiles and functional signatures of monocytes and NK cell responses to mycobacteria.

Aim 3: Determine prevalence, predictors, and effect of anti-mycobacterial immunity in HIV+ children. Hypothesis: ART increases innate and adaptive anti-mycobacterial immune responses and deficits in antimycobacterial immunity will predict Mtb infection. **Aim 3a.** Determine and compare pre- and post-ART anti-mycobacterial immunity in HIV+ children. **Aim 3b.** Identify immune predictors of Mtb infection and markers of TB disease in HIV+ children.

R21MTBPREG: *Nested study to measure the effect* the effect of pregnancy on innate and adaptive immune responses to Mtb using previously approved PBMC samples that permit the study of immune responses pre-, during, and post-pregnancy.

De-identified specimens from this cohort (MITIPS) which includes HIV-infected and HIV-uninfected pregnant women will be used to address the aims below in a new R21 project. Additional samples from the Partners Cohorts will be used including pre-pregnancy samples. The study procedures remain unchanged and additional UW co-investigators (Shah, Lingappa have been added. Consent for maternal immune responses to TB is part of the parent proposal. The following aims include more detailed maternal immune characterization than in the parent grant.

Aim 1. Determine impact of peripartum stage, in context of HIV, on Mtb-specific T cell responses. _Hypothesis:_ Known LTBI+ pregnant women, with or without HIV, will induce fewer Mtb-specific CD4+ IFNγ+ cells overall (Aim 1a), and the quality and magnitude of induced memory T cell responses (Aim 1b) will shift away from a TH1-type T cell response compared to the same women before or after pregnancy. _Approach_: **Aim 1a**: Utilizing flow cytometry on longitudinally collected paired samples, we will compare frequency of ESAT6/CFP-10-specific IFNγ+CD4+ cells as a marker of Mtb infection from pre-pregnancy, pregnancy, and post-pregnancy timepoints in HIV-infected and HIV-uninfected women using generalized linear models to assess association between peripartum stage and frequency of Mtb-specific T cell responses. **Aim 1b:** Expanded antigen-specific CD4+ and CD8+ T cell memory response will be evaluated using an agnostic analysis of T cell functionality (COMPASS) to investigate effect of peripartum stage on CD4+ T cell polyfunctionality including HIV status.

Aim 2. Determine the mechanisms by which pregnancy and HIV together influence innate immune responses to Mtb. _Hypothesis:_ Macrophages collected from both LTBI+ and LTBI- HIV-infected and HIV-infected pregnant women, compared to those collected from the same women before or after pregnancy, will demonstrate diminished proinflammatory cytokine responses to Mtb and increased intracellular Mtb replication. _Approach:_ We will compare concentrations of innate immune cytokines produced (**Aim 2a**) and the degree of Mtb intracellular replication (**Aim 2b**) from monocyte-derived macrophages collected and stored longitudinally before, during, and after pregnancy, following live *ex vivo* Mtb infection, using generalized linear models.

6)

a) INTRODUCTION/BACKGROUND:

HIV-infected individuals and infants have an increased risk of progression from Mtb infection to TB disease.[3] Pregnant and postpartum periods have also been associated with increased risk of TB.[4] Whether maternal HIV increases susceptibility to Mtb *infection* among peripartum women and their infants, and the dual roles of HIV and peripartum stage on latent TB testing are unknown. We propose to determine the impact of maternal HIV on maternal peripartum and infant Mtb infection incidence using prospective parallel longitudinal cohorts of HIV-infected and uninfected mothers and their HIV-exposed and unexposed infants. We will also evaluate the effect of maternal HIV status and peripartum stage on LTBI test performance.

b) LITERATURE REVIEW:

<u>TB contributes significant morbidity and mortality to HIV-infected pregnant women and their children.</u>[2] TB is the leading cause of mortality of HIV-infected individuals,[8] and the third leading cause of death among women of child-bearing age in high burden areas.[2] Maternal HIV/TB is associated with adverse infant outcomes including prematurity, small for gestational age, vertical HIV transmission, neonatal TB, and death.[2,9] Recent estimates indicate the global TB burden in pregnancy is higher than previously thought, with >200,000 pregnant women with active TB in 2011.[1] Epidemiologic data suggest a ~2-fold increased risk of TB during late pregnancy/early postpartum.[4] In a recent pediatric TB model, 7.5 million children were projected to have Mtb infection in 2010, of whom >650,000 developed TB.[10] Pediatric TB-related mortality is underreported due to detection difficulties,[11] and is a top cause of respiratory death among children in sub-Saharan Africa necropsy studies.[12] Children of HIV-infected parents are at high risk of TB exposure and disease.[13,14] **Preventing TB in HIV-infected women and their children is a key step in reducing HIV-related TB morbidity and mortality.**

<u>The influence of HIV in pregnancy on Mtb infection acquisition is not well-defined and could be synergistic (Figure 1).</u> HIV and pregnancy are both associated with progression from latent to active TB.[2-4] **However, it is unclear if HIV infection or pregnancy predisposes individuals to acquiring Mtb *infection*.**[7,15-17] Peripartum women may be at increased risk for Mtb infection due to hormonal and immunologic changes.[2,4,18] Increasing levels of progesterone throughout pregnancy favor transition from Th-1 to Th-2 T-cells responses, speculated to increase risk of influenza,[19,20] and potentially Mtb infection.[2] Th-1 responses reach a nadir late in the 3rd trimester, and rebound soon after delivery.[21] This early postpartum rebound has been described as analogous to an immune reconstitution inflammatory syndrome.[18] In HIV, progression to active TB is due in part to decreases in the number and function of CD4 T-cells, impaired granuloma formation, and altered macrophage response.[22] The binary concept of latent vs. active TB, especially in the context of HIV, is evolving to a continuum paradigm reflecting the complex interactions between host immune status, bacillary load, symptom development and progression to TB disease.[3] It is unclear whether HIV-infected individuals are also at increased risk of *acquiring Mtb infection* secondary to immunocompromise; some studies suggest that a substantial proportion of incident TB cases in HIV-infected individuals are due to new TB strains (i.e. clustered infections) as opposed to reactivation of previous infection.[23,24] **It is plausible that concurrent HIV and pregnancy related immunocompromise may amplify susceptibility to Mtb infection.**

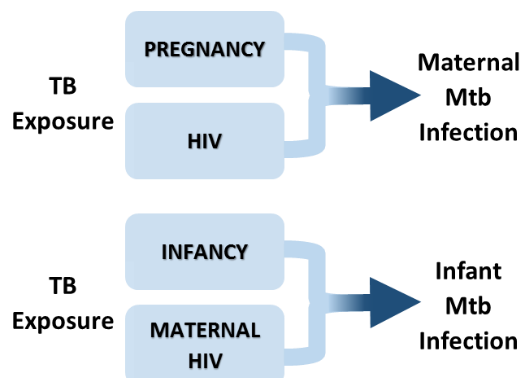


Figure 1: *Hypothesized synergy between HIV/pregnancy and maternal HIV/infancy on risk of Mtb infection acquisition.*

The effect of maternal HIV on infant Mtb risk is not well-characterized. With successful prevention of maternal to child transmission (PMTCT) programs, the population of HIV-exposed but uninfected (HEU) children is growing. These children have increased risk of mortality compared to HIV-unexposed children,[25] and altered immune responses to immunizations and infections, with conflicting data regarding decreased cytokine response to Bacille Calmette-Guerin (BCG) vaccine.[26,27] It is unclear if altered BCG response is linked with Mtb infection risk or progression. **HEU children may be at increased risk of Mtb infection due to altered immunity and TB exposure associated with maternal HIV.**

Table 1: IGRA positive conversions in HIV-infected and/or peripartum individuals

Study	Location	Population	Conversion rate	Conversion cofactors
Aichelburg 2014[15]	Vienna, Austria	HIV+ adults attending outpatient HIV clinic in low TB incidence setting	9% over 24 months	High TB incidence birth country, injection drug use
Pullar 2014[28]	Norway	HIV+ adults attending outpatient HIV clinic in low TB incidence setting	7% over 12 months	Unknown
Jonnalagadda, LaCourse 2015[29]	Nairobi, Kenya	HIV+ pregnant women tested at 32 weeks gestation and 1 year postpartum	17% over ~14 months	HIV+ partner, flush toilet, maternal illness and cough during follow-up
Mathad 2014[7]	Pune, India	HIV- pregnant women tested at pregnancy, delivery and postpartum, nested longitudinal cohort	38% between pregnancy and postpartum (unpub)	Not identified for longitudinal cohort. Cross-sectional cohorts: postpartum stage, urban/periurban residence
LaCourse 2016 (unpub)	Western Kenya	HIV+ pregnant women tested in pregnancy and 6 weeks postpartum	4% between pregnancy and 6 weeks postpartum 13.4/100 person-years	Not identified for conversion due to limited power.

Serial detection of TB-immune responses using IGRA can contribute to estimation of Mtb infection. Historically, diagnosis of Mtb infection was based on TST, but this method can produce false positive results with cross-reactivity to BCG and non-tuberculosis mycobacteria (NTM), and false negative results due to impaired cellular immunity.[30] IGRA measures *in vitro* release of interferon-gamma (IFN-γ, a primarily Th-1 response) after stimulation by Mtb-specific antigens, therefore is not cross-reactive with BCG or most non-tuberculosis mycobacteria, and may be less influenced by anergy.[30,31] Multiple studies in HIV-infected adults have assessed TST before and after initiation of ART.[28,32,33] In this context, TST is unable to discriminate new Mtb infection from immune reconstitution of TST response following ART. Combined IGRA and TST testing can provide information regarding incremental value of a new test using latent class models that yield more realistic estimates of Mtb infection by treating both the index test (IGRA) and reference test (TST) as imperfect tests.[34,35] Although a high proportion of IGRA reversions (positive to negative) have been reported in healthcare workers[36,37] and HIV-infected individuals in low TB burden settings,[15,28,38] reversion appears less frequent in high TB burden settings, particularly with baseline positive IGRA well above the cut-off threshold.[39-41] There are few estimates of Mtb infection using serial IGRA among HIV-infected and/or peripartum individuals in high burden settings (Table 1). QuantiFERON-TB Gold (QFT) IGRA, measures the amount of INF-γ released by primarily CD4+ T helper lymphocytes after TB-specific antigens (ESAT-6, CFP-10 and TB7.7) stimulation. QuantiFERON-TB Gold Plus (QFT-Plus) measures INF-γ released by CD8+ cytotoxic T lymphocytes as well, after stimulation with the same antigens, which may have increased sensitivity in populations with lower CD4 counts including HIV.[42,43] QFT IGRA correlated better with TB contact compared to TST among HIV-infected and uninfected children in Cape Town, South Africa.[44] **Longitudinal IGRA evaluation provides an opportunity to estimate incident Mtb among HIV-infected peripartum women and their HIV-exposed children.**

<u>Data are limited on Mtb infection incidence among HIV-infected peripartum women and their children.</u> Among HIV-infected women in Kenya with negative IGRA during pregnancy, 17.4% had an IGRA conversion at 12 months postpartum[29] (Table 1). Notably, IGRA conversion was substantially above the positive threshold. Among those with positive IGRA, the majority remained positive throughout the postpartum period, suggesting stability in positive postpartum IGRA responses.[45] In a study of 6-month old HIV-exposed Kenyan infants, 10.9% were IGRA positive, suggesting a Mtb cumulative infection rate of >20% at 1 year.[46] In these peripartum and pediatric cohorts, most IGRA conversions occurred without an identified TB contact. In South Africa and Botswana, among 3-4 month old HEU children screened for TB exposure before isoniazid prophylaxis trial entry, >10% had a TB contact.[13] In this trial, even after negative study entry screens for TB exposure, 7.3% of HEU infants developed TB disease and 2.6% were diagnosed with Mtb infection by TST.[14] In a contemporary cohort of pregnant HIV-infected women in Kenya, 3.8% converted from both negative TST/IGRA to IGRA+ by 6 weeks postpartum, for an estimated incidence of detected LTBI infection of 13.4/100 person-years (LaCourse, unpub). **These data indicate Mtb infection risk is high among HIV-infected peripartum women and their children, despite lack of obvious contact.**

<u>Detecting LTBI, particularly recent infection, can identify those individuals who may most benefit from preventive therapy.</u> Although a recent trial of ART and isoniazid preventive therapy (IPT) vs. ART alone for the prevention of TB showed benefit irrespective of TST response,[47] most studies demonstrate greater IPT efficacy among those with positive TST.[48] A 2010 systematic review of RCTs reported 62% reduction in active TB among HIV-infected TST positive adults not on ART.[48] This TB risk was further reduced to 72% with longer duration IPT (36 months) in Botswana.[49] The WHO recommends HIV-infected adults without evidence of active TB receive IPT, and that benefit is greatest for those with positive TST.[50] Immediate ART plus IPT resulted in fewer TB cases vs. deferred ART + IPT or immediate ART without IPT in the Ivory Coast, with greater benefit among those who were IGRA positive.[51] Following IGRA conversion, South African adolescents had an 8-fold higher risk of developing active TB within 2 years compared to non-converters.[52] Children <1 year have a 30-50% risk of progression from Mtb infection to disease.[5] **Identification of early Mtb infection is important, due to the substantially increased risk of developing TB within the first 1-2 years following infection.**

<u>TB cases in HIV-infected individuals may be due to new infection and TB risk remains high despite ART.</u> Molecular fingerprinting studies indicate TB cases in HIV-infected individuals in sub-Saharan Africa are more likely due to new infection as opposed to reactivation.[23,24] Although ART decreases risk of active TB by 67% (with TB risk declining with increase in CD4 and ART duration),[53] the risk of active TB remains significantly elevated despite CD4 recovery on ART[54] and even among those initiating ART at CD4>350,[55] compared to HIV-uninfected individuals. Susceptibility to TB may be related to qualitative T-cell dysfunction and may partially explain why HIV-infected individuals on ART remain at risk of TB even after CD4 count recovery.[56-58] These studies, coupled with universal ART coverage modeling data, indicate that ART alone will be insufficient to prevent future HIV-associated TB.[59] **Given the high proportion of TB due to recent transmission among HIV-infected individuals, including those on ART, early identification of recent Mtb infection is an important component of TB prevention and control strategies in PMTCT programs.**

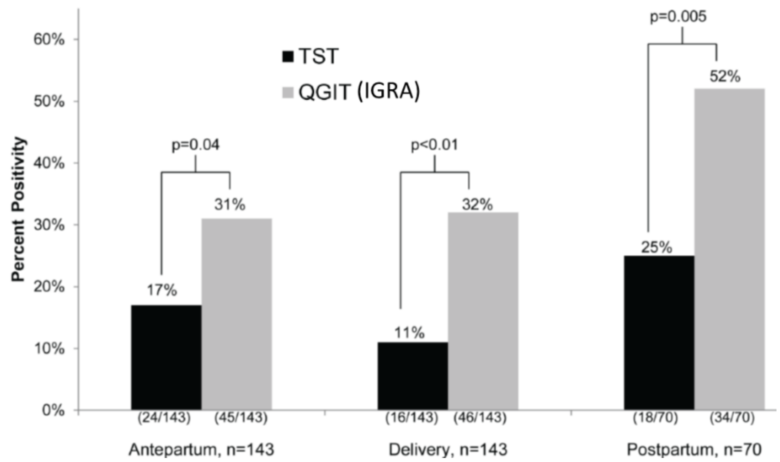


Figure 2*: Cross-sectional comparison of TST/IGRA positivity by pregnancy stage in HIV-uninfected women. IGRA positivity was significantly higher than TST at each stage. (Source: Mathad, Gupta PlosOne 2014)*

<u>The effect of both HIV and peripartum stage on LTBI diagnostic test performance is unknown.</u> In a meta-analysis of IGRA in HIV-infected individuals, pooled sensitivity of IGRA Quantiferon Gold (QFT) in low-income countries was 60% (95% CI 47–75%).[6] In more recent studies, agreement between TST and QFT in HIV-infected individuals was 55-73% (kappa 0.29-0.51).[60,61] In pregnancy, cellular expression of IFN-γ is reduced in the later stages of pregnancy with postpartum rebound.[21] A significant decrease in in-vitro lymphocyte response to tuberculin purified protein derivative was seen in previously TST positive women in the US during late pregnancy and delivery, which returned to early pregnancy levels 24 hours postpartum.[62] Among HIV-*negative* women in India, the proportion of positive IGRA was significantly higher throughout the peripartum period with increased IGRA positivity and IGRA+/TST- discordance postpartum (Figure 2).[7] In a cross-sectional study in India, there was discordance between TST and IGRA regardless of HIV status, with higher discordance among HIV-infected women (kappa 0.22 vs. 0.40) indicating a reliance on TST for LTBI screening would result in >50% fewer HIV-infected pregnant women being treated with IPT (29% IGRA+ vs. 11% TST+, p=.01) (Mathad & Gupta, CROI, 2014). These studies longitudinally evaluated HIV-negative peripartum women and cross-sectionally compared HIV-infected and uninfected pregnant women in India**.**

In our recently completed pilot study of HIV-infected peripartum women in western Kenya, more women were QFT+ than TST+ in pregnancy (n=96, 35.4% vs. 13.5%, p=0.001) and 6 weeks postpartum (n=88 29.6% vs. 14.8%, p<0.001) (LaCourse, unpub). Among 18 consistently QFT+ women, 8 (44%) converted from TST- to TST+, with improved test agreement postpartum (56.9%, κ= 0.20 to 82.4%, κ=0.60, 95% CI 0.42-0.77). Intriguingly, mean QFT mitogen (4.46 vs. 7.64 IU/mL, p<0.001) and Mtb-Ag (1.03 vs. 1.54 IU/mL, p=0.03) responses were lower among all women retested in pregnancy vs. postpartum, (mitogen: 4.46 vs. 7.64 IU/mL, p<0.001, Mtb-Ag: 1.03 vs. 1.54 IU/mL, p=0.03), and specifically among persistently QFT+ women (Mtb-Ag: 3.46 vs 4.48 IU/mL, p=0.007). QFT indeterminate rate was higher in pregnancy (16%) compared to postpartum (0%) due to lower mitogen response. These lower QFT Mtb-Ag and mitogen responses in pregnancy compared to postpartum suggest pregnancy-associated immunologic changes may influence LTBI test performance in HIV-infected women. Larger cohorts with longer duration of follow-up are needed to probe correlates of test discordance and conversion as well as to investigate whether maternal HIV status impacts LTBI diagnostics. **There are no published studies longitudinally comparing TST and IGRA in women who are HIV-infected and pregnant; the combined effects of pregnancy and HIV on LTBI tests are not known.**

7) RATIONALE:

HIV and pregnancy are associated with increased risk of progression to TB disease, but the risk of peripartum maternal and infant Mtb *infection* in the setting of maternal HIV is unknown.

Identifying Mtb infection is important for potential interventions to decrease progression to TB disease in these high-risk groups.

Our preliminary data indicate IGRA conversion is high among peripartum HIV-infected Kenyan women (17% between pregnancy and 12 months postpartum),[29] and Mtb infection prevalence is substantial in their children (10% at 6 months of age).[46] Positive IGRA during pregnancy was associated with a 3 to 5-fold increase in postpartum maternal and infant TB and mortality.[63] In our recently completed pilot study of pregnant HIV-infected women, TST missed >60% of Mtb infection detected by IGRA (LaCourse, IAS 2015). However, lower mean QFT Mtb-Ag and mitogen levels and higher rates of indeterminates (due to low mitogen levels) in pregnancy compared to postpartum, suggest that interferon-gamma release assays are also likely impacted by pregnancy-related immunologic changes. These data demonstrate significant risk of Mtb infection and TB disease in HIV-infected mothers and their infants.

8) HYPOTHESIS & STUDY QUESTIONS:

Hypothesis 1: HIV will be associated with increased risk of IGRA conversion by 12 months postpartum.

Hypothesis 2: Mtb infection prevalence will be higher among HIV-exposed infants due to immunologic differences following maternal HIV exposure and/or increased exposure to active TB.

Hypothesis 3: IGRA will identify twice as many women with Mtb infection as TST. TST/IGRA discordance will be higher in HIV-infected women, particularly during pregnancy.

9) OBJECTIVES

a) BROAD OBJECTIVES:

The broad objective of this study is to determine the impact of maternal HIV on maternal peripartum and infant Mtb infection incidence and to evaluate the effect of maternal HIV status and peripartum stage on LTBI test performance. Defining the risk of Mtb infection in these populations is important to strengthen TB prevention efforts in maternal child health (MCH) settings.

b) SPECIFIC OBJECTIVES:

1) To estimate the risk of Mtb infection in HIV-infected and uninfected peripartum women
2) To estimate the risk of Mtb infection in HIV-exposed and unexposed infants peripartum women
3) To determine the LTBI diagnostic test performance (QFT-Plus and TST) in HIV-infected and uninfected peripartum women

10) STUDY DESIGN AND METHODOLOGY:

Overall Strategy: Using prospective parallel longitudinal cohorts we will compare Mtb infection incidence (measured by IGRA) between HIV-infected and uninfected pregnant women and Mtb infection prevalence between HIV-exposed and unexposed children. We will evaluate maternal HIV status and peripartum stage effect on LTBI test performance (Table 2).

Table 2: Overall Study Strategy

Study Design:	Observational, prospective parallel longitudinal cohorts
Primary Outcomes:	AIM 1: Maternal peripartum Mtb incidence (IGRA) AIM 2: Infant Mtb prevalence (IGRA) AIM 3: Maternal peripartum IGRA/TST discordance
Population:	HIV-infected and uninfected pregnant women and their infants
Exclusions:	Women with active TB in past 1 year or on enrollment
Target enrollment:	Aim 1: ~330[a] pregnant women (~165 HIV+/165 HIV-) Aim 2: 400 infants (200 HIV-exposed, 200 unexposed) Aim 3: 400 pregnant women (200 HIV+/200 HIV-) irrespective of enrollment IGRA/TST status
Follow-up duration:	Mothers: ~15 months (pregnancy[b] - 1 year postpartum) Infants: 12 months (birth - 1 year)
Sampling framework:	Consecutive enrollment of pregnant women in antenatal care and their infants, Kisumu County Hospital, Ahero Sub-district and Bondo District Hospitals, Nyanza region of western Kenya. Additional sites if needed for enrollment goals: Siya Country Referral Hospital, Ranchuonyo sub-County Hospital, Lumumba Health Center, and Jaramogi Oginga Odinga Teaching and Referral Hospital (JOOTRH)

[a]Aim 1 excludes women with +IGRA on enrollment of the 400 women enrolled
[b]Women will be enrolled between 20-34 weeks gestation

<u>AIMS 1 & 2:</u> Longitudinal assessment of Mtb infection risk among HIV-infected and HIV-uninfected peripartum women and their children. **<u>Aim 1: Maternal cohorts (Mtb incidence):</u>** To estimate maternal Mtb incidence, we will identify women with <u>*negative* IGRA</u> at enrollment (~330 women), and serially test them at 6 weeks and 1 year postpartum with IGRA. **<u>Aim 2: Infant cohorts (Mtb prevalence):</u>** We will serially test infants born to <u>all enrolled mothers</u> (200 HIV-exposed, 200 HIV-unexposed) at 6 weeks and 12 months with IGRA.

<u>Aim 3:</u> Prospective study of IGRA/TST concordance among peripartum HIV-infected and HIV-uninfected women. We will measure TST/IGRA concordance by peripartum stage in 200 HIV-infected and 200 HIV-uninfected pregnant women in pregnancy, 6 weeks and 1 year postpartum.

b) STUDY AREA DESCRIPTION

This study will be conducted in Nyanza Province at 3 public sector hospitals: Kisumu District Hospital, Bondo District Hospital, and Ahero sub-District Hospital. We have enrolled women and infants in longitudinal studies at these sites for >4 years. We also enroll at Siya Country Referral Hospital, Ranchuonyo sub-County Hospital, Lumumba Health Center, and Jaramogi Oginga Odinga Teaching and Referral Hospital (JOOTRH) if needed to meet enrollment goals. The area is suitable for this research study because of the high HIV-1 prevalence in the region (19-26% antenatal prevalence), high clinic turnover (approximately 200 new pregnant women per month) and the relevance of the research to the local population. The three hospitals are close in proximity to the KEMRI/CDC laboratory, which will process and store specimens collected during the study.

Kenya is a high TB burden country (annual TB incidence of 268/100,000).[64] Nyanza has a high antenatal HIV prevalence (19-26%), and TB-HIV co-infection.[65] The proportion of TB cases with HIV is ~68%.[66] In our prior study, the burden of culture-confirmed pulmonary TB was ~2.4% among HIV pregnant women at sites in this area. The cumulative Mtb infection of HIV-exposed infants at 6 months is >10%.[46] A large study from AMPATH (Western Kenya) estimated TB disease annual incidence among HIV-infected children (median age 1 year) to be 17%.[67]

c) STUDY POPULATION:

The study population will comprise HIV-infected and HIV-uninfected pregnant women seeking antenatal care services at three public hospitals in Nyanza Province and their children. The population attending these clinics is reflective of women living in Nyanza Province; the source population is rural, diverse in ethnicity, and of generally low socioeconomic status.

Inclusion Criteria:

- Pregnant women ≥16 years of age (between 20-34 weeks gestation) and their infants will be eligible for enrollment.
- Pregnant and plans to remain in the area with their infants until at least 12 months postpartum
- Willing to have serial visits at MCH clinic with serial Mtb infection testing and TB symptom screening until 12 months postpartum

Exclusion Criteria:

- Not pregnant
- Women with TB in the past year or found to have TB on enrollment
- Resides outside the clinic catchment area
- Intends to move from the clinic catchment area during pregnancy and 12 months postpartum period
- Is unable or unwilling to participate in serial visits to the MCH clinic during pregnancy and the 12 month postpartum period

d) SAMPLE SIZE DETERMINATION AND FORMULAS USED:

<u>AIMS 1 & 2:</u> Longitudinal assessment of Mtb infection risk among HIV-infected and HIV-uninfected peripartum women and their children.

Aims 1 & 2 Sample size and power calculations:

<u>Aim 1:</u> We expect ~165 women will have initial negative IGRA in each cohort of 200 women. We will have 80% power to detect a ~6.0-9.6% increase in proportion of IGRA positivity at ~15 months of follow-up in HIV-infected peripartum women if IGRA conversion is 1-5% among HIV-uninfected mothers. (Table 3). **Expected outcomes: Aim 1:** We have >80% power to detect a ~6% increase in Mtb infection HIV-infected women compared to HIV-uninfected women (7% vs. 1% at 1 year postpartum), <u>which is likely adequate given our pilot study which found 17% IGRA conversion at 1 year postpartum in HIV-infected peripartum women.</u>

Table 3: AIM 1 sample size estimates for maternal Mtb incidence, assuming power 80%, 2-sided α=0.05

Proportion of IGRA conversion in HIV-uninfected	No. per maternal cohort*	Min detectable difference in proportion of IGRA conversion in HIV-infected
1%	165	6.0%
	150	6.5%
	140	7.0%
3%	165	7.8%
	150	8.3%
	140	8.9%
5%	165	9.0%
	150	9.6%
	140	9.8%

* excludes women IGRA+ at enrollment, and potential lost to follow-up

<u>Aim 2:</u> With 200 children in each infant cohort we will have 80% power to detect ~5.2 to 9.0% increase in prevalence of IGRA positivity at 12 months of follow-up in HIV-exposed infants, if IGRA conversion is 1-5% in infants of HIV-uninfected mothers (Table 4). **Expected outcomes: Aim 2:** We have >80% power to detect a ~5% increase in Mtb infection between HIV-exposed and HIV-unexposed infants (6.2% vs. 1%), <u>which is likely adequate given our pilot data demonstrating 20% IGRA positive among HIV-exposed children at 1 year of age.</u> If Mtb infection incidence difference is smaller, we will still gain novel information that can be correlated to maternal/infant cofactors.

Table 4: AIM 1 sample size estimates for infant Mtb prevalence, assuming power 80%, 2-sided α=0.05

Proportion of IGRA conversion in HIV-unexposed	No. per infant cohort	Min detectable difference in proportion of IGRA conversion in HIV-exposed
1%	200	5.2%
	165	6.0%
3%	200	6.8%
	165	7.8%
5%	200	8.0%
	165	9.0%

<u>Aim 3:</u> Prospective study of IGRA/TST concordance among peripartum HIV-infected and HIV-uninfected women.

Aim 3 Sample size and power calculations:

<u>Aim 3:</u> With 200 women in each cohort, and assuming the true κ value is 0.50, a two-sided 95% confidence interval for the κ statistic would be +/-0.16 for an estimated LTBI prevalence of 20%. Precision will improve with higher LTBI prevalence and higher or lower kappa. **Expected outcomes: <u>Aim 3:</u>** <u>We expect that IGRA will identify 2-fold as many women with Mtb infection compared to TST.</u> Our pilot data indicates that TST identifies <60% of HIV-infected women with positive IGRA. We expect κ will be low (0.20-0.40), and discordance will be higher among HIV-infected compared to HIV-uninfected women, and higher during earlier compared later postpartum periods.

e) SAMPLING METHOD:

Since the study aims to measure incidence and prevalence of Mtb infection among peripartum women and their infants, we aim to obtain a sample of subjects who are representative of the general population. We will consecutively enroll eligible pregnant women and their subsequent infants from maternal health and prevention of maternal to child health clinics. Recruiting from maternal-health clinics will help ensure representativeness of the study population, because the existing public sector infrastructure serves the majority of women in the population who become pregnant and includes in-built frequent follow-up to 12 months postpartum for delivery of childhood immunizations and infant growth monitoring.

f) DEFINITION OF CASES/CONTROLS IF APPLICABLE:

Not applicable.

g) RECRUITMENT AND CONSENTING PROCEDURES:

<u>Recruitment:</u> HIV-infected and uninfected pregnant women presenting for antenatal care at Kisumu District Hospital, Bondo District Hospital, and Ahero sub-District Hospital will be informed about the study and offered participation by antenatal clinic staff/nurses and referred to the study staff for more information about the research. We also recruit at Siya Country Referral Hospital, Ranchuonyo sub-County Hospital, Lumumba Health Center, and Jaramogi Oginga Odinga Teaching and Referral Hospital (JOOTRH) if needed to meet enrollment goals. Patients who are interested in learning more about the study will be able to complete the informed consent process and the study enrollment visit on the same day. Potential subjects recruited for this trial are limited

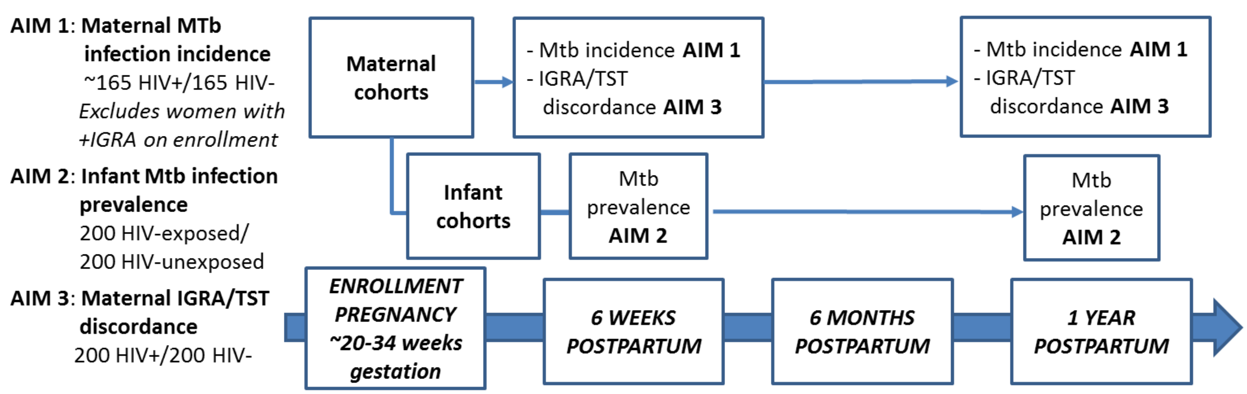
to those persons who are pregnant, ages 16 or older, receiving antenatal services, and their children who are born at the end of the pregnancy during which the subject is enrolled. Potential subjects will have access to all available antenatal, postnatal, pediatric, HIV, and TB services regardless of their decision to participate in the research. Access to antenatal services or maternal child health services will not be delayed based on inclusion in the study.

Following informed consent and study screening procedures, eligible pregnant women will be offered enrollment into the study. Informed consent will occur in a one-on-one counseling session with a study staff person in either Kiswahili, Dholuo or English according to participant's preference. During the informed consent counseling, the counselor will describe the study in detail, review the consent form, including reviewing expected benefits and possible risks of participation, and answer all questions a subject may have. For women who are not literate, the consent will be read to them in the appropriate language of their choosing with a witness independent of the study present. For women who cannot write, an inked thumbprint will be used in lieu of signature. Women will provide consent for their infants. Subjects will be notified that they can withdraw consent for participation in the study at any time. A copy of the consent form in the language of preference will be offered to the participant. Once patients consent to study participation, they will be asked to sign an informed consent form. Informed consent will occur in a one-on-one counseling session with a study staff person and will occur in either Kiswahili, Dholuo or English according to participant's preference.

h) DATA COLLECTION PROCEDURES (clinical and non-clinical, field, data collection instruments):

Follow-up schedule: For the proposed study, we will incorporate Study visits to coincide with scheduled MCH visits. Mothers will have study visits at enrollment, 6 weeks, 6 months and 12 months postpartum. TST and IGRA will be performed on enrollment, 6 weeks and 12 months postpartum. Infants will have study visits at 6 weeks (enrollment), 6 months, and 12 months with IGRA performed at 6 weeks and 12 months and TST performed at 12 months. Please see Figure 3 for Study Flow.

Figure 3: Study Flow



Clinical Procedures:

Maternal cohorts: Pregnant women attending ANC care will be recruited, and enrolled if eligible, after written informed consent. At enrollment, household locator information, HIV care medical identification number, and cell-phone contacts will be obtained to facilitate tracing. A study nurse will administer a standardized questionnaire that addresses sociodemographic, clinical, obstetric and HIV-related factors, TB exposure and history, and maternal and household member TB symptoms (using WHO symptom screen) (Table 5). Mothers with suspected TB on enrollment will be referred to the TB program and if found to have active TB, will be ineligible for participation. At each visit mothers will undergo physical examination with BMI calculation; medical records will be used to abstract data on ART regimen, other medications, maternal HIV viral load and CD4 cell counts by trained study staff. HIV-infected women found to have positive TST or IGRA testing will be referred for IPT. Please see Table 6: Study visits and planned procedures.

Infant cohorts: Infants will be enrolled at the 6 weeks immunization visit where infant medical records and MCH cards will be used to abstract maternal PMTCT ART, infant PMTCT prophylaxis, infant HIV status, birth weight, BCG vaccination date, intercurrent illnesses, and vaccines (Table 5). At each visit infant examination will include growth measures, questionnaire addressing infant feeding (including breastfeeding status), and symptom clinical review (including cough, fever, weight loss). Detailed TB exposure information[68] will be recorded for both mothers and infants including relationship, duration of contact to TB suspect, as well as reported TB symptoms among household members. Please see Table 6: Study visits and planned procedures.

Study participants will be reimbursed approximately 300 Kenyan Shillings (KSH) for transport and effort at each study visit. Payment will be administered at the conclusion of the study visit.

Table 5: Data Collection
Source: Locator survey
Detailed information for locating subjects including address, directions and mobile phone number.
Source: Standardized Questionnaire
Anthropometric: Weight, height, MUAC
Sociodemographic: maternal age, education level, rooms in household, number of household members, household water/electricity source
Pregnancy: gestational age, pregnancy history including history of complication.
TB: TB symptom screen, TB infection or disease and treatment history, family history of TB disease, contact TB history including history of TB disease or symptoms of TB disease in household members and known contact with TB patients over last 2 years
HIV: HIV status, treatment history, history of opportunistic infections, partner/spouse's HIV status (if known), isoniazid preventive therapy,
General health: medication and other general medical conditions, breastfeeding and infant feeding
Source: MCH/PMTCT/HIV/TB medical records
Anthropometric: Weight, height
Pregnancy: gestational age, pregnancy-complications, routine pregnancy labs, medications
TB: TB infection or disease and treatment history, TB w/u lab results, TB treatment outcome
HIV: CD4 count, viral load, date of HIV diagnosis, ART history, current medications, co-trimoxazole prophylaxis, isoniazid preventive therapy, h/o opportunistic infections
General health: medication and other general medical conditions, breastfeeding and infant feeding, immunizations

Table 6: Study visits and planned procedures

	Antenatal		Postpartum				
Maternal cohorts	**VISIT 1**	**48-96 hrs after**	**VISIT 2**	**48-96 hrs after**	**VISIT 3**	**VISIT 4**	**48-96 hrs after**
	Pregnancy		***6 weeks postpartum***		***6 month postpartum***	***12 month postpartum***	
Enrollment	x						
Sociodemographic survey	x		X		x		
Health history	x		X		x		
Physical exam	x		X		x	x	
TB exposure screen	x		X		x	x	
TB symptom screen	x		X		x	x	
Blood draw (15 ml)	x		X			x	
Breast milk			X				
TST placed	x		X			x	
TST read		x		x			x
Referral for HIV testing*			X		x	x	
Infant cohorts			***6 weeks***		***6 months***	***12 months***	**48-96 hrs after**
Enrollment			X				
Sociodemographic survey			X		x	x	
Health history			X		x	x	
Physical exam			X		x	x	
TB exposure screen			X		x	x	
TB symptom screen			X		x	x	
Blood draw (5 ml)			X			x	
TST placed						x	
TST read							x
Referral for HIV testing**			X		x	x	

*For women who are HIV-uninfected on enrollment
** For children who are HIV-exposed

Laboratory/testing procedures:

Maternal cohorts: Mothers will have study visits at enrollment, 6 weeks, 6 months and 12 months postpartum. TST and IGRA will be performed on enrollment, 6 weeks and 12 months postpartum (Figure 3, Table 6). Breast milk will be collected at 6 weeks postpartum. HIV-negative women will be referred to MOH clinics for HIV testing at 6 weeks, 6 and 12 months postpartum.

Infant cohorts: Infants will have study visits at 6 weeks (enrollment), 6 months, and 12 months with IGRA performed at 6 weeks and 12 months (Figure 3, Table 6). Due to the high rates of BCG cross-reactivity after recent immunization, we will not perform TST in infants until 12 months of age. HIV-exposed children will be referred for HIV PCR testing at 6 weeks, and HIV testing at 1 year per national guidelines. In the case of changing HIV-testing guidelines we will refer mothers and infants according to the most up-to-date guidelines at the facilities where they are enrolled.

Blood: Maternal (15 ml) and infant (5 ml) blood will be collected for IGRA assays and flow cytometry. For IGRA QFT-Plus assays, blood is collected in collection tubes (nil, mitogen, TB antigen 1 [ESAT-6 and CFP-10 CD4 peptides], TB antigen 2 [ESAT-6, CFP-10 CD4 and CD8 peptides]) and processed within 16 hours of collection per manufacture recommendations.[42] A response of ≥0.35 IU/ml to TB antigens in either TB 1 or TB 2 (with nil <8 IU/ml and positive mitogen control) will be considered positive. Blood for IGRA will also be drawn in the event of a concern for active TB diagnosis. For flow cytometry blood is processed to separate into PBMC and plasma.

Breast milk collection**.** Up to 30 mls of breast milk will be collected from mothers at 6 weeks postpartum and evaluated for maternal antibodies to TB.

TST: TST will be placed and read by a study nurse within 48-96 hours.[69,70] Trained study personnel will inject 0.1 ml (5 international units) of the designated PPD intradermally to the volar surface of the designated forearm using the Mantoux method. The amount of swelling at the site (induration) will be interpreted at 48-96 hours following TST placement by study staff. TST of $\geq$5 mm will be considered positive in HIV-infected women, and $\geq$10 mm among women and infants without HIV. TST conversion will be defined as $\geq$5 mm of induration regardless of previous results in HIV-infected and an increase of >10 mm in HIV-uninfected women.[71]

TST is contraindicated only for persons who have had a severe reaction (e.g., necrosis, blistering, or ulcerations) to a previous TST; this is a rare occurrence (less than 1%). Prior to TST placement, subjects will be asked if they have had a severe reaction in the past. Subjects with a history of severe reaction will not have a TST placed but will remain in the study. Subjects who experience a dermatologic adverse effect from the TST will be treated in the study clinic for care and management.

All Specimens will be handled using universal precautions, treated as potential Group 3 pathogens in lab, and will be handled by staff trained in Good Laboratory Practices.

R01PDTB:

NK and T-cell assays: For the ongoing MITIPS study PBMC separation and cryopreservation is conducted at the CDC-KEMRI laboratory in Kisumu, Kenya. We have successfully used cryopreserved maternal and infant PBMCs from other studies processed in this laboratory in flow cytometric studies, including studies of Mtb immune responses.

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Phenotype of monocytes and NK cells: We will use PBMCs from infants to evaluate expression of the following markers by flow cytometry: CD3, CD14, CD16, CD40, CD56, CD69, HLADR, TLR2 (CD282), TLR4 (CD284), KIRs (NKB1, CD158a, CD158b), NKG2C, NKG2D, NKp46 and perforin. Cells will be stained with a fixable live/dead viability dye. Monocytes will be defined as CD14+HLA-DR+ cells, and further subdivided into classical (CD14+CD16-), intermediate (CD14+CD16+) and non-classical (CD14-CD16+) monocytes. Monocytes will be evaluated for expression of CD40, TLR2, and TLR4. NK cells will be defined as CD3-CD16+/-CD56+/- lymphocytes, and further subdivided into CD56neg, CD56dim and CD56bright NK cell subsets. NK cells will be evaluated for expression of TLR2, KIRs, CD69, HLA-DR, NKG2C, NKG2D, NKp46, and perforin.

Functional capacity of monocytes and NK cells: Functional profiles of NK cells and monocytes will be interrogated by ICS following stimulation of PBMCs from HUU, HEU, and HIV-infected infants with a panel of Ags: Mtb whole cell lysate, heat-killed C. albicans, heat-killed S. aureus, and Escherichia coli LPS. PBMCs will be plated in two separate 96-well plates, one for evaluation of monocyte and one for evaluation of NK cell function. The plate for monocyte function will be incubated at 37°C for 1 hr, after which Brefeldin A will be added and incubation continued for an additional 5 hrs. Recombinant human IL-2 and anti-CD107a Ab will be added to all wells of the 96-well plate for NK cell function and the plate incubated at 37°C for a total of 24 hrs, with Brefeldin A, monensin added during the final 5 hrs of the incubation. After 6 hr (for monocytes) or 24 hr (for NK cells) incubation, cells will be harvested, fixed and permeabilized, and stained with a fixable live/dead stain and fluorescently conjugated mAbs: CD3, CD14, CD16, CD56, CD69, HLA-DR, KIRs (NKB1, CD158a, CD158b), CD107a, IFN-γ, TNF-α, IL-1β, IL-6, and IL-10.

Data Analysis for NK and T-cell response comparisons between HEU and HUU: We will first assess expression of each immune marker (or functional readout) by monocytes and NK cells from each individual. Data from HUU infants will serve as a reference for monocyte and NK cell phenotype and function in the absence of HIV exposure and infection. For each marker, we will conduct an ANOVA to compare percentages of monocytes and NK cells expressing that marker between groups. Associated p-values will be adjusted using the Benjamini-Hochberg false discovery rate method. Next, we will study the combined pattern of multiple molecules to generate monocyte and NK cell signatures that change with maternal HIV exposure or infant HIV infection. An aggregate model will be determined using a regularized logistic regression. We will utilize paired analyses to analyze differences within infants at 6 weeks and 12 months. From these analyses, we will determine: (i) if there are unique phenotypic/functional signatures of innate immune cells that are present in HEU or HIV-infected infants, which are distinct from signatures associated with HUU infants; (ii) if there is evidence of dampened trained immunity following BCG vaccination by decreased monocyte and NK cell cytokine production to mycobacterial and non-mycobacterial antigens (C. albicans, S. aureus, and LPS) in HEU and/or HIV-infected infants, (iii) whether HIV-associated changes in BCG-induced trained immunity are transient (present only at 6 weeks) or persist through 12 months of age; and (iv) if there are unique phenotypic signatures of monocytes and NK cells that correlate with the functional profile of these cell subsets responding to Mtb Ags.

Maternal immune activation will be assessed in blood samples collected from HIV-infected and HIV-uninfected mothers in MITIPS at 6 weeks postpartum. Plasma will be isolated from blood and stored at −80°C until use in ELISA and multiplex bead-based assays (Luminex) for measurement of molecules associated with immune activation, including TNF-α, IL-1β, IL-6, IFN-α, IFN-γ, sCD14, sCD163, lipopolysaccharide (LPS), endotoxin core antibody (EndoCAb), neopterin, D-dimer, and high-sensitivity C-reactive protein (hs-CRP). Data analysis: Correlates of infant anti-mycobacterial innate immune responses: Based on maternal-child characteristics noted in our recent PMTCT cohorts above, we propose to assess the impact of pre-pregnancy ART, IPT, infant growth and preterm birth on anti-mycobacterial immune responses. In addition, the MITIPS cohort will have data available on maternal LTBI QFT assays and TST during pregnancy and postpartum. We will build multivariable linear regression models including these maternal and infant factors that potentially influence anti-mycobacterial responses. Linear regression models will be built to assess quantitative cytokine levels (in response to mycobacterial stimulation) as the outcome, and covariates above as potential predictors. Impact of anti-mycobacterial immunity at 6 weeks on Mtb infection in HEU and HUU: We will include Mtb-specific antigens in infant flow cytometry assays at 6 weeks and 12 months to determine Mtb infection. We will conduct a nested case-control study comparing cases who acquire Mtb (ESAT-6/CFP-10 positive at 12 months but negative at 6 weeks) comparing anti-mycobacterial immunity at 6 weeks in case and controls overall, and stratified by HEU and HUU cohorts, to determine if there are differences in early innate immune responses responsible for protection from Mtb infection. For these analyses, we will compare cases and controls for cytokine expression and polyfunctional responses in monocytes and NK cells after stimulation with mycobacterial Ags. We will also use COMPASS to compare polyfunctional indexes (Bayesian hierarchical unbiased) in the cases to controls to discern potential protective innate immune phenotypes.

PBMCs from HUU and HEU will be utilized to evaluate chromatin accessibility and transcriptomic signatures of sorted populations of monocytes and NK cells (n=16 infants per group, with equal numbers of male and female infants per group; selected HEU will have the same maternal ART (TDF/3TC/EFV) regimen and maternal viral suppression). Chromatin accessibility and transcriptome assays described below will be conducted on paired infant samples collected at 6-week and 12- month time points.

<u>Lab Methods:</u> Chromatin accessibility profiles of monocytes and NK cells: DNA accessibility represents a surrogate readout of an epigenetic state (active vs. repressed) and can be used to map activities associated with changes in epigenetic modifications and potential regulatory activity of a region. Chromatin accessibility in monocytes and NK cells will be determined using the Assay for Transposase-Accessible Chromatin sequencing (ATAC-seq)87,98. PBMCs will be thawed and stained with CD3, CD14, CD16, CD56 and sorted for purified monocyte and NK cell subsets on a FACSAria II analyzer. Monocytes will be sorted as live CD14+HLADR+ cells; NK cells will be sorted as live CD3–CD16+/–CD56+/– lymphocytes. Sorted cells (5,000 cells each for monocytes and NK cells per sample) will be suspended in a one-step Tn5/permeabilization reaction mix that is consistent across cell types and conditions. Tagmented DNA will be isolated and PCR amplified with i5 and i7 dual indexing primers. Amplified libraries will be quality checked on a Bioanalyzer for a distinct size distribution and sequenced on a HiSeq2500 using 50bp paired-end Illumina chemistry. Sequence reads are mapped to the hg38 reference human genome using Bowtie100 and accessible regions/peaks determined by MACS2. Accessible regions are annotated to their nearest gene using HOMER and custom R/Boiconductor scripts, and significant differentially accessible regions (DAR) determined with edgeR. Quality control guidelines as recommended by Encyclopedia of DNA elements (ENCODE) consortium will be used (e.g., fraction of reads in peaks (FRiP)). Transcriptome of monocytes and NK cells: It is critical to have paired transcription data from samples evaluated for chromatin accessibility changes, as chromatin accessibility only provides information relating to the potential of a regulatory region or promoter. Genes with "open" promoters are not necessarily expressed and may be primed for expression and awaiting the right signal. RNA-seq will be conducted to determine transcriptional profiles of monocytes and NK cells. Monocytes and NK cells will be sorted as described above (1,000 cells each of monocytes and NK cells per sample) and deposited into 96-well PCR plates containing RLT buffer and

immediately frozen. RNA will be recovered using magnetic SPRI beads. RNA quality/integrity will be determined using a Bioanalyzer. RNA-seq libraries will be generated using the Takara SMART-seq v4 cDNA synthesis kit. Final sequencing libraries will be constructed with the Illumina NexteraXT kit, which inserts sequencing adapters and indexes each sample with a unique barcode. Approximately 30 million uniquely mapping sequence reads are required to achieve reliable transcriptome coverage. To control for batch and sample variation, External RNA Controls Consortium (ERCC) synthetic RNAs will be spiked into each sample at the onset of sample prep. Quality control of sequence reads will be assessed using the FastQC tool. The reads that pass QC will be independently aligned against the human reference transcriptome hg38 with TopHat2 using the UCSC KnownGene reference transcriptome table. RNA-seq data will be normalized to the transcript's length, resulting in fragments per kilobase transcript per million reads (FPKM). Differentially expressed genes (DEGs) from RNA-seq data will be identified with edgeR. Aim 2b. Determine the association between chromatin accessibility, transcriptome profiles and functional signatures of monocyte and NK cell responses to mycobacteria. It will be critical to determine the functional significance of transcription and accessibility of key genes identified above on the innate immune response to Mtb. To this end, we will integrate analysis of RNA expression and chromatin accessibility data with phenotypic and functional data. We will evaluate gene expression and DAR maps of chromosomal regions of key phenotypic markers and innate immune effector molecules evaluated previously following stimulation of PBMCs with mycobacterial Ags.

Since many additional effector molecules are involved in innate immune response to Mtb, we anticipate the RNA-seq and ATAC-seq studies will identify additional genes and DARs. To further explore functional relevance of these differences in the innate immune response to Mtb, we will prioritize the most differentially expressed gene transcripts and genes with the most differentially accessible regions and evaluate corresponding protein expression in Mtb Ag-stimulated PBMCs by flow cytometry. For molecules in which Abs are not readily available, or if expression is difficult to detect by flow cytometry, we will evaluate transcript expression in Mtb Ag-stimulated PBMCs by RTPCR. These analyses will thus allow us to determine epigenetic mechanisms (i.e., differential chromatin accessibility) that underlie the phenotype and functional capacity of infant innate immune responses to Mtb. <u>Data Analysis:</u> Analyses and integration of ATAC-seq, RNA-seq, and immunological data: All analyses will be performed using the R/Bioconductor packages111, HOMER software, and custom analysis scripts. First order analyses will include uniform processing and mapping of sequence reads and quantitation of transcription and accessibility enrichment, as well as quality control. Second order analyses will focus on defining differences in like data sets (i.e., RNA vs RNA; ATAC vs ATAC). For each data set, we will compare paired infants' monocyte and NK cells at 6 weeks and 12 months. We will also compare HUU subjects' monocyte and NK cells to responses from HEU and HIV-infected infants. Sex differences have been reported in human innate immune cell function, thus we will also compare accessibility and transcriptome signatures in male and female infants. Together, these analyses will determine if a unique subset of DEGs and constitutive and differentially accessible regions (CAR and DAR, respectively) specifically denotes innate immune cell function in the three groups of infants. In total, the differences identified will serve to highlight genes, potential regulatory regions, and functional pathways with a goal of identifying unique groups of DARs and DEGs that define the phenotype and functional capacity of innate immune cells in HUU, HEU and HIV-infected infants. Third order analyses will integrate RNA expression, chromatin accessibility, and phenotypic and functional data derived to identify direct effects of chromatin accessibility on the transcriptome and immunologic phenotype. We will determine if DAR occur near genes that drive key immunologic, metabolic, cell survival, or other functional pathways through functional gene ontogeny (GO) pathway annotation approaches. Transcription factor binding motifs in DAR will be identified with HOMER. Additionally, the PageRank algorithm, which integrates epigenetic and transcriptome data to rank transcription factors by their importance to a cell type's (e.g., monocytes and NK cells) gene expression program will be employed. This information will define active vs. inactive transcription factor regulatory networks that are collectively regulated during the innate immune response to Mtb. The overlap of these datasets provides the functional consequences (or lack thereof) of the epigenome. Thus, the relationship between DAR, DEGs, and transcription factor binding motifs could identify novel transcription factor properties that are dysregulated in innate immune cells of HEU and/or HIV-infected children and provide further insight mechanisms of impairment of innate immune function in HIV-exposed and infected children.

Aim 3a. Determine and compare pre- and post-ART anti-mycobacterial immunity in HIV+ children

Cohort characteristics: The Pediatric Urgent Start of HAART (PUSH) cohort includes 181 HIV+ children randomized to urgent (<48 hours post-enrollment) or post-stabilization (at 7-10 days post-enrollment) ART[120]. Median age was 1.9 years (IQR 0.8, 4.8), median CD4% 15% (IQR 9, 22), and median viral load 5.7 $\log_{10}$ copies/mL (IQR 5-6.3). Children were enrolled at 4 sites in Western Kenya and Nairobi and had PBMC cryopreservation at baseline, 2 months and 6 months post-ART. In this cohort, children had sputum and gastric aspirate collection for TB culture and Xpert, stool collection for Xpert, and urine LAM[121,122]. Fourteen children had microbiologically confirmed TB at enrollment, 78 had unconfirmed TB and 89 had unlikely TB.

Table 3. AIM 3 Detectable differences

Available PBMC samples Pre- and post-ART by TB status		Minimum detectable differences in post- vs. pre-ART
Confirmed + Unconfirmed TB	(n=50)	4.0%
Unlikely TB	(n=50)	4.0%
ESAT-6/CFP-10+baseline	(n=30)	5.3%
ESAT-6/CFP-10+baseline	(n=20)	6.6%
ESAT-6/CFP-10+baseline	(n=10)	9.9%

Sample size and power: We anticipate increased anti-mycobacterial responses post-ART. With 80% power, 10% SD, and a sample size of 50 we could detect a difference of 4%-9.9% in post-ART vs. pre-ART in paired analyses stratified by TB disease or infection status (Table 3).

Laboratory methods: Among children with available paired PBMC specimens, we will assess anti-mycobacterial immune responses. **Mycobacteria-specific T cell frequency and phenotype in HIV-infected children:** Multi-parameter flow cytometry will be used to quantify and phenotype mycobacteria-specific CD4 T cells in HIV-infected children before and 6-months after starting ART. PBMCs will be stimulated overnight with Mtb Ags (Mtb whole cell lysate, PPD, and CFP-10/ESAT-6 peptide pool). HCMV pp65-specific CD4 T cell will be evaluated in the same individuals to determine phenotypic changes that are unique to mycobacteria-specific CD4 T cells, compared to CD4 T cells specific for other chronic infections. The frequency of CD4 T cells expressing IFN-γ, TNF-α, IL-2, and CD40L will be determined by flow cytometry. Ag-specific T cells will be phenotyped for: (i) immune activation (CD38, HLA-DR, and Ki67); (ii) differentiation status and memory profile (CD45RA, CCR7, CD27, CD28); and (iii) apoptosis (Bcl-2, CD95, Caspase 3). **Anti-mycobacterial innate immune cell responses**: Monocyte and NK cell responses to Mtb Ags, pre- and post-ART, will be evaluated as described in Aim 1a.

Data analysis**:** For each immune marker, we will first compare the post-ART time point to the baseline (pre-ART), using a t-test for paired data, to determine whether ART induces changes in expression. P-values will be [123]corrected for multiple testing. We will then build a composite model to use a subset of molecules to characterize overall effects of ART on mycobacteria-specific CD4 T cell, monocyte and NK cell phenotype and function. Since data are paired, we will use a regularized conditional logistic regression to build the model. We will use COMPASS[90] to identify specific polyfunctional populations of Ag-specific CD4 T cells, monocytes, and NK cells that correlate with viral load, CD4 count and other parameters of HIV disease progression.

<u>R21MTBPreg:</u>

Aim 1a. Compare frequency of mycobacterial-specific IFNγ-producing CD4+ T cells by pregnancy stage. <u>LTBI testing</u>: We will first perform flow cytometry on stored PBMCs from the Partners cohort to determine LTBI status at pre-pregnancy time points, and on known LTBI+ participants (from real time IGRA testing) from MITIPS. We will use short-term assays (6 hours) to measure T cell memory responses Mtb antigens peptide pools (CFP-10/ESAT-6) and controls (medium alone, PHA). We will then measure proportion of CD4+ T cells that produce IFNγ after pooled peptide stimulation when compared with media alone. Participants will be considered LTBI+ if frequency of cells double in the peptide stimulation group compared with negative control.

<u>Analysis Plan:</u> We will use generalized linear models to assess association between peripartum stage and outcome of ESAT6/CFP-10-specific IFNγ+CD4+ cell frequency in LTBI+ individuals between pre-pregnancy/pregnancy (Partners), and pregnancy/postpartum (MITIPS), controlling for time between samples including HIV status stratification. A subset of women from Partners have samples from pre-pregnancy, pregnancy, and postpartum. *Exploratory*: We will estimate incidence of Mtb infection (LTBI- to LTBI+ conversion) from pre-pregnancy to pregnancy (Partners), and pregnancy to postpartum (MITIPS).

Aim 1b. Frequency and magnitude of overall CD4 and CD8 T cell response to Mtb before, during, and after pregnancy. The effect of pregnancy on overall Mtb-specific memory T cell response is not known. Multiple T cell characteristics are important for Mtb control, including "polyfunctional" CD4+ cells or "polycytotoxic" CD8+ cells, increased numbers of "central memory" T cells (CCR7+, CD45RA-), IL-17 production, and a recently described memory T cell phenotype (CXCR3+CCR6+) found in dominant Mtb-specific CD4 phenotypes in LTBI+ individuals. Further, certain T cell phenotypes may be detrimental to TB control, including excess FoxP3+ regulatory T cells, nonspecific T cell activation (HLA-DR+) and exhaustion (PD1+ or CD160+). We will evaluate how pregnancy influences these characteristics in the context of LTBI and HIV.

<u>Analysis Plan:</u> Using the previously screened LTBI+ samples we will measure frequency of single and combined expression of IFN-γ, IL-2, TNF-α and IL-17 in viable CD4 and CD8 T cells; expression pattern of HLA-DR, CD38, CD45RA, CCR7, CXCR3, CCR6 in viable cytokine+ (i.e., specific) CD4 and CD8 T cells; and expression patterns of PD-1, CTLA-4, CD160, and FoxP3 on/in these cells. We will use COMPASS (to determine overall antigen-specific T cell activation and correlate longitudinally between peripartum timepoints. We will measure functionality scores (FS) and polyfunctionality scores (PFS) of all cytokine and activation markers (IL-2, TNF, IFN-γ, IL-17, HLA-DR, CD38, CD154), and overall T cell responses using multiple markers. We will separately compare lineage markers to evaluate how proportions of memory cell phenotypes change over the peripartum period. We will use generalized linear models to compare FS and PFS from pre-pregnancy to pregnancy timepoints and pregnancy to postpartum timepoints controlling for time between samples and stratified by HIV status. We hypothesize Mtb-specific CD4 IFNγ, CD8 IFNγ, and/or polyfunctional responses will be less frequent and contain fewer central memory ($CD45RA^-CCR7^+$) cells but greater total Foxp3+ cells during pregnancy than pre- or postpartum.

Aim 2. Determine the mechanisms by which pregnancy and HIV together influence innate immune responses to Mtb.

Rationale: <u>The influence of pregnancy on macrophage function, including Mtb recognition, cytokine responses, and intracellular replication is not well understood.</u> Macrophages are critical for Mtb control, and their function is altered in a complex fashion over the course of pregnancy and postpartum. Monocytes from pregnant women are increased overall, and demonstrate further increased numbers after influenza infection, compared to nonpregnant women. Macrophages treated with progesterone developed both increased IFNα and diminished TNFα, IL-6 and IL-12 to TLR4 stimuli, which all may be broadly detrimental to Mtb control in macrophages. Tissue-resident macrophages demonstrate impaired phagocytosis in cows,[67] suggesting possible alterations in critical cellular functions for intracellular macrophage control after Mtb infection. Further, HIV infection also induces increased inflammation in macrophages that may impair Mtb immune responses. *We hypothesize pregnancy is associated with decreased proinflammatory cytokine responses and increased intracellular Mtb replication after Mtb infection, when compared to pre- or post-pregnancy*. We will isolate monocytes from LTBI+/- women and measure cytokine production and replication, comparing monocytes during pregnancy with those before and after pregnancy stratified by HIV-infection status. We have experience in conducting all assays proposed in this Aim. Together, these experiments will delineate which cells, inflammatory responses, and effector mechanisms are altered by pregnancy and deepen our understanding of why pregnant women are at increased risk for Mtb progression and infection.

2a. Macrophage cytokine response after Mtb infection: Using cryopreserved PMBC samples from LTBI+/- samples in both cohorts, we will isolate monocytes by plate adherence followed by CD14+ magnetic bead column isolation, which our lab performs regularly. Next, we will infect monocytes overnight with live Mtb (MOI 1) and measure cytokine and chemokine protein levels with multiplex bead assays (Luminex), which permits simultaneous measurement of >30 cytokines and chemokines. In particular, we will measure proinflammatory cytokines (TNFα, IL1α, IL-1β, IL-6, IL-10, IL-12), anti-inflammatory cytokines (IFNα, IL-1ra, IL-10), and chemokines (IL-8, MIP-1α and MIP-1β) critical for Mtb control. We hypothesize macrophages isolated during pregnancy will secrete increased anti-inflammatory IFNα and decreased TNFα and IL-1β after Mtb infection, as previously observed after TLR stimulation of macrophages during pregnancy.

2b. Mtb intracellular replication in macrophages from pregnant women. We will also measure the effects of pregnancy on Mtb intracellular replication in HIV-infected and HIV-uninfected women. Thawed, isolated monocytes will be plated on tissue culture plates and differentiated into macrophages by administering M-CSF for 5 days. Subsequently, we will infect macrophages from women at different pregnancy stages (pre-pregnancy vs. pregnancy and postpartum vs. pregnancy) with a Mtb luminescent strain (MOI 1) and follow intracellular replication for 7 days. We demonstrated previously relative luminescence is a reliable and reproducible proxy for bacterial CFU counts. We anticipate macrophages isolated in pregnancy will demonstrate increased intracellular replication compared to those pre- or post-pregnancy.

i) VARIABLES: dependent, independent, confounders

Several research questions will be addressed in this study. The main study variables and their associated aims are detailed in Table 7.

Table 7. Study variables used to address the specific aims.

Aim	Outcome (Dependent variable)	Exposure (Independent variable)	Measurement	Potential Co-factors
Aim 1	Maternal Mtb infection incidence	Maternal HIV status	IGRA	Maternal age, CD4, viral load, ART use, partner HIV status, TB exposure, household TB symptoms, IPT, peripartum stage, household crowding, employment, magnitude of IFN-γ response
Aim 2	Infant Mtb infection prevalence	Infant HIV exposure status	IGRA	Maternal Mtb infection status, prevalent vs. incident maternal Mtb infection, maternal/infant IPT, CD4, and ART; household crowding, TB exposure, paternal HIV status, infant HIV status, magnitude of IFN-γ response

Aim 3	Maternal LTBI diagnostic performance	Maternal HIV status	IGRA TST	Maternal age, CD4, ART use (among HIV-infected), peripartum stage, time since TB exposure, magnitude of IFN-γ response, prevalent vs. incident maternal Mtb infection

j) MATERIALS:

Equipment: No funds are requested for equipment. Funds will be used to purchase necessary supplies. Lab testing will occur at the internationally accredited KEMRI/CDC lab with available equipment.

Personnel: The grant award includes support for UW investigators, clinic personnel, the data team, and a study coordinator. Study personnel working in Kenya will be hired through UON/KNH or UW-Kenya according to standard procedures.

k) TRAINING PROCEDURES:

Drs. Sylvia LaCourse, John Kinuthia and Daniel Matemo will supervise training of clinical personnel in study procedures. This will include research ethics, LTBI/TB counseling and testing, specimen collection, and completion of study questionnaires and report forms. We will offer PMTCT and ANC staff one hour of training on active TB, LTBI and the impact of TB during pregnancy and in early childhood.

We will ensure that study personnel are trained in communicating important study results to MCH providers. Significant results include: the presence of symptoms concerning for active TB according to Kenya National Guidelines, the results of sputum smear evaluation (AFB-smear and –culture), and the results of tuberculin skin testing. For subjects diagnosed with active TB, we will facilitate referrals to the National Treatment Programme for prompt initiation of treatment. As we anticipate that our study may increase referrals for LTBI prophylaxis and treatment of active TB, we will discuss our study with the staff at the hospital's TB and HIV comprehensive care clinics prior to study enrollment.

l) QUALITY ASSURANCE PROCEDURES:

Clinical care: The study will adhere to Government of Kenya guidelines for the care of pregnant/postpartum women and their infants. Data collected as part of the study will be abstracted from the mother's "Mother & Child Health Booklet" as well as the MCH clinic's medical records. Counseling and testing for HIV will be performed in accordance with government-approved MCH guidelines. HIV-infected study participants will receive their HIV medicines at their local hospital's Comprehensive Care Clinic/MCH clinic, enabling reporting of antiretroviral therapy and follow-up in accordance with the national AIDS strategy.

Procedures to minimize risk of HIV, vertical transmission of HIV
HIV-uninfected women will be counseled in ways to reduce their risk of HIV acquisition, including condom use. HIV-infected women will be counseled on the prevention of mother to child transmission per Kenyan guidelines. Previously HIV-negative women and HEU infants found to be HIV-infected during the study will be immediately referred to appropriate HIV services. HIV evaluation and treatment is free per Kenyan National HIV guidelines.

Procedures to minimize risk of progression from latent to active TB
HIV-infected women with evidence of latent TB, or development of new Mtb infection (by either positive TST or IGRA) during the study will be referred to the HIV comprehensive care clinic on site to receive isoniazid preventive therapy per Kenyan guidelines. Children identified as having

close contact with a person with TB will also be referred to the TB program clinic for isoniazid preventive therapy per Kenyan guidelines. All subjects identified as having potential active TB will be referred to the TB program for further investigation as well as treatment as required per Kenyan National TB guidelines including CXR and Xpert as necessary. Isoniazid prevention therapy and TB evaluation and treatment is free per Kenyan National TB guidelines.

<u>Procedures to minimize risk of pregnancy complications</u>
Women with high-risk pregnancies, or risks of complications will be referred to deliver at their closest MCH-linked maternity center (Kisumu County Hospital, Bondo District Hospital or Ahero sub-District Hospital, Siya Country Referral Hospital, Ranchuonyo sub-County Hospital, Lumumba Health Center, and Jaramogi Oginga Odinga Teaching and Referral Hospital (JOOTRH))). Women or children developing other unexpected health problems that are not addressable within the study clinic will be referred to the appropriate clinic within the hospital.

<u>Procedures to ensure newly diagnosed HIV-infected women receive HIV care</u>
Enrolled HIV-uninfected pregnant women will be referred for HIV tests through MCH trained HIV counselors at enrollment, 6 weeks, 6 months and 12 months postpartum. If any women are found to be HIV-infected they will be referred for HIV care (including prevention of mother to child transmission of HIV services) through the HIV care clinic.

<u>Procedures to ensure newly diagnosed HIV-infected children receive HIV care</u>
Enrolled HIV-unexposed infants referred for HIV tests through MCH trained HIV counselors at 6 weeks and 12 months of age. If any of these children are found to be HIV-infected they will be referred immediately for HIV care through the Pediatric HIV care clinic.

<u>Adherence to protocol</u>: Weekly reporting of enrolment, follow-up, medical complications, laboratory results, and specimen collection will enable us to monitor that the study is running according to approved protocols. Frequent reporting will also enable us to quickly respond to any problems that arise during the study.

<u>Laboratory quality control</u>: The KEMRI/CDC laboratory that will process the specimens and conduct most of the laboratory tests is ISO certified, and participates in external quality control verification.

11) ETHICAL CONSIDERATIONS

a) Consent explanation

- Title: Impact of maternal HIV on Mycobacterium tuberculosis infection among peripartum women and their infants

- Introduction: “*We are asking you and your child to be in a research study. The purpose of this form is to give you the information you will need to help you decide whether you and your child will be in the study or not. Please read the form carefully. You may ask questions about the purpose of the research, what we would ask you to do, the possible risks and benefits, your rights as a volunteer, and anything else about the research or this form that is not clear. When we have answered all your questions, you can decide if you want to be in the study or not. This process is called “informed consent.” This form serves as a both as a record of your consent to be in the study and as a parental permission form. We will give you a copy of this form for your records.”*

<u>The word “you” in this form refers to you and your child</u>.

What you should know about this study:

- *This form explains what would happen if you join this research study.*
- *Please read it carefully. Take as much time as you need.*
- *Please ask the research team questions about anything that is not clear.*
- *You can ask questions about the study at any time.*
- *If you choose not to be in this study, it will not affect any other care received at clinic.*
- *If you say 'Yes' now, you can still change your mind later.*
- *You can quit the study at any time.*
- *You would not lose benefits or be penalized if you decide not to take part in the study or to quit the study later.*

- Objectives of the study: ***"****The goal of any research study is to answer questions. We (the research team listed on the front of this form and our staff) are doing this research study to answer the following question:*

- Does having HIV increase the risk of getting Mycobacterium tuberculosis (MTB) infection in peripartum women and their infants?

Infection with the organism called MTB Mycobacterium tuberculosis (MTB) causes the disease called tuberculosis (TB). Once infected with MTB, some people go on to get TB disease but not all. Young children, people with HIV, and women who are pregnant more likely to get TB disease because their body defenses are sometimes weak. Pregnancy may also increase the risk of developing TB. However, we do not know if HIV increases the risk of getting MTB infection in pregnancy or in general."

- Benefits:

"Potential Benefits for You:

Being in this study might benefit you in the following ways:

- *Close monitoring of TB symptoms which will prompt referral to appropriate screening and treatment facilities*
- *Referral for serial testing of HIV (if currently negative) which may identify newly infected women and their infants.*
- *HIV-infected women with positive TSTs as well as infants found to have recent TB contacts, may benefit from referral for isoniazid prophylaxis, as well as from focused questioning regarding TB risk factors and prompt referral to the TB program if concern for active TB arises.*

Potential Benefits for Others:

- *The research will provide important information regarding whether HIV in pregnancy increases susceptibility to MTB infection in peripartum women and their infants, as well as if HIV affects how well tests for TB infection work.*
- *This information could benefit other women and their children in areas of high HIV/TB burden."*

- Risks:

"What are the potential harms or risks if I join this study?

There are potential harms or risks if you take part in this study. Some are common and some are rare.

Potential Harms and Discomforts (from the most common, to the most rare):

- *Local irritation due to blood draw.*
- *Local irritation due to TST*

- *Loss of confidentiality*

We do not anticipate any study-related physical adverse effects to participants. We believe the risk of loss of confidentiality is low due to measure we have in place to protect your privacy."

- Compensation mechanism: *"Study participants will be reimbursed approximately 300 Kenyan Shillings (KSH) for transport and effort at each study visit. Payment will be administered at the conclusion of the study visit."*

- Alternative treatments: ***"****Whether or not you decide to participate in this research study, you can continue to receive your regular mother-child health care at this clinic."*

- Voluntarism:

"*Whether or not you decide to participate in this research study, you can continue to receive your regular mother-child health care at this clinic."*

"When we have answered all your questions, you can decide if you want to be in the study or not. This process is called "informed consent."

- *"If you choose not to be in this study, it will not affect any other care received at clinic.*
- *If you say 'Yes' now, you can still change your mind later.*
- *You can quit the study at any time.*
- *You would not lose benefits or be penalized if you decide not to take part in the study or to quit the study later."*

"You have the option to take part in this research study because you are pregnant. We will enroll women both with and without HIV."

"If you join this study, you can decide to stop ***at any time, for any reason****. If you decide to stop you would need to talk with site investigators so you leave the study in a safe way."*

"Whether or not you decide to participate in this research study, you can continue to receive your regular mother-child health care at this clinic."

- Type of specimens and amount to be obtained:
"Blood collection. *A study clinician will collect up to 15 mls of blood (teaspoon) from you during enrollment in pregnancy, 6 weeks, and 12 months postpartum. We will also collect up to 5 mls of blood (teaspoon) from your infant at 6 weeks and 12 months of age which will be tested for MTB infection. We will draw an additional 15 mls of blood (teaspoon) from you or 5ml from your infant if you or your infant develop active TB for TB infection testing."*

"Breast milk collection. *We will collect up to 30 mls of breast milk from you at 6 weeks postpartum."*

- **Follow up schedules if applicable/ expected time in study**: "*We would follow you from the time you enroll in pregnancy until 1 year after you deliver. We would also follow your infant from the time they are born until they turn 1 year of age.*

Visit schedule. *You would be seen during pregnancy on enrollment and you and your infant would be seen together at 6 weeks, 6 months and 12 months postpartum. These visits are* *matched with the Kenyan recommended schedule of maternal and pediatric well child/immunization visits."*

- Information on researchers and telephone contacts in case to be contacted

Name	Position	Department	Telephone Numbers
Sylvia LaCourse	Principal Investigator	Medicine, UW	+1-206-616-7217
Grace John-Stewart	Co-investigator	Global Health, UW	+1-206-543-4278
John Kinuthia	Co-investigator	Research & Programs, KNH	+254-722-799-052
Elizabeth Maleche-Obimbo	Co-investigator	Pediatrics, UoN	+254-202-720-947
Daniel Matemo	Study Coordinator	Obstetrics and Gynecology, UoN	+254-722-322-378
Barbara Richardson	Co-investigator	Global Health, UW	+1-206-744-2425

Emergency telephone number: *Dr. John Kinuthia**:** +254-722-799-052*

- Information on the KNH/OUN/ERC in case they need to contact the committee (phone numbers)

"If I have questions about my rights as a research subject, I can call the *Kenyatta National Hospital Ethics and Research Committee, at 2726300 Ext. 44102*.

- Any other necessary information about the study:

"Interviews and questionnaires. *During enrollment, we will ask you questions about your health and the health of people in your household. For example, we will ask you about your HIV and TB history, and whether you or your infant have symptoms of TB. If you have symptoms or test results concerning for TB you may be referred to the local TB Control Programme for further evaluation. If you are referred to the TB Control Programme we would like to access your TB evaluation and treatment records.*

These questions will help us understand what may affect a woman's and her infant's risk of getting MTB infection. We will keep the answers to these questions private."

Additional procedures:
"Tuberculin skin test (TST). *A study clinician will a small needle to put some testing material, called tuberculin, just under your skin on your arm at all of your study visits. We will also do the test for your infant at 12 months of age. We will ask you to return to the clinic in 2-3 days to check the result by measuring if there is a reaction on your skin. TST is a test that is used to diagnose MTB infection, but does not mean you have TB disease. The TST test cannot give you TB, it can only tell us if you have been potentially exposed to TB in the past.*

Future research*. Information that we collect from you, your medical record, and lab results, and specimens may used in future research.*

Missed visit. *It is very important that you come to your scheduled post-partum and infant visits. If you cannot make your appointment, please call the study staff. If you miss your appointment, the study staff will try to contact you. They will do this by trying to call you. They may also talk to the contact people that you list. If they talk to these people, they will not tell them why they are trying to reach you. If study staff cannot reach you, they may visit your household. If this occurs, they will dress in plain clothes and keep the reason for their visit private."*

Additional removal from study procedures:
"The research study clinicians could also decide to take you out of this study. This might happen if we find out that it is not safe for you to continue in the study. It may also happen if you cannot come to enough of the study visits. If we ask you to leave the study we would always explain why and this would not affect other care received at the facility in any way."

Funding of study:

"The study team and/or the University of Washington and Kenyatta National Hospital are receiving financial support from the National Institutes of Health in the United States".

Confidentiality:
"We will keep your identity as a research subject confidential. All of your and our infants test results, medical records, and answers to questions will be kept private. No identifying information of any kind will be released to any other person or agency that is not working on this study, without your permission in writing. We will not publish or discuss in public anything that could identify you. Any specimens you provide, and your medical information will be identified by a code number. All of your information, including the link between your name and code number will be kept in a secure location at the clinic only. Once the study is completed, we will maintain the link for 6 years. After this time we will remove your name and all identifying information from the study files. The study team may share identifiable information about you in the case the study team becomes aware of possible harm to yourself or others.

Although we will make every effort to keep your information confidential, no system for protecting your information can be completely secure. It is still possible that someone could find out you were in this study and could find out information about you. Government or university staff may review studies such as this one to make sure they are being done safely and legally. If a review of this study takes place, your records may be examined. The reviewers will protect your privacy. The study records will not be used to put you at legal risk of harm.

Study records may be reviewed by:
- *University of Washington, including the Institutional Review Board*
- *Kenyatta National Hospital and University of Nairobi, including the Ethics and Research Committee*
- *US National Institutes of Health"*

- Research-related injury:
"If you think you or your infant has a medical problem or illness related to this research, contact Dr. John Kinuthia: +254-722-799-052 right away. He will treat you or refer you for treatment. If you or your child is injured as a result of being in the study, you will be offered free care at the study clinic. If you require medical care that the study clinic cannot provide, we will refer you to the appropriate organizations to receive care for the injury. The costs of the treatment may be billed to you or the National Hospital Insurance Fund (NHIF) just like other medical costs, or it may be covered by the UW's discretionary Human Subject's Assistance Program (HSAP) depending on a number of factors. The researcher may request HSAP coverage by following established procedures. If you wish to request HSAP coverage yourself, you may contact the researchers listed on the first page, or the UW Human Subjects Division at hsdinfo@uw.edu or +1-206-543-0098. Ask the researchers if you would like information about the limits and conditions of the HSAP. The UW does not normally provide any other form of compensation for Injury. However, the law may allow you to seek payment for injury-related expenses if they are caused by malpractice or the fault of the researchers. You do not give up any legal rights by signing this consent form."

- Possible storage of specimen for further analysis with the permission from the KNH/UON/ERC:

"We would like to save your samples at the KEMRI/CDC, University of Nairobi, the University of Washington, Emory University, or the Fred Hutchinson Cancer Research Center for future HIV and/or TB related research and maternal and infant health. This may include testing for other factors which may affect whether a person is more or less likely to get infections, or things that may affect infant and maternal health (mother's health during postpartum period with special emphasis on HIV-related illnesses, infant health with special emphasis on HIV-exposure, and TB exposure).

Information we get from you, and your samples, may be shared with other investigators studying HIV, TB, or mother and child health. We will not share your name or any identifying information with them. An Institutional Review Board or Independent Ethics Committee, which looks at study application to ensure the safety and rights of research participants, must approve future research studies in which we will use your or your baby's samples to obtain information about both of you. Permission from the University of Nairobi's Ethics Committee will be sought before any of these samples are used for future research. These tests are for research and are not useful for your or your baby's clinical care. Before your samples or your baby's samples leave the clinic, they will be assigned a code and your name or your baby's name will not be on them. We will store these samples for ten years after completion of the study. Storage of samples past this time period will only occur with approval from an Institutional Review Board and Ethics Committee.

If you do not want to have your or your baby's samples saved for future research, you can still be in this study and your or your baby's samples will be destroyed once testing for the study is completed. If you agree to store your or your baby's samples now, but change your mind before the end of the study, let the study staff know and we will make sure that your or your baby's samples do not get stored for future research. We will not sell your or your baby's samples. Tests done on your or your baby's samples may lead to a new invention or discovery. We have no plans to share any money or other benefits resulting from any potential invention or discovery with you."

12) DATA MANAGEMENT AND STATISTICAL ANALYSIS PLANS:

Data management plan

Collection and storage of study data. On enrollment, a study chart will be compiled that will contain signed consent forms. With the exception of the locator form and data abstraction from medical records, which will use a paper-based CRF, all other data will be entered directly into password protected tablets using the secure password protected RedCap database sponsored by the UW Institute of Translational Health Science. All data in the database is de-identified and coded by de-identified study ID. The mobile RedCap application allows for data entry onto a tablet without requiring an internet connection at the time of data collection. At the end of each day, data from the table is uploaded to the web-based RedCap online database.

Data will be entered directly by study staff, and the data manager will be responsible for daily upload of data to the online RedCap database. The data manager will discuss each day with the study staff if there are any issues with data entry so that any data entry error is corrected quickly.

The locator form is stored in a locked file cabinet at the study site. The de-identified paper-based forms used only for data abstraction for medical records are de-identified and kept in patient files in a locked file cabinet. Participant files will be accessible only to researchers. Patient identifier and locator information will be kept securely under lock and key.

All laboratory results are de-identified using Study ID. De-identified IGRA QFT-Plus results performed at the KEMRI/CDC lab will be provided in electronic form to be uploaded directly into the RedCap database. Additionally de-identified scanned paper copies of the results will be provided by KEMRI/CDC will be sent directly to the study data clerk from the laboratory to confirm results in the electronic form are correct.

Data checking and validation. The database will be designed with variable range limits to minimize the possibility of inaccurate entries. Correct data entry will be validated by line-listing entered data against printed clinical files.

Monitoring of study protocol and clinical care. The data manager will oversee the generation of weekly reports that will summarize the number of patients seen, medical complications, laboratory results, and specimen collection. These weekly reports of de-identified data will ensure quality assurance for data. This will enable us to ensure that the study is running according to protocol, and to share the data between study investigators in Kisumu, Nairobi, and Seattle.

Statistical analyses

AIM 1 & 2 Outcomes and Analyses:

Aim 1 primary outcome: We will compare the incidence of maternal Mtb infection, defined by positive IGRA conversion between HIV-infected and HIV-uninfected peripartum women. Women with positive IGRA at enrollment or who develop active TB will be excluded from the Aim 1 incidence analysis.

Aim 2 primary outcome: We will compare the prevalence of infant Mtb infection, defined by positive IGRA at 6 weeks or 12 months, between HIV-exposed and unexposed children. Infants found to have active TB will be excluded from the Aim 2 primary analyses.

Additional Aim 1& 2 primary outcome: We will identify maternal cofactors associated with maternal incident Mtb infection, and maternal and infant cofactors associated with infant Mtb prevalent infection (including age, gestational age, ART, others listed Table 7).

Aim 1 primary analyses: We will use generalized estimating equation (GEE) models with a log link and independent correlation structure to compare the primary outcome of incident maternal Mtb infection incidence between the two maternal cohorts. Potential cofactors for maternal Mtb incidence will be evaluated using multivariable GEE models.

Aim 2 primary analyses: We will use Chi square (χ^2) tests to compare prevalence of infant Mtb infection at 6 weeks and 12 months between HIV-exposed and unexposed infants. Cofactors for infant Mtb will be evaluated using multivariable logistic regression models. Cofactor analysis will be include maternal and infant infection status and will be stratified by maternal and infant HIV status to assess any effect modification of HIV infection.

Aim 1 & 2 secondary outcomes #1: We will compare the incidence of a combined endpoint of TB and death between HIV-infected and uninfected peripartum women, and HIV-exposed and unexposed infants using methods described in above Aim 1 & 2 primary analysis.

Aim 1 & 2 secondary outcomes #2: We will compare median magnitude of maternal IGRA responses between HIV-infected and uninfected pregnant women, and between HIV-exposed and unexposed infants using non-parametric tests among IGRA converters and non-converters. Among women with positive baseline IGRA, serial data from 6 weeks and 12 months will be available to estimate stability of IGRA results and reversion. Sensitivity analyses will be performed to estimate incidence using the subset with evidence of persistent detection or above a specified higher threshold level of detection. These data can inform new models to estimate Aim 1 incidence data.

AIM 3 Outcomes and Analyses:

Aim 3 primary outcome & analyses: We will estimate the degree of IGRA/TST agreement at different peripartum stages, using Cohen's kappa (κ) coefficient where $\kappa > 0.75$ represents excellent agreement, $\kappa = 0.4\text{-}0.75$ fair to good agreement, and $\kappa < 0.4$ poor agreement. We will compare the proportion of positive tests at each peripartum stage between HIV-infected and HIV-uninfected women using χ^2 testing. Potential cofactors for positive tests and discordant TST/IGRA (i.e. TST+/IGRA-, TST-/IGRA+) will be evaluated by with multivariable logistic regression models.
Aim 3 secondary outcome & analyses: We will estimate the proportion of reversions for both TST and IGRA, defined as a positive test followed by a negative test. The total proportion of reversion among HIV-infected and uninfected women will be compared by χ^2.

13) STUDY LIMITATIONS AND HOW TO MINIMIZE THEM:

Aims 1 & 2: Limitations/Alternative Approaches: Our sample size and power calculations are based on previous Mtb infection risk estimates in HIV-infected peripartum women and their infants. Total number of incident maternal and infant Mtb infections will limit our power to discern potential cofactors; with only cofactors with appreciable increased risk detected. With fewer outcomes (due to attrition, etc.) minimal detectable difference in proportions will increase. We expect <10% lost to follow-up based on recent studies. This exploration provides an opportunity to define potentially influential risk factors important for defining clinical suspicion, vigilance, or preventive testing/screening approaches. We anticipate data from Aims 1 & 2 will inform larger Mtb infection correlate studies in HIV-infected women and their children which could evaluate relevant or potential cofactors in more detail. Routine TB culture will not be performed, and subclinical TB is possible. All women and infants will be closely monitored for TB symptoms at each study visit. Symptomatic participants will be referred to the TB program for evaluation per national guidelines, which includes Xpert (rapid TB PCR) as first line for HIV-infected individuals. Longitudinal follow-up (~15 months for mothers, 12 months for infants) will aid in TB disease identification. We have used maternal and infant IGRA positivity estimates, based on TB-SPOT.TB IGRA on cryopreserved specimens in a prior PMTCT cohort. New cohorts may have different Mtb infection prevalence/incidence. Maternal PMTCT regimens (Option B+ maternal ART, infant nevirapine) differ from historical cohorts (short-course zidovudine) which may modify maternal and infant Mtb infection risk. To address reliability and reproducibility of low-level IGRA QFT-Plus positive results (≥0.35 and <0.60 IU/ml),[72] we will perform secondary analyses and compare our results with two definitions of positive QFT (≥0.35 manufacture's cut-off vs. ≥0.60 IU/ml). Women may receive empiric IPT within the programmatic setting irrespective of TST/IGRA results. Data from TB contact tracing studies and controlled trials of IPT indicate IGRA response is retained after IPT.[73,74]

Aim 3: Limitations/Alternative Approaches: The total number of positive and discordant maternal TST/IGRA will limit our statistical power to discern potential cofactors. Positive TST may reflect boosting of response with serial testing, or to previous BCG or NTM exposure as opposed to Mtb infection.[75] Most boosting occurs 1-5 weeks after testing.[75] In our study, testing will occur at longer intervals therefore TST conversion will less likely be due to boosting. IGRA are less effected by boosting, but may occur due to previous TST. Although a single cutoff is used to identify positive IGRA, we will perform analyses for discordance using multiple IGRA cut-offs. Women may receive empiric IPT within programmatic settings irrespective of TST/IGRA results. TB contact tracing and controlled IPT trials indicate IGRA response is retained after IPT.[73,74]

14) TIMELINE / TIME FRAME:

Enrollment will start in Year 1 and continue until the beginning of Year 5. A study time-line is outlined below.

Table 8: Timeline

Timeline

	Year 1				**Year 2**				**Year 3**				**Year 4**				**Year 5**			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Protocol/ CRF development	■	■	■																	
Ethical approval		■	■																	
Staff hiring/ training			■	■																
Recruitment/ follow-up				■	■	■	■	■	■	■	■	■	■	■	■	■				
Data analysis					■			■	■				■	■	■	■	■	■		
Manuscript prep/submission									■	■	■	■		■	■	■	■	■	■	■
Results dissemination																			■	■

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16) BUDGET (total budget period)

This proposal is part of an NIH K23 Mentored Patient-Oriented Research Career Development Award. The budget for the grant which supports staff, equipment, and supplies for the research proposed in this protocol is below.

A) PERSONNEL- SALARIES AND DISBURSEMENTS: $103,900

B) PATIENT COSTS: $1,000

C) SUPPLIES AND EQUIPMENT: $78,500

D) ANIMAL ACQUISITION: NOT APPLICABLE

E) TRAVEL AND ACCOMODATION: $37,875

F) TRANSPORT (VEHICLES, REPAIR, FUEL): $4,750

G) OPERATING EXPENSES (POSTAGE, REPORT WRITING ETC): $**1,450**

H) CONSULTANCY IF APPLICABLE: NOT APPLICABLE

I) MISCELLANEOUS: $16,927

J) CONTINGENCY (%): NOT APPLICABLE

17) APPENDICES/ATTACHMENTS

I) REFERENCE LIST OF ABBREVIATIONS
II) **PROTECTION OF HUMAN SUBJECTS**

APPENDIX I List of Abbreviations and Acronyms

AIDS	Acquired Immunodeficiency Syndrome
AFB	Acid-fast bacilli
ALT	Alanine transaminase
ANC	Antenatal care
ART	Antiretroviral therapy
BCG	Bacille Calmette Guerin
BMI	Body mass index
CDC	Centers for Disease Control and Prevention
CXR	chest radiograph
DNA	deoxyribonucleic acid
ERC	Ethical Review Committee
HEU	HIV-exposed uninfected
HIV	Human Immunodeficiency Virus
HLA	human leukocyte antigen
IGRA	interferon gamma release assays
IPT	isoniazid preventive therapy
INH	isoniazid
IRB	Institutional Review Board
KEMRI	Kenya Medical Research Institute
KNH	Kenyatta National Hospital
KRTC	Kenya Research and Training Center
LTBI	latent tuberculosis infection
LFT	liver function test
MCH	maternal child health
MOH	Ministry of Health
Mtb	Mycobacterium tuberculosis
PID	Patient identification number
PBMCs	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PMTCT	prevention of mother to child transmission
PPD	Purified protein derivative
NIAID	(United States) National Institute of Allergy and Infectious Diseases
NIH	(United States) National Institutes of Health
QFT-Plus	Quantiferon Plus
SAE	serious adverse event
TB	tuberculosis
TST	tuberculin skin test
UoN	University of Nairobi
UW	University of Washington, Seattle
VL	Viral load (HIV-1 RNA copies/ml)
WHO	World Health Organization

APPENDIX II: PROTECTION OF HUMAN SUBJECTS

PROTECTION OF HUMAN SUBJECTS

A. Human Subjects Involvement, Characteristics, and Design

We propose to determine the impact of maternal HIV on the risk of maternal peripartum and infant Mtb infection through prospective parallel longitudinal cohorts of mother-infant pairs using an observational study design. We will compare Mtb infection incidence between HIV-infected and uninfected pregnant women, and Mtb infection prevalence between HIV-exposed and unexposed children. We will also evaluate the effect of maternal HIV status and peripartum stage on the performance of LTBI tests.

A1. Justification

Tuberculosis remains the leading cause of morbidity and mortality among HIV-infected individuals globally. Four out of every five TB cases among people with HIV occur in Africa. Kenya is one of the 22 countries designated by the World Health Organization as a high TB burden country, with an annual TB incidence of 268/100,000. Western Kenya, where this research will take place, has one of the highest antenatal prevalences of HIV in Kenya (19-26%), and 68% of new TB case are among those with HIV. With successful prevention of maternal to child transmission (PMTCT) programs, the population of HIV-exposed but uninfected (HEU) children is growing. These children have increased risk of mortality compared to HIV-unexposed children, and remain at higher risk for TB due to either TB exposure and/or altered immunity associated with maternal HIV.

The aim of this proposed study is to examine the influence of maternal HIV on maternal peripartum Mtb infection incidence and infant Mtb infection prevalence, and compare diagnostic performance of LTBI tests (TST and IGRA) in HIV-infected and HIV-uninfected women in pregnancy and postpartum. TB causes significant morbidity and mortality in women and their children in high TB burden areas. HIV-infected individuals and infants are at high risk of progression from Mtb infection to TB disease. Pregnant and postpartum periods have also been associated with increased risk of TB. We do not know whether maternal HIV increases susceptibility to peripartum maternal or infant Mtb *infection*. Detecting Mtb infection, particularly recent infection, can identify those individuals who may most likely benefit from preventive therapy.

We are enrolling HIV-infected pregnant women and their children to further understand the risk of Mtb infection, in a population at high risk of progression to active TB with resulting poor maternal and infant outcomes. Comparing Mtb infection incidence in parallel cohorts of HIV-infected and HIV-uninfected peripartum women and Mtb infection prevalence in HIV-exposed and HIV-unexposed infants will allow us to identify whether maternal HIV is associated with increased risk of Mtb infection in these populations.

Additionally, we will identify latent TB diagnostic test characteristics in HIV-infected and HIV-uninfected peripartum women. It is important that this research is performed with peripartum women and their infants as pregnancy and infancy have distinctly different risks of active TB, and potentially Mtb infection. This research could not be conducted in a US setting due to the low burden of HIV/TB and because the results would not be relevant to mothers and their infants in high HIV/TB burden settings.

A2. Study Population

We plan to enroll 200 HIV-infected and 200 HIV-uninfected pregnant women from antenatal care at the Ahero Sub-district and Bondo District Hospitals in the Nyanza region of western Kenya. We will also enroll from Siya Country Referral Hospital, Ranchuonyo sub-County Hospital, Lumumba Health Center, and Jaramogi Oginga Odinga Teaching and Referral Hospital (JOOTRH) if necessary to ensure enrollment goals. **For Aim 1**, we wish to identify the incidence of Mtb infection, therefore we will exclude women found to have prevalent latent TB on enrollment (by IGRA) or active TB, from this analysis. We anticipate approximately 20% of women will have positive IGRA at enrollment based on estimates from our previous TB in Pregnancy studies. We wish to enroll ~165 women in each Aim 1 maternal cohort to account for a conservative estimated 10% lost to follow-up to ensure 150 women with outcomes. For **Aim 2** we will enroll all children born to the women enrolled in the study irrespective of maternal enrollment LTBI testing results. We anticipate there will be approximately 400 infants born to mothers enrolled in the study (~200 HIV-exposed, and 200 HIV-unexposed children). For **Aim 3** we will include mothers irrespective of enrollment LTBI testing results (~200 HIV-infected, and 200 HIV-uninfected peripartum women).

A3. Sampling plan, recruitment and retention strategies and inclusion/exclusion criteria.

Sampling plan

We will consecutively enroll 200 HIV-infected and 200 HIV-uninfected pregnant women in antenatal care at the Ahero Sub-district and Bondo District Hospitals in western Kenya who are interested and willing to in participate in the research and their infants (200 HIV-exposed, 200 HIV-unexposed). Participation in the study will involve agreeing to physical exams, surveys and labs/procedure from enrollment in pregnancy to 12 months postpartum (Appendix 5).

Eligibility

Women are eligible for the overall study if they are pregnant (between 20-34 weeks gestation) and at least 16 years old, have not been treated for active tuberculosis in the last year, and are willing to return for follow-up visits. Women who are 16 years of age and pregnant are able to provide study consent, as their pregnancy emancipates them for participation in research under Kenyan law.

Enrollment and consent

Potential participants will be informed about the study by antenatal clinic staff/nurses and referred to the study staff for more information about the research. Following informed consent and study screening procedures, pregnant women will be offered enrollment into the study. Informed consent will occur in a one-on-one counseling session with a study staff person in either Kiswahili, Dholuo or English according to participant's preference. A copy of the consent form in the language of preference will be offered to the participant. For women who are not literate, the consent will be read to them in the appropriate language of their choosing with a witness independent of the study present. For women who cannot write, an inked thumbprint will be used in lieu of signature. Women will provide consent for their infants.

Procedures

Maternal cohorts: Physical exams and sociodemographic and health surveys will be performed on enrollment in pregnancy, 6 weeks, 6 months and 12 months postpartum. Sociodemographic and health surveys will include questions about age, marital status, HIV, TB (including known TB contacts and other potential TB exposures), obstetrical history, educational status, household crowding, and World Health Organization TB symptoms screen (cough, fever, weight loss, night sweats) of both the participant as well as reported household member symptoms, and intercurrent illness. TSTs will be placed and blood will be drawn on enrollment, 6 weeks, and 12 months postpartum. TSTs will be read within 48-96 hours of placement by a study nurse. Breast milk will be collected at 6 weeks postpartum. HIV-uninfected women at enrollment will also be offered HIV testing per national protocols through maternal child health clinic HIV counselors at enrollment into the study, 6 weeks, 6 months and 12 months postpartum.

Infant cohorts: Physical exams and sociodemographic and health surveys will be performed on enrollment at 6 weeks of age, 6 and 12 months. Information regarding infant PMTCT prophylaxis, birth weight, BCG vaccination date, intercurrent illnesses, and other immunizations will be collected on enrollment. At each visit infant examination will include growth measures and a questionnaire addressing infant feeding (including breastfeeding status) and symptoms (including cough, fever, weight loss, failure to thrive) will be performed. Blood will be drawn at 6 weeks and 12 months of age. Due to the high rates of BCG cross-reactivity after recent immunization, we will not perform TSTs in infants until 12 months of age.

Timing of visits is designed to align with routine ante/postnatal care, maternal ART and infant immunization visits.

Retention

Women will be asked to give contact information to study staff as well as contact information for a trusted person who may be able to locate them. Women will use a locator map to describe their primary residence and how to get there. The study staff and the subject will craft a detailed plan for each subject describing how best to contact them and allowing the subject to share any relevant information (for example, if the subject has not disclosed her HIV status or pregnancy). Study staff may contact subjects outside of clinic with their permission, but will never identify themselves by other than a first name and will not leave messages. Study information will not be discussed by telephone. Home visits may also occur with the subject's permission. At every step of the retention process, discretion and participant confidentiality will be the top priority. Subjects will be reimbursed for their transportation for each visit and all study related services are provided free of charge. Retention in our prior maternal child cohort studies has been high (>90%). Travel reimbursement, and aligning visits with routine medical care (child immunization and maternal ART visits) is likely to contribute to high retention in this proposed study.

A6. Justification for involvement of children and pregnant women (Vulnerable populations)

Please see justification described in A1. above. This research can only be done by enrolling HIV-infected and HIV-uninfected peripartum women and their infants, in order to understand the role of HIV in pregnancy on the risk of maternal and infant MTb infection, as well as to understand the role of pregnancy stage and HIV on latent TB diagnostics.

A7. Assignment to study groups

There is no randomization component to this study. Subjects in both maternal cohorts will have the same study testing, however HIV negative women will be additionally referred for HIV testing during enrollment, and at 6 weeks, 6 months and 12 months postpartum at the onsite MCH clinics. HIV-exposed children will receive HIV PCR testing at 6 weeks, and at 1 year HIV testing will be performed per national guidelines.

A8. Collaborative sites/study location/research site

The proposed study will be conducted in the Nyanza Province of western Kenya at the antenatal, postpartum, and pediatric clinics located at the Ahero Sub-district and Bondo District Hospitals. The region has high antenatal HIV prevalence (19-26%), and high antenatal clinic attendance (~200 new pregnant women per month). These study sites have been involved with numerous UW-associated studies including those focused on HIV and TB in pregnancy over the past 4 years. The study location in western Kenya is important due to the high prevalence of both HIV and TB. This study will provide data which will aid in determining recommendations for TB prevention efforts in maternal and child health settings. Studies of this nature have not been done and are vital to identify those who may most benefit from preventive therapy as well as ascertain the timing and type of recommended latent tuberculosis test. This study is an extension of ongoing research with HIV-infected pregnant women and our research group routinely works with HIV-infected women and their children in Kenya. Our study team members are acutely sensitive to protecting participants' interests during the conduct of this research. Both HIV-infected and HIV-uninfected women will be enrolled in this study, therefore we do not anticipate that participation in the study will risk revealing a woman's HIV status to other women in the clinical setting.

Study staff will work in conjunction with antenatal and pediatric staff to identify potential participants. Study staff will help any women or infants with suspected tuberculosis to access care at TB clinics, as well as HIV care clinics if necessary. This insures that care for all women and children in this maternal and child health setting is not compromised by the presence of the study, and should ensure that subjects do not feel pressure to participate in the study to receive antenatal, postnatal, pediatric, TB or HIV-related services. Study staff have been working side by side with clinic staff at these sites for >4 years. Study staff are well trained in recruitment and are knowledgeable in recruitment without persuasion/coercion.

B. Sources of Materials

B1. Biological specimens

Blood will be drawn from patients for TB immunologic studies including whole blood for IGRA and for PBMCs and plasma separation for flow cytometry (mothers (15 ml): pregnancy, 6 weeks postpartum, 1 year postpartum; infants (5 ml): 6 weeks, 1 year of age). HIV-uninfected women will be referred to HIV counselors for HIV testing at 6 weeks, 6 months and 12 months postpartum. HIV exposed infants will be referred for HIV PCR testing at 6 weeks, and at 1 year HIV testing will be performed per national guidelines.

Breast milk (30 mls) will be collected from mothers at 6 weeks postpartum for maternal antibodies to TB.

B2. Data collection

At the enrollment visit we will collect basic demographics and clinical characteristics including age, marital status, obstetrical history, economic status, educational status, and household crowding. Women will be interviewed and their medical chart reviewed in order to collect data regarding HIV status, TB history (including reported TB contacts) as well as demographic information.

<u>Data management</u>

Participant files will be accessible only to researchers and will be stored in a location outside the clinic in a locked office. Study databases will not include patient identifiers. Data will be stored in a secure password-protected online RedCap database sponsored by UW Institute of Translational Health Science. A participant list with patient identification and study ID will be kept in a locked file cabinet. All patient specimens will be marked by a de-identified study ID. Participant files will be accessible only to researchers and will be stored the research office in a location outside the clinic in a locked office. Study databases will not include patient identifiers. Written consent or a witnessed thumbprint if not literate will be obtained from all participants prior to enrollment. Consent will be provided in Kiswahili, Dholuo and English languages.

C. Potential Risks

C1. Confidentiality

The primary risk to the subjects is the potential for loss of confidentiality. Disclosure of HIV status is a non-negligible risk. If the patient's HIV status was known, this could lead to psychological harm or affect the standing of the subject in the community. We believe the risk of such disclosures is low, however, due to this risk, we will implement the protections to ensure security. Each subject will be given a number upon entry to the study; recorded survey data and study information will include only a study number and will not contain the subjects' names. A subject could conceivably incur psychological harm or loss of confidentiality through our attempts to call them or reach them through retention protocols. However, we will only call subjects on a number that they have given permission to use, and we will never discuss the subject of the research study in any telephone conversation. If we reach someone other than the participant on the telephone, we will only leave a name and callback number, not disclose information about the purpose for the call or identify association with the research project. As is standard practice in Kenya, health workers sometimes will visit a participant's home for retention purposes. This is done with great discretion and the purpose of the home visit is never discussed with anyone other than the study participant. This protocol will be described in detail to subjects at study entry, and subjects may decline to provide contact information for anyone other than themselves. All study personnel have been trained in Good Clinical Practice and confidentiality.

C2. Specimen collection

Risk of pain and bruising from phlebotomy: (Maternal and infant cohorts) Phlebotomy will be performed by nurses trained in optimal phlebotomy practices in both adults and children and will be performed when subjects are sitting or lying down. At each visit where blood is scheduled 15 ml of blood will be collected from mothers, and 5 ml of blood will be collected from infants. We do not anticipate any study-related physical adverse effects to participants.

C3. TST Placement

Risk of pain from TST placement: TST is contraindicated only for persons who have had a *severe* reaction (e.g., necrosis, blistering, anaphylactic shock, or ulcerations) to a previous TST; this is a rare (<1%) occurrence. TST use is uncommon in the study area in Kenya. Prior to TST placement, subjects will be asked if they have had a severe reaction to TST in the past. TST will not be placed in study participants who have had a previous *severe* reaction). For mothers TST will be placed at enrollment, 6 weeks and 12 months postpartum regardless of previous positive reaction to TST. For infants TSTs will be placed at 12 months of age. TST will be read at 48-96 hours after placement by study staff.

D. Adequacy of Protection Against Risks

D1. Recruitment and informed consent

Potential subjects recruited for this trial are limited to those persons who are pregnant, ages 16 or older, receiving antenatal services, and their children who are born at the end of the pregnancy during which the subject is enrolled. Potential subjects will have access to all available antenatal, postnatal, pediatric, HIV, and TB services regardless of their decision to participate in the research.

Patients who are interested in learning more about the study will be able to complete the informed consent process and the study enrollment visit on the same day. Access to antenatal services or maternal child health services will not be delayed based on inclusion in the study. Once patients consent to study participation, they will be asked to sign an informed consent form. Informed consent will occur in a one-on-one counseling session with a study staff person and will occur in either Kiswahili, Dholuo or English according to participant's preference. During the informed consent counseling, the counselor will describe the study in detail, review the consent form, including reviewing expected benefits and possible risks of participation, and answer all questions a subject may have. Subjects will be notified that they can withdraw consent for participation in the study at any time. A witnessed thumbprint if not literate will be obtained from all participants prior to enrollment. Mothers will provide consent for their infant's enrollment.

<u>Alternatives to study participation</u>

If patients are not interested in the study, they will not be enrolled into the study.

D2. Protections against risk

<u>Protecting confidentiality in the research clinic</u>

At the enrollment visit, subjects will be asked questions that that include sensitive topics such as HIV status, TB exposure, household symptoms, and pregnancy history. The survey will be administered orally by the study nurse in a private setting. The written responses will go in the study subject's folder identified only by their study ID number. The subject numbers will be linked to names and demographic information in a separate file to allow linkage with data from the medical record. This file will be stored separately from the patient file. Data entry personnel will only have access the anonymous survey, with the study participant number, not the list linking subject numbers to names. Only study investigators will have access to the list linking names to subject numbers. All specimens collected from participants will be labeled with subject's number. The list linking subject numbers to names will be kept in a locked file cabinet in a locked office. De-identified data will be kept on a secure, password and firewall-protected computer that is located in a locked study office near the clinics.

Protecting confidentiality during retention efforts

Our retention protocol has been used in other research studies at the same clinic and is designed carefully and explicitly to protect the privacy of study subjects. All participants in the study will create their own retention plan. They are asked questions such as "If we need to reach you, what is the best way to do it?" which gives them to opportunity to create a plan. An example of such a plan is that some women may prefer study staff to visit their workplace but not their home; some may give permission to try to reach a sister, but not a partner/husband. In this way the retention plan is personalized for each woman's specific situation. Study staff in charge of retention are highly sensitive to each subject's privacy and are also part of the plan created to ensure that study participation is safe for the subjects. Regardless of a subject's preferences, during retention efforts, our study workers will never identify themselves as part of a research project, health care or the health center. They will identify themselves as "Subject's friend X is trying to reach them" and leave a number to call. In this way subjects can feel confident that none of our study employees will inadvertently disclose protected information or create suspicion in the home.

Procedures to minimize pain and bruising from phlebotomy

Phlebotomy will be performed by nurses trained in optimal phlebotomy practices for both adults and children and will be performed when subjects are sitting or lying down.

Procedures to minimize adverse effects from TST placement

TST placement will be performed by nurses trained in optimal TST placement and will be performed when subjects are sitting or lying down. Prior to TST placement subjects will be asked regarding any previous severe reaction to TST (e.g., necrosis, blistering, anaphylactic shock, or ulcerations). If a subject has had a previous severe reaction to TST, it will not be placed during the course of the study.

Procedures to minimize risk of HIV, vertical transmission of HIV

HIV-uninfected women will be counseled in ways to reduce their risk of HIV acquisition, including condom use. HIV-infected women will be counseled on the prevention of mother to child transmission per Kenyan guidelines. Previously HIV-negative women and HEU infants found to be HIV-infected during the study will be immediately referred to appropriate HIV services. HIV evaluation and treatment is free per Kenyan National HIV guidelines.

Procedures to minimize risk of progression from latent to active TB

HIV-infected women with evidence of latent TB, or development of new Mtb infection (by either positive TST or IGRA) during the study will be referred to the HIV comprehensive care clinic on site to receive isoniazid preventive therapy per Kenyan guidelines. Children identified as having close contact with a person with TB will also be referred to the TB program clinic for isoniazid preventive therapy per Kenyan guidelines. All subjects identified as having potential active TB will be referred to the TB program for further investigation as well as treatment as required per Kenyan National TB guidelines including CXR and Xpert as necessary. Isoniazid prevention therapy and TB evaluation and treatment is free per Kenyan National TB guidelines.

Procedures to minimize risk of pregnancy complications

Women with high-risk pregnancies, or risks of complications will be referred to deliver at their closest MCH-linked maternity center (Bondo District Hospital or Ahero sub-District Hospital). Women or children developing other unexpected health problems that are not addressable within the study clinic will be referred to the appropriate clinic within the hospital.

Procedures to ensure newly diagnosed HIV-infected women receive HIV care

Enrolled HIV-uninfected pregnant women will be referred for HIV tests through MCH trained HIV counselors at enrollment, 6 weeks, 6 months and 12 months postpartum. If any women are found to be HIV-infected they will be referred for HIV care (including prevention of mother to child transmission of HIV services) through the HIV care clinic.

Procedures to ensure newly diagnosed HIV-infected children receive HIV care

Enrolled HIV-unexposed infants referred for HIV tests through MCH trained HIV counselors at 6 weeks and 12 months of age. If any of these children are found to be HIV-infected they will be referred immediately for HIV care through the Pediatric HIV care clinic.

E. Potential Benefits of the Proposed Research to Human Subjects and Others

The proposed research will provide important information regarding the extent that HIV in pregnancy increases susceptibility to Mtb infection in peripartum women and their infants, as well as the role of HIV and peripartum stage on the performance of latent TB diagnostics. The risks to the individual subjects are low. Benefits to participants include close monitoring of TB symptoms which will prompt referral to appropriate screening and treatment facilities and serial testing of HIV which may identify newly infected women and their infants. The subjects from the HIV-infected maternal cohort with evidence of Mtb infection, as well as infants found to have TB contacts, will benefit from referral for isoniazid prophylaxis, as well as from focused questioning regarding TB risk factors and prompt referral to the TB program if concern for active TB arises. The information from the results of this study could benefit other peripartum women and their children in areas of high HIV/TB burden.

F. Importance of the Knowledge to be Gained

The proposed research will help define the effects of HIV in pregnancy on the risk of MTb infection in peripartum women and their infants. For the HIV-uninfected mother cohort, the research will further characterize latent TB test characteristics in pregnancy and evaluate MTb infection risk in their infants in a high TB burden setting. As millions of peripartum women and their children live in areas of high HIV and TB burden, knowledge derived from this study could improve TB prevention strategies within maternal and child health settings throughout sub-Saharan Africa, as well as in other high HIV/TB burden areas.

G. Data and Safety Monitoring Plan

The principal investigator (PI) will take responsibility for data safety and monitoring as part of the general oversight and scientific leadership of the study. Commensurate to the non-interventional nature of the study, as well as low risk to participants, we do not plan to have an independent data and safety monitoring board. The PI will report all unanticipated problems involving risks to the subjects or others to the University of Washington IRB and the Kenyatta National Hospital Ethical Research Committee in writing within 48 hours of the occurrence of the adverse event. The PI will also submit a research summary, safety summary, and adverse event information to the IRB and ERC annually.

H. ClinicalTrials.gov

This trial does not fit criteria for mandatory registration with ClinicalTrials.gov.