THE BURDEN OF PERINATAL TUBERCULOSIS IN HIV-INFECTED MOTHERS AND THEIR INFANTS

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DECLARATION

I, Katrina Jo Downing, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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ABSTRACT

South Africa is one of six countries worldwide that has the highest national burden of tuberculosis (TB) and the largest number of HIV-infected people in the world. HIV infection, *Mycobacterium tuberculosis* (*M.tb*) infection and TB disease is most common during a woman's reproductive age, particularly in South African women. HIV co-infection increases the risk of TB disease either by facilitating reactivation of a latent TB infection or by favouring the progression of a recently acquired TB infection towards active disease in HIV-infected patients. Globally, HIV-TB co-infected adults are 19 times more likely to develop TB disease than HIV-uninfected adults, in the absence of preventive therapy. In South Africa 61% of TB cases are reported to be HIV-infected. HIV-infected pregnant women with latent TB infection are more likely to progress to active TB disease and women in the early postpartum period are twice as likely to develop TB as non-pregnant women, usually at 3 months post-delivery. More pregnant women die from TB disease than from any other pregnancy or childbirth related causes, particularly in South Africa. This risk is greater in HIV-infected, pregnant women, who account for 29.7% of pregnant women attending public antenatal clinic services in South Africa. Infants of pregnant women with TB have increased risks of mortality and morbidity compared to infants of women without TB, and these risks are even higher in pregnant women co-infected with HIV and TB.

The risk of *M.tb* exposure, infection and TB disease in HIV-exposed, uninfected infants is high. An analysis is presented on the relationships between socio-demographic and clinical risk factors and *M.tb* infection and TB disease in HIV-infected mothers and HIV-exposed infants examined in the setting of an infant TB vaccine clinical trial. Prevalence of maternal *M.tb* infection and the incidence rate of maternal TB disease and infant *M.tb* infection and TB disease in this cohort is also investigated.

The protocol (Part A) outlines the study design and the methodology of the research for this sub-analysis. The literature review (Part B) provides an overview of recent and current literature on the prevalence and incidence rate of *M.tb* infection and TB disease in HIV-infected pregnant and post-partum women and their HIV-exposed infants in resource-limited settings, particularly in sub-Saharan Africa and specifically in South Africa. Literature on the risk factors associated with the exposure and progression to *M.tb* infection and TB disease in these susceptible populations is described. The results of the sub- analysis are presented as a manuscript (Part C). The main findings are the incidence rate of maternal TB was 1.36/100 person-years and incidence rate of infant *M.tb* infection and TB was 2.47 and 3.62/100 person-years respectively. Maternal CD4 count $\geq$350 cells/mm^3 was strongly associated with QFT positivity that may have affected the estimate of maternal *M.tb* infection. Infant *M.tb* infection was driven by new household TB contact(s) as was infant TB disease in addition to higher QFT values (IU/ml) and maternal smoking.

Determining which pregnant or postpartum HIV-infected women and their infants are at the highest risk of becoming *M.tb* infected and developing TB disease, by improving active TB screening of mother-infant pairs, could be an important public health means to reducing the burden of disease and death caused by TB, particularly in HIV endemic areas of South Africa where Prevention of Mother to Child Transmission coverage is greater than 95%.

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PART A: PROTOCOL

1. PROTOCOL SUMMARY

South Africa is one of six countries worldwide that has the highest national burden of tuberculosis (TB) and the largest number of people in the world [1]. More women of childbearing age die from TB than from any other pregnancy or childbirth related causes in developing countries. This risk is greater in HIV-infected, pregnant women [2,3], who account for 29.7% of pregnant women attending public antenatal clinic services in South Africa [4]. Infants of pregnant women with TB have increased risks of mortality and morbidity compared to infants of women without TB, and these risks are even higher in pregnant women co-infected with HIV and TB [5].

The risk of *M.tb* exposure, infection and TB disease in HIV-exposed, uninfected infants is high [6]. A better understanding of the risk factors associated with *M.tb* infection and TB disease in pregnant or postpartum HIV-infected women and their HIV-exposed infants will aid efforts to reduce the burden of disease and death caused by TB in these populations, particularly in HIV endemic areas of South Africa where Prevention of Mother to Child Transmission (PMTCT) coverage is greater than 95%. Improving active maternal, infant and household TB screening and providing TB preventive chemotherapy and testing new effective TB vaccine candidates in HIV-exposed infants in the first year of life is imperative to reduce the burden of TB in this population.

No participants will be recruited for this dissertation. The dataset for this sub-analysis was generated during an ethics approved, double blind, randomized, placebo-controlled, Phase II clinical trial entitled “Phase II Randomised Controlled Trial to Evaluate Safety and Immunogenicity of MVA85A and Selective, Delayed Bacille Calmette-Guerin (BCG) Vaccination in Infants of HIV-infected Mothers” (protocol number G1100570/1). The specific aims of the clinical trial were to evaluate the safety and immunogenicity of MVA85A, given at birth, and BCG given at 8 weeks of age to HIV-exposed, uninfected infants. All participants were followed for 365 days for safety and immunogenicity endpoints.

Statistical analysis of the clinical trial data showed that administering MVA85A to HIV-exposed infants within 96 hours of birth as a prime vaccine was safe and induced an early modest antigen-specific immune response [7].

The sub-analysis is summarised in the table below.

Title	The Burden Of Perinatal Tuberculosis In HIV-infected Mothers And Their Infants
Study design	Sub analysis of data generated during a double blinded, randomised, controlled trial. HIV-exposed infants were randomised 1:1 to receive single dose, intradermal MVA85A vaccine or Candin® control by intradermal injection within 96 hours of birth. Infants confirmed HIV-uninfected by HIV DNA PCR received BCG at 8 weeks of age.
Study Population and setting	Infants (n=248) born to HIV-infected mothers receiving antiretroviral therapy (ART) or PMTCT in TB and HIV endemic areas of South Africa (Worcester (South African Tuberculosis Vaccine Initiative (SATVI), University of Cape Town (UCT)) and Khayelitsha (Desmond Tutu TB Centre (DTTC), Stellenbosch University (SUN)).
Study Objectives	• To determine the prevalence of and risk factors for *M.tb* infection in HIV-infected, pregnant women • To determine the incidence rate of and risk factors for *M.tb* infection in infants of HIV-infected mothers • To determine the incidence rate of and risk factors for TB disease in infants of HIV-infected mothers • To estimate the postpartum incidence rate of and risk factors for TB disease in HIV-infected mothers
Study outcome measures	• The QuantiFERON TB assay cannot accurately differentiate between *M.tb* infection and active TB disease or distinguish *M.tb* reactivation from reinfection with the organism. However, since there is no gold standard for latent *M.tb* infection, the QuantiFERON TB assay is an acceptable but imperfect test that detects a host cellular immune response to *M.tb* antigens *in vitro* and is therefore an indirect marker of TB exposure [8]. *M.tb* infection in HIV-infected mothers is defined in this sub-analysis as a positive QuantiFERON TB assay result • *M.tb* infection in HIV-exposed infants in the clinical trial was assessed by QuantiFERON TB assay and not by the Tuberculin Skin Test since prior BCG vaccination and exposure to environmental mycobacteria can cause false positive results in the Tuberculin Skin Test but not in the QuantiFERON TB assay. Incident *M.tb* infection is defined in this sub-analysis as a positive QuantiFERON TB assay result • Incident TB Disease in HIV-infected mothers is defined as mothers who received TB drug treatment. Incident TB disease is defined in HIV-exposed infants based on the clinician's decision to treat for TB, including microbiological, radiological and clinical criteria for diagnosis. This definition included confirmed, unconfirmed and unlikely TB [9]

2. INTRODUCTION

2.1. Burden of disease

Tuberculosis (TB) remains one of the world's biggest public health threats and accounted for 10.4 million new active TB cases in 2015, of which 1.2 million (11%) were among people living with HIV, and 1.4 million deaths worldwide [1].

The national TB and HIV burden in South Africa ranks as one of the highest in the world. TB incidence in infants and children is very high: the estimated annual incidence of TB in HIV-uninfected children aged 0-4 in Cape Town, South Africa, in 2009 was 511 cases per 100 000 population [10] and was highest in children aged 12-23 months [11]. HIV-infected infants were at a 24.1 fold higher risk of pulmonary TB and a 17.1 fold higher risk of disseminated TB, compared with HIV-uninfected infants [12]. TB is the leading cause of death in South Africa, accounting for 7.2% of all deaths [13].

The HIV status of pregnant women is of critical importance to their health and that of their unborn children. HIV infection was associated with an estimated 70% of maternal deaths in South Africa [14] and almost 50% of all deaths of children younger than 5 years [15]. The majority of infants and children who are HIV-infected were infected through mother-to-child transmission (MTCT). The HIV prevalence in pregnant women who attended public antenatal clinic services in South Africa was 29.7% in 2013, which has remained relatively stable since 2004 [4]. The South African antiretroviral intervention programme to prevent MTCT (PMTCT) has proven to be hugely successful with 95% coverage of HIV-infected pregnant women attending public antenatal clinic services [16] and a significant reduction in the MTCT rate (at 6 weeks post-partum) from 8% in 2008 to 1.5% in 2015 [16,17]. However, despite this decrease, there were still a significant number of new HIV infections in children in South Africa [18].

2.2. Burden of TB in pregnant women and their infants

As with HIV infection, TB is most common during a women's reproductive age of 15-29 years [2] and is a major cause of maternal morbidity and mortality, especially in HIV-infected women [3]. HIV-infected pregnant women with latent TB infection (LTBI) are more likely to progress to active disease [3] than HIV-uninfected pregnant women. Pregnant women with TB/HIV co-infection have poorer outcomes than those who are only TB or HIV-infected [19]. TB and HIV infection account for more maternal deaths during pregnancy and the postpartum period in Sub-Saharan Africa than obstetric causes [2], accounting for 26.3% of maternal deaths in South Africa between 2011 and 2013 [20]. Postpartum women are twice as likely to develop TB as non-pregnant women [21] with median onset 3 months post-delivery [22].

Infants of pregnant women with TB have increased risks of adverse outcomes compared to infants of women without TB, including perinatal mortality, low birth weight and intrauterine growth restrictions [23] and these outcomes are higher in

infants born to pregnant women co-infected with HIV and TB than in women infected with TB or HIV only [5].

2.3. Risk factors associated with *M.tb* infection and TB Disease in pregnant and postpartum women and their infants

In endemic areas, most TB transmission to adults is outside the household, whereas transmission in young children is from household contact with an infectious TB case [25–27]. The risk of *M.tb* infection and TB disease in children, including those younger than 2 years of age, is greater if the household exposure is to a sputum smear positive source case, due to greater bacillary loads of these contacts, than to sputum smear-negative cases [28]. Furthermore, mothers who are TB and HIV co-infected have a 30% higher rate of infecting their children than those with TB alone [29].

The risk of progression from *M.tb* infection to TB disease is determined by microbiological, immunological factors and age [24,28]. Adult TB disease results from either recent infection or reactivation of latent or previously acquired infection. Pregnancy or the postpartum period affects the course of TB due to the partial suppression of the Th1 proinflammatory immune response that can mask symptoms of TB and increase the susceptibility to new infection and reactivation.

The risk of TB disease, either reactivation of a latent infection or recent infection, is greater in HIV-infected patients [30]. Globally, HIV-infected adults infected with TB are 19 times more likely to develop TB than HIV-uninfected adults, in the absence of preventive therapy [31]. In South Africa 61% of TB cases are reported to be HIV-infected [32].

In contrast to TB disease progression in adults, childhood TB disease results from recent infection. From studies prior to the chemotherapy era, it was shown that HIV-infected infants have a 50% lifetime risk of progression to disease following infection [28]. Progression to TB disease can occur in children at any age but is most common in those younger than 5 years of age and adolescents [23]. Progression to TB is usually within 12 months of *M.tb* infection and 50% of infants will develop TB disease during the first year of life if their mothers have active TB disease, in the absence of Isoniazid Preventive Therapy (IPT) or if BCG unvaccinated. Young children (under 2 years of age), HIV-infected children and severely malnourished children infected with TB have a more frequent and greater risk of progressing to active TB as well as developing serious forms of TB like disseminated TB and TB meningitis [23].

Besides contact with a source case and HIV infection other risk factors for exposure the following to and infection with *M.tb* and TB disease in infants and children include: [33–38]

- Poverty
- Composition of the household, as the risk of TB contact increases with more adults living in the same house
- The number of living and sleeping areas in the house
- Do infants and children sleep with adults or other children

- Presence of cigarette smokers in the house
- Indoor pollution from biofuels used for heating or cooking that may interfere with mucosal integrity and mucosal immunity in children
- Poor ventilation and dark spaces where *M.tb*-containing droplets stay airborne for longer and survive longer than those exposed to sunlight)
- Alcohol abuse: adults attending informal bars are at increased risk of coming into contact with other TB sources
- Helminth (worm) infestation in infants
- Genetic susceptibility and race/ethnicity (certain host genes may play a role in determining susceptibility)
- Age
- Malnutrition
- Diabetes, cancer or silicosis and other immuno-suppressive treatment
- BCG unvaccinated
- Migration

2.4. Risk of *M.tb* infection and TB disease in HIV-exposed, uninfected infants

HIV-exposed, uninfected infants are at an increased risk of exposure to *M.tb* and are at a high risk of developing active TB disease following infection [23], whether they are HIV-infected or not [39]. In addition to the risk factors described above, specifically household contact [6] and adverse environmental and socio-economic conditions, other risk factors include duration of breastfeeding and quality of breast milk, severity of maternal HIV disease, immune dysfunction [40] and delay in BCG vaccination [7,41]

A better understanding of the risk factors associated with *M.tb* infection and TB disease in HIV-infected mothers and their HIV-exposed infants could allow for informed public health planning and targeted use of available healthcare resources in an effort to prevent *M.tb* transmission, *M.tb* infection and TB disease. Improving active maternal, infant and household TB screening and providing TB preventive chemotherapy and testing new effective TB vaccine candidates in HIV-exposed infants in the first year of life is imperative to reduce the burden of TB in this population.

2.5. Context of the dissertation

The dataset used for this dissertation was generated during a double blind, randomized, placebo-controlled, Phase II clinical trial entitled “Phase II Randomised Controlled Trial to Evaluate Safety and Immunogenicity of MVA85A and Selective, Delayed Bacille Calmette-Guerin (BCG) Vaccination in Infants of HIV-infected Mothers” (protocol number G1100570/1). The rationale for this study was that infants of HIV-infected mothers are at an increased risk of exposure to *M.tb* and are at a high risk of developing active TB disease [6,39] and are therefore more in need of a safe and effective TB vaccine than other infants and children. BCG vaccination protects against severe forms of childhood tuberculosis such as miliary TB and TB meningitis and therefore BCG vaccination is almost universally given soon after birth in tuberculosis endemic regions such as sub-Saharan Africa [42]. However, BCG vaccination of HIV-infected infants is associated with severe adverse events

including local, regional and disseminated BCG disease and BCG Immune Reconstitution Inflammatory Syndrome (IRIS) [41,43,44]. Based on this significant risk that outweighs the benefits of BCG vaccination in this population, the WHO revised their recommendations in 2007 [45] to no longer give BCG to HIV-infected children, even if they are asymptomatic, and to HIV-exposed infants with unknown HIV status but who are symptomatic. HIV-exposed infants whose HIV status is unknown and who do not have symptoms suggestive of HIV infection should receive BCG, as outlined in the guidance document [45,46]. However, most HIV-exposed infants are infected peri- or postpartum and are usually not symptomatic at birth. Furthermore, at the time of the trial, diagnosis of HIV infection in infants in developing countries, particularly South Africa, was between 6-8 weeks of age, which is usually after routine BCG has been administered, although in 2015, South Africa implemented HIV DNA PCR testing in all HIV-exposed infants at birth [47]. The BCG Working Group of the International Union against Tuberculosis and Lung Disease (IUATLD), together with the WHO, recognized the challenges for the practical implementation of the revised WHO guidelines, specifically the ability of infant vaccination programmes, particularly those in high TB endemic countries with limited resources, to allow selective, delayed BCG vaccination in HIV-exposed infants from birth until 10-14 weeks, following a negative HIV PCR result [41]. Implementation of this strategy would reduce disseminated BCG disease in HIV-infected infants yet allow HIV-exposed uninfected infants to benefit from BCG vaccination. However, delaying BCG vaccination until exclusion of perinatal HIV infection would put infants at high risk of acquiring TB in the first weeks of life [7]. Given this risk and that of BCG-associated adverse events in HIV-infected infants, a TB vaccination strategy that included a priming vaccination with a novel TB vaccine at birth followed by routine BCG vaccination of those infants known to be HIV-uninfected was thought to be a safe and effective alternative to delayed BCG vaccination alone for HIV-exposed infants in settings with a high burden of HIV and TB.

The clinical trial was conducted at two South African TB vaccine trial sites near Cape Town. The trial started on 16 November 2012 and follow-up was completed on 12 May 2015. Immunological data analysis was completed in September 2016.

Information about this trial was disseminated to HIV-infected mothers attending routine antenatal PMTCT services by posters, pamphlets. Mothers provided antenatal and postnatal written informed consent. A total of 248 HIV-exposed infants were randomized in a 1:1 ratio to receive either a single dose of MVA85A vaccine or Candin® control by intradermal injection within 96 hours of birth. Assignment to the study arms was double blinded Those infants confirmed to be HIV-uninfected by HIV PCR at 6 weeks of age were given BCG at 8 weeks of age and were followed for safety outcomes as per the study protocol. Infants who were confirmed to be HIV-infected by HIV PCR did not receive BCG. All participants were followed-up for 365 days at scheduled visits for the safety endpoints as well as immunology endpoints.

A requirement for inclusion in the study was participation in the national PMTCT programme. Mothers and infants continued to attend PMTCT and Antiretroviral Therapy (ART) health services during the trial, from recruitment to the end of follow-up. Although the South African ART policies changed in 2013, the guidelines for HIV-

exposed infants remained unchanged from those of 2010. Safe infant feeding counseling and support is part of the postnatal follow-up of HIV-infected mother-infant pairs that is offered at the 6 week EPI visit [48]. Infant feeding choices were documented during the trial at the screening visit.

Statistical analysis of the clinical trial data showed that administering MVA85A to HIV-exposed infants within 96 hours of birth as a prime vaccine was safe and induced an early modest antigen-specific immune response [7]

3. RESEARCH QUESTION AND OBJECTIVES

3.1. Research Question

What is the burden of and risk factors for TB in HIV-infected mothers and HIV-exposed infant in the first year of life?

3.2. Hypothesis

Infants of HIV-infected mothers who are TB infected during pregnancy have a higher rate of *M.tb* infection and disease in the first year of life compared to infants of mothers who are not TB infected during pregnancy.

3.3. Objectives

1. To determine the prevalence of and risk factors for *M.tb* infection in HIV-infected, pregnant women
2. To determine the incidence rate of and risk factors for *M.tb* infection in infants of HIV-infected mothers
3. To determine the incidence rate of and risk factors for TB disease in infants of HIV-infected mothers
4. To estimate the postpartum incidence rate of and risk factors for TB disease in HIV-infected mothers

4. METHODS

4.1. Study Design

No participants were recruited for this sub-analysis (dissertation). The dataset for this sub-analysis was generated during a double blind, randomized, placebo-controlled, Phase II clinical trial entitled the “Phase II Randomised Controlled Trial to Evaluate Safety and Immunogenicity of MVA85A and Selective, Delayed Bacille Calmette-Guerin (BCG) Vaccination in Infants of HIV-infected Mothers (protocol number G1100570/1)” as described in section 3.5 above.

4.2. Study Population and setting

For the purposes of this sub-analysis, the study population will be 248 infants born to HIV-infected mothers receiving ART or PMTCT prophylaxis that met the inclusion criteria and had none of the exclusion criteria of Protocol G1100570/1.

The study was conducted at two South African TB vaccine trial sites:

4.2.1 South African Tuberculosis Vaccine Initiative (SATVI), University of Cape Town (UCT)

SATVI has a TB clinical trial site in Worcester in the Boland area. The incidence of TB in this area in 2007 was 680 per 100 000 overall and 1500 per 100 000 in children younger than 2 years of age. At the time of the study, more than 98% of HIV-infected, pregnant women who attended local antenatal services participated in the PMTCT programme. HIV prevalence in pregnant mothers in the Cape Winelands district in 2009 was 13.2% and 15% in 2013. Mother to child transmission ranged from 3.2% - 5.6%. The PMTCT and ART programme for women and children is well established in the area.

4.2.2 Desmond Tutu TB Centre (DTTC), Stellenbosch University (SUN)

SUN has a TB clinical trial site situated in an area of Khayelitsha (Site C), which is the largest partially informal township of Cape Town located approximately 35km from the city centre. It has an estimated population of 500 000 [49]. The incidence of TB in this area in 2009 was 1500 per 100 000 overall and 3290 per 100 000 in children less than 18 months of age. Khayelitsha has the highest HIV prevalence in the Western Cape Province [49]. The maternal HIV prevalence has increased from 24.7% in 2002 to 34.3% in 2012, which was higher than the national antenatal rate of 29.5% [49]. The PMTCT programme has very good coverage in the area with a MTCT rate of approximately 2.5% in 2010. HIV care including ART is delivered to mother and infants at the clinical trial site.

4.3 Inclusion and Exclusion Criteria (stated verbatim as per Protocol G1100570/1)

4.3.1 Inclusion Criteria

- “HIV-infected mother receiving either ART, or started on PMTCT prophylaxis”
- “Maternal antenatal and post-natal written consent”
- “Maternal age 18 years or older at the time of consent”
- “Infant age < 96 hours”
- “Infant birth and residence in the study area”
- “Mother contactable and able to attend follow-up visits”

4.3.2 Exclusion Criteria

- “Neonatal Apgar score <7 at 5 minutes”
- “Infant birth weight 2,000g or > 4,500g”
- “Estimated infant gestational age < 32”
- “Neonatal respiratory distress”
- “History or evidence of infant congenital abnormality, or immunosuppressive condition, other than HIV infection”
- “Any maternal or infant condition or systemic illness that in the opinion of the investigator is likely to affect safety and immunogenicity of studyvaccine”
- “Infant BCG vaccination prior to enrolment”
- “Residence in a household, or frequent close contact, with an adult diagnosed with active TB who has not yet completed TB treatment”
- “Mother with active TB who has not yet completed TB treatment”
- “Unknown or negative maternal HIV status”

- "Intention to leave the study area and/or unable to attend follow-up visits"

4.4 Measures and procedures

Clinical trial staff collected and recorded data at scheduled visits on "Days 7, 28, 42, 56, 63, 84, 112 and 365" as outlined in protocol number G1100570/1. Maternal and infant socio-demographic, clinical, maternal-HIV data and active TB surveillance data were recorded at these visits and will be included in sub-analysis (Tables 1).

4.4.1 QuantiFERON (QFT) assay

QuantiFERON-TB Gold (QFT, Qiagen) was performed on all mothers at enrolment and on infants who successfully completed one year of follow-up, if not previously investigated as described in section 5.4.4. The QFT assay is an indirect test for latent tuberculosis infection. The assay was performed in the SANAS accredited laboratories at SATVI according to the manufacturer's instructions (Qiagen QFT package insert) [50]. This whole blood interferon-gamma (IFN-γ) release assay (IGRA) measures cell mediated immune responses to the *M.tb*-specific ESAT-6, CFP-10 and TB7.7 (p4) peptides that are associated with *M.tb* infection. Heparinized whole blood is added to the following tubes:

- TB antigen tube: Contains *M.tb*-specific proteins
- Negative control tube: No TB antigens or other additives (Nil tube) to determine non-specific IFN-γ production.
- Positive control tube: Contains a non-TB antigen (Mitogen) for IFN-γ production that contains a non-TB antigen.

Production of IFN-γ from blood stimulated by the *M.tb* antigens is detected by ELISA and measured in IU/ml. Results are interpreted as:

- Negative: IFN-γ value minus that of the Nil tube = < 0.35 IU/ml.
- Indeterminate: A low IFN-γ response to Mitogen and to the TB antigens.
- Positive result: IFN-γ value in the TB Antigen tube $\geq$ 0.35 IU/ml

The QFT assay results were recorded on the database as IFN-γ values in IU/ml (Quantitative result) and qualitatively as positive, (TB antigen minus nil IFN-γ value $\geq$ 0.35 IU/ml as per the manufacturer's instructions), negative (IFN-γ value < 0.35 IU/ml) or Indeterminate. In this sub-analysis, QFT Indeterminate results for both mothers and their infants were included as QFT negative results.

4.4.2 Study Outcomes (endpoints)

The outcomes (or endpoints) of the dissertation are defined as follows:

4.4.3 Maternal and Infants *M.tb* infection outcomes

QFT cannot accurately differentiate between *M.tb* infection and active disease or distinguish reactivation from reinfection [8]. However, since there is no gold standard for LTBI, QFT is an acceptable but imperfect test that detects a host cellular immune

response to *M.tb* antigens *in vitro* and is therefore an indirect marker of *M.tb* exposure [8].

Although QFT has not been validated as a diagnostic test for *M.tb* infection in infants and children younger than 2 years of age, a study done by the SATVI group showed that both TST and QFT had excellent concordance in children younger than 3 years of age in a high TB burden setting and equivalent performance of both tests in the diagnosis of TB disease [11]. Since TST is confounded by prior BCG vaccination and exposure to most environmental mycobacteria, whereas the QFT assay is not, QFT was used as an efficacy endpoint definition of *M.tb* infection in BCG-vaccinated infants at the SATVI site under a tuberculosis vaccine trial setting [51].

In this sub-analysis, *M.tb* infection in HIV-infected mothers and HIV-exposed infants is defined as a positive QFT result.

4.4.4 Maternal and Infant TB Disease outcomes

Participants with suspected TB disease were investigated at local hospitals. Maternal incident TB disease is defined as mothers who receive TB drug treatment. Incident TB disease is defined in infants based on the clinician's decision to treat for TB, including microbiological (notably TST or QFT), radiological or clinical criteria for diagnosis. This definition includes confirmed, unconfirmed and unlikely TB [9]

5. DATA MANAGEMENT AND STATISTICAL ANALYSIS

5.1. Data Management

Management, validation and verification of the clinical trial data was per protocol and the Data Management plan. The clinical trial Data Manager will extract the dataset for this sub-analysis from the clinical trial database as a Microsoft Excel spreadsheet. Any queries relating to this dataset will be directed to the Data Manager, who will provide additional data if required.

5.2. Statistical Analysis

The dataset used for this sub-analysis will be analysed using STATA version 12.0 (DataCorp, USA). Variables to be analysed for independent associations in HIV-infected mothers and their HIV-exposed Infants are shown in Tables 1 and 2 respectively. Variables were selected for analysis based on their possible associations with maternal and infant *M.tb* infection and TB disease and for their clinical or biological significance, particularly in HIV-infected, postpartum mothers and their HIV-exposed infants, as reported in several studies in the literature and described in sections 3.3 and 3.4 above. Categorical and numerical variables will be coded in the sub-analysis as shown in Tables 1.

Table 1. Variables to be analysed in HIV-infected mothers and HIV-exposed infants

Variable name	Variable Type	Variable coding	Variable explanation
Maternal Age	Numerical (Continuous)	Years	Age at time of enrolment
Number of other adults in household	Numerical (Discrete)	Count	Number of other adults living in the household
Number of other infants in the household	Numerical (Discrete)	Count	Number of infants besides the enrolled infant living in the house
Number of sleeping rooms	Numerical (Discrete)	Count	Number of rooms in the house used for sleeping
Highest Educational level	Categorial (Nominal)	1 = Primary school 2 = Junior High school 3 = Senior High School	Level of education from Grade 1- 7 is classified as primary school level; Grades 8-10 as junior high school and Grades 11-12 as senior high school
Employment status	Categorical (Binary)	1 = employed 0 = unemployed	Whether mother was employed or unemployed
Employment type	Categorical (Nominal)	1 = Formal sector 2 = Informal sector 3 = Farm	Whether mother was employed in the formal, informal sector or worked on a farm
ART treatment	Categorical (Binary)	1 = ART 0 = PMTCT	Type of antiretroviral therapy: prevention of mother to child transmission (PMTCT) programme or lifelong ART (ART)
Household contact (Baseline)	Categorical (Binary)	1= Yes; 0 = No	Household or close contact with TB at enrolment
New TB household contact	Categorical (Binary)	1= Yes; 0 = No	Household or close contact with TB since enrolment
Maternal CD4 count	Numerical (Continuous)	Cells/mm^3	Recent maternal absolute CD4 count
Maternal Viral load	Numerical (Continuous)	Copies/ml	Measure of amount of HIV genetic material (RNA) per milliliter of blood
Maternal Smoking	Categorical (Binary)	1 = Yes; 0 = No	Smoking status of mother
Maternal and infant Qualitative QFT	Categorical (Binary)	1 = Positive 0 = Negative	QFT positive or negative (including Indeterminate results)
Maternal and infant QFT IFN-γ	Numerical (Continuous)	IU/ml	Quantitative value of IFN-γ in the TB antigen minus nil tube
Maternal IPT	Categorical (Binary)	1 = Yes; 0 = No	Whether mother was currently on Isoniazid treatment at baseline
Maternal TB Disease	Categorical (Binary)	1 = Yes; 0 = No	Whether mother was being treated for TB

Infant Gestational age	Numerical (Continuous)	weeks	Estimated number of weeks that the infant was in the uterus
Birth weight	Numerical (Continuous)	grams	Weight of infant at birth
Length	Numerical (Continuous)	cm	Length of the infant at birth
Head circumference	Numerical (Continuous)	cm	Head circumference measurement at birth
Gender	Categorical (Binary)	1= Male 0 = Female	Gender of infant
Feeding choice	Categorical (Binary)	1 = Breast Milk 0 = Formula	Whether infant was breast fed, formula fed or a mixture of breast and formula feeding
Infant HIV Status	Categorical (Binary)	1= HIV-infected 0 = HIV-uninfected	HIV status of the infant
Infant TB Disease	Categorical (Binary)	1 = Yes; 0 = No	Based on clinician's decision to treat for TB

Objective1: The prevalence of *M.tb* infection in the mothers will be estimated as the number of mothers with a positive QFT at the time of enrollment divided by the total number of mothers whose infants were enrolled into the study.

Objective 2: The incidence rate of *M.tb* infection in infants will be calculated as person years of follow-up using the number of infants with a positive QFT at Day 365 divided by the total number of infants who successfully completed one year of follow-up.

Objective 3: The incidence rate of TB disease in infants will be calculated as person years of follow-up using the total number of infants who successfully completed follow-up divided by the number of infants who were treated for TB during follow-up as the numerator.

Objective 4: The incidence rate of TB disease in mothers will be calculated as person years of follow-up of infants using the total number of infants who successfully completed follow-up divided by the number of mothers who were treated for TB during follow-up as the numerator.

Descriptive statistics of maternal and infant baseline demographic and clinical data for continuous data will include number of observations, mean and standard deviation for normally distributed data or median and interquartile range (IQR) for skewed data and number of observations, their frequencies and percentages for categorical variables.

Univariate associations of socio-demographic and clinical risk factors with maternal and infant QFT positivity and TB disease will be assessed by Chi-squared or Fisher's Exact tests and odds ratios (OR) with 95% confidence intervals (95% CI). Those risk factors with a P value <0.05 or that are deemed to be of biological precedence with or without a P value <0.25 will be considered for multivariable logistic regression

models to explore independent associations between these risk factors and QFT positivity or TB disease in mothers and infants.

The QFT manufacturer's recommended threshold for a positive test result of $\geq$ 0.35 IU/ml is under debate. It is unclear whether higher IFN-γ values represent more recent *M.tb* infection, greater aerosolized inoculum, or sustained infection [52,53]. For these reasons, it is of interest to investigate the association of risk factors with *M.tb* infection (measured by maternal and infant continuous QFT IFN-γ (IU/ml) values), particularly those $\geq$0.35 IU/ml, in linear regression models. Non-normally distributed dependent variables will be log transformed and coefficients interpreted as percentages. Regression diagnostics will be run to check whether the assumptions of linearity, normality of residuals (errors), homogeneity of variance and independence underlying the regression model are met justified and to identify outliers and influential points.

Table 2 is an example of how results will be presented for *M.tb* infection (QFT positivity) in HIV-infected mothers; in HIV-exposed infants; and for TB Disease in HIV-infected mothers; and in HIV-exposed infants. Results will be presented graphically where appropriate.

Table 2. Dummy table of univariate and multivariable models of socio-demographic and clinical risk factors associated with *M.tb* infection (QFT positivity) in HIV-infected mothers

Variable name	QFT +	QFT -	Univariate analysis		Multivariable analysis	
			OR (95% CI)	P value	OR (95% CI)	P value
Age (Median; IQR)						
Number of other adults in household (Median, IQR)						
Number of other children in household (Median, IQR)						
Number of Sleeping rooms (Median, IQR)						
Highest Educational level (n,%) Primary (Reference) Junior High Senior High						
Employment status: (n,%) Employed (=1) Unemployed						
Employment type: (n,%) Formal (Reference) Informal Farm						
ART treatment: (n,%) PMTCT ART (=1)						
Household TB contact (baseline; n,%)						
Previous TB Treatment (n,%)						
CD4 count (cells/mm^3) (median, IQR)						
CD4 count category (n, %) < 350 $\geq$ 350 (=1)						
Smokes (n, %)						
IPT treatment (n, %)						

6. ETHICS AND COMMUNICATION

No participants will be recruited for this sub-analysis as the research will be conducted using an existing dataset that was generated during the clinical trial. The protocol (number G1100570/1) received approval from the University of Cape Town, Faculty of Health Sciences Human Research Ethics Committee (HREC) on 6 March 2012 (013/2012), the Stellenbosch University HREC on 23 May 2012, the Oxford Tropical Research Ethics Committee on 4 April 2012 and the South African Medicines Control Council (MCC) on 26 March 2012 (Appendix A: Copies of HREC and MCC approval letters). Ethics approval from UCT's Faculty of Health Sciences HREC will be sort for the use of the data set in this sub-analysis.

The ethical principles of beneficence, non-maleficence, autonomy and justice of the clinical trial were described in the study protocol. The clinical trial was conducted in accordance with the Declaration of Helsinki (version 2008), Good Clinical Practice (ICH-GCP), South African Good Clinical Practice Guidelines and the regulations of the MCC. All study staff, including this candidate, was trained on the study protocol and were certified in Good Clinical Practice (ICH-GCP).

The potential benefits, risks and measures to minimize risk in this dissertation are addressed as follows:

6.1. Beneficence

There will be no direct benefit for the participant's whose data will be analysed in this dissertation. However, determining the risk factors associated with *M.tb* infection, TB transmission and TB disease in pregnant or postpartum HIV-infected women and their infants will potentially benefit this population to reduce the burden of disease and death caused by TB. This could be achieved by improving TB control strategies, such as maternal, infant and household screening and providing necessary TB interventions such as preventive chemotherapy and developing effective vaccine strategies.

6.2. Non-maleficence

There is no anticipated harm or discomfort to the participants as data that was previously collected during the approved clinical trial will be analysed in this dissertation. Maintaining participant confidentiality is of great importance. Access to the clinical trial database is password restricted to the data management team and certain study staff designated by the Principal Investigator. The clinical trial data manager will extract the data set for this dissertation from the clinical trial's database as a Microsoft Excel document. Any additional information that may be required from the database will be done so through the data manager. The participant identification numbers that will be included in the data set are unique and coded. The candidate will not have access to any information that could identify the participant. There will therefore not be any breach of participant confidentiality. Participant data will be reported in such a way that no participant might be recognized from any publication.

6.3. Respect for Autonomy

Information about the clinical trial was disseminated to mothers attending routine antenatal PMTCT services either via posters and pamphlets or group information

sessions. Mothers of potential participants received information about the study from a study staff member before signing a written informed consent document. After the birth of their infants, mothers who still wanted their infants to participate in the study signed the informed consent document a second time to reaffirm their decision. Those mothers who no longer wanted their infants to participate in the study were given the opportunity to withdraw consent, without prejudice, by not re-signing the informed consent form. Participation was voluntary. Since no participants will be recruited for this dissertation there is no need to re-affirm participation by written informed consent will not be re-affirmed. Participant data used in this dissertation will remain de-identified to maintain participant confidentiality.

6.4. Justice

TB-HIV co-infection is a major cause of maternal mortality and morbidity during pregnancy and the postpartum period, which heavily impacts on the mortality and morbidity of their infants. Although it has been shown that the rate of TB exposure in HIV-exposed, uninfected infants is high [6], currently, there are limited data on the risk of *M.tb* infection and TB disease in HIV-exposed, uninfected infants in the era of expanded ART access and effective PMTCT. These vulnerable populations have the greatest need for improved TB control strategies and TB interventions.

7. REPORTING AND PUBLICATION

The findings from this dissertation will be reported to the sponsor (UCT) and relevant stakeholders. A manuscript will be published in an accredited scientific journal.

8. REFERENCES

1. World Health Organization. Global tuberculosis report 2016 [Internet]. 2016. Available: www.who.int/tb/publications/global_report/en/

2. Adhikari M, Jeena P, Bobat R, Archary M, Naidoo K, Coutsoudis A, et al. HIV-Associated Tuberculosis in the Newborn and Young Infant. Int J Pediatr. 2011;2011: 354208. doi:10.1155/2011/354208

3. Bates M, Ahmed Y, Kapata N, Maeurer M, Mwaba P, Zumla A. Perspectives on tuberculosis in pregnancy. Int J Infect Dis. 2015;32: 124–127. doi:10.1016/j.ijid.2014.12.014

4. Shisana O, Human Sciences Research Council, United States, Centers for Disease Control and Prevention (U.S.), editors. South African national HIV prevalence, incidence and behaviour survey, 2012. Cape Town, South Africa: HSRC Press; 2014.

5. Mathad JS, Gupta A. Tuberculosis in Pregnant and Postpartum Women: Epidemiology, Management, and Research Gaps. Clin Infect Dis Off Publ Infect Dis Soc Am. 2012;55: 1532–1549. doi:10.1093/cid/cis732

6. Cotton MF, Schaaf HS, Lottering G, Weber HL, Coetzee J, Nachman S, et al. Tuberculosis exposure in HIV-exposed infants in a high-prevalence setting. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2008;12: 225–227.

7. Nemes E, Hesseling AC, Tameris M, Mauff K, Downing K, Mulenga H, et al. Safety and Immunogenicity of Newborn MVA85A Vaccination and Selective, Delayed Bacille Calmette-Guerin (BCG) for Infants of HIV Infected Mothers: A Phase 2 Randomized Controlled Trial. Clin Infect Dis Off Publ Infect Dis Soc Am. 2017; doi:10.1093/cid/cix834

8. Pai M, Denkinger CM, Kik SV, Rangaka MX, Zwerling A, Oxlade O, et al. Gamma Interferon Release Assays for Detection of Mycobacterium tuberculosis Infection. Clin Microbiol Rev. 2014;27: 3–20. doi:10.1128/CMR.00034-13

9. Graham SM, Ahmed T, Amanullah F, Browning R, Cardenas V, Casenghi M, et al. Evaluation of tuberculosis diagnostics in children: 1. Proposed clinical case definitions for classification of intrathoracic tuberculosis disease. Consensus from an expert panel. J Infect Dis. 2012;205 Suppl 2: S199-208. doi:10.1093/infdis/jis008

10. Wood R, Lawn SD, Caldwell J, Kaplan R, Middelkoop K, Bekker L-G. Burden of New and Recurrent Tuberculosis in a Major South African City Stratified by Age and HIV-Status. Cattamanchi A, editor. PLoS ONE. 2011;6: e25098. doi:10.1371/journal.pone.0025098

11. Moyo S, Isaacs F, Gelderbloem S, Verver S, Hawkridge AJ, Hatherill M, et al. Tuberculin skin test and QuantiFERON® assay in young children investigated for tuberculosis in South Africa. Int J Tuberc Lung Dis. 2011;15: 1176–1181. doi:10.5588/ijtld.10.0770

12. Hesseling AC, Cotton MF, Jennings T, Whitelaw A, Johnson LF, Eley B, et al. High Incidence of Tuberculosis among HIV-Infected Infants: Evidence from a South African Population- Based Study Highlights the Need for Improved Tuberculosis Control Strategies. Clin Infect Dis. 2009;48: 108–114. doi:10.1086/595012

13. Statistics South Africa. Mortality and causes of death in South Africa, 2015 [Internet]. Available: http://www.statssa.gov.za/publications/P03093/P030932015.pdf

14. National Department of Health, South Africa. Saving mothers 2008-2010: Fifth report on the confidential enquiries into Maternal Deaths in South Africa.

15. Bamford L, Stephen C, Patrick M. “Saving Children 2009”: Evaluating quality of care through mortality auditing. SAJCH. 2011;5: 28–32.

16. South Africa Global AIDS Response Progress Report (GARPR) 2015.

17. National Department of Health, South Africa. Joint Review of HIV, TB and PMTCT Programmes in South Africa - April 2014 | Health Systems Trust [Internet]. [cited 29 Jan 2017]. Available: http://www.hst.org.za/publications/joint-review-hiv-tb-and-pmtct-programmes-south-africa-april-2014

18. UNAIDS. South Africa HIV profile 2014 [Internet]. Available: http://www.unaidsrstesa.org/wp-content/uploads/2015/05/UNAids-Profile-South-Africa.pdf-18-Feb.pdf

19. Sobhy S, Babiker Z, Zamora J, Khan K, Kunst H. Maternal and perinatal mortality and morbidity associated with tuberculosis during pregnancy and the

postpartum period: a systematic review and meta-analysis. BJOG Int J Obstet Gynaecol. 2016; n/a-n/a. doi:10.1111/1471-0528.14408

20. National Department of Health, South Africa. Saving Mothers 2011-2013: Sixth report on the confidential enquiries into maternal deaths in South Africa.

21. Zenner D, Kruijshaar ME, Andrews N, Abubakar I. Risk of tuberculosis in pregnancy: a national, primary care-based cohort and self-controlled case series study. Am J Respir Crit Care Med. 2012;185: 779–784. doi:10.1164/rccm.201106-1083OC

22. Gupta A, Nayak U, Ram M, Bhosale R, Patil S, Basavraj A, et al. Postpartum tuberculosis incidence and mortality among HIV-infected women and their infants in Pune, India, 2002-2005. Clin Infect Dis Off Publ Infect Dis Soc Am. 2007;45: 241–249. doi:10.1086/518974

23. National Department of Health, South Africa. Guidelines for the management of Tuberculosis in children, South Africa, 2013, National Department of Health [Internet]. Available: www.kznhealth.gov.za/family/National-Childhood-TB-Guidelines-2013-ZA.pdf

24. Lienhardt C. From exposure to disease: the role of environmental factors in susceptibility to and development of tuberculosis. Epidemiol Rev. 2001;23: 288–301.

25. Crampin AC, Glynn JR, Traore H, Yates MD, Mwaungulu L, Mwenebabu M, et al. Tuberculosis transmission attributable to close contacts and HIV status, Malawi. Emerg Infect Dis. 2006;12: 729–735. doi:10.3201/eid1205.050789

26. Middelkoop K, Bekker L, Myer L, Dawson R, Wood R. Rates of Tuberculosis Transmission to Children and Adolescents in a Community with a High Prevalence of HIV Infection among Adults. Clin Infect Dis. 2008;47: 349–355. doi:10.1086/589750

27. Verver S, Warren RM, Munch Z, Richardson M, van der Spuy GD, Borgdorff MW, et al. Proportion of tuberculosis transmission that takes place in households in a high-incidence area. The Lancet. 2004;363: 212–214. doi:10.1016/S0140-6736(03)15332-9

28. Marais BJ, Rabie H, Cotton MF. TB and HIV in children – advances in prevention and management. Paediatr Respir Rev. 2011;12: 39–45. doi:10.1016/j.prrv.2010.09.002

29. Gupta A, Bhosale R, Kinikar A, Gupte N, Bharadwaj R, Kagal A, et al. Maternal Tuberculosis: A Risk Factor for Mother-to-Child Transmission of Human Immunodeficiency Virus. J Infect Dis. 2011;203: 358–362. doi:10.1093/jinfdis/jiq064

30. Santin M, Muñoz L, Rigau D. Interferon-γ Release Assays for the Diagnosis of Tuberculosis and Tuberculosis Infection in HIV-Infected Adults: A Systematic Review and Meta-Analysis. PLOS ONE. 2012;7: e32482. doi:10.1371/journal.pone.0032482

31. World Health Organization. TB/HIV Factsheet 2016 [Internet]. Available: www.who.int/tb/challenges/hiv/factsheets/en/

32. World Health Organization. Global tuberculosis report 2015 [Internet]. Geneva, Switzerland: World Health Organization; 2015. Available: apps.who.int/iris/bitstream/10665/191102/1/9789241565059_eng.pdf

33. Hargreaves JR, Boccia D, Evans CA, Adato M, Petticrew M, Porter JDH. The Social Determinants of Tuberculosis: From Evidence to Action. Am J Public Health. 2011;101: 654–662. doi:10.2105/AJPH.2010.199505

34. Lin H-H, Chiang Y-T, Chuang J-H, Yang S-L, Chang H-Y, Ezzati M, et al. Exposure to Secondhand Smoke and Risk of Tuberculosis: Prospective Cohort Study. PLOS ONE. 2013;8: e77333. doi:10.1371/journal.pone.0077333

35. Lin H-H, Ezzati M, Murray M. Tobacco Smoke, Indoor Air Pollution and Tuberculosis: A Systematic Review and Meta-Analysis. Novotny TE, editor. PLoS Med. 2007;4: e20. doi:10.1371/journal.pmed.0040020

36. Middelkoop K, Mathema B, Myer L, Shashkina E, Whitelaw A, Kaplan G, et al. Transmission of Tuberculosis in a South African Community With a High Prevalence of HIV Infection. J Infect Dis. 2015;211: 53–61. doi:10.1093/infdis/jiu403

37. Seddon JA, Shingadia D. Epidemiology and disease burden of tuberculosis in children: a global perspective. Infect Drug Resist. 2014;7: 153–165. doi:10.2147/IDR.S45090

38. Singh M, Mynak ML, Kumar L, Mathew JL, Jindal SK. Prevalence and risk factors for transmission of infection among children in household contact with adults having pulmonary tuberculosis. Arch Dis Child. 2005;90: 624–628. doi:10.1136/adc.2003.044255

39. Madhi SA, Nachman S, Violari A, Kim S, Cotton MF, Bobat R, et al. Primary isoniazid prophylaxis against tuberculosis in HIV-exposed children. N Engl J Med. 2011;365: 21–31. doi:10.1056/NEJMoa1011214

40. Evans C, Jones CE, Prendergast AJ. HIV-exposed, uninfected infants: new global challenges in the era of paediatric HIV elimination. Lancet Infect Dis. 2016;16: e92–e107. doi:10.1016/S1473-3099(16)00055-4

41. Hesseling AC, Cotton MF, Fordham von Reyn C, Graham SM, Gie RP, Hussey GD. Consensus statement on the revised World Health Organization recommendations for BCG vaccination in HIV-infected infants. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2008;12: 1376–1379.

42. Zwerling A, Behr MA, Verma A, Brewer TF, Menzies D, Pai M. The BCG World Atlas: A Database of Global BCG Vaccination Policies and Practices. PLOS Med. 2011;8: e1001012. doi:10.1371/journal.pmed.1001012

43. Hesseling AC, Caldwell J, Cotton MF, Eley BS, Jaspan HB, Jennings K, et al. BCG vaccination in South African HIV-exposed infants – risks and benefits. South Afr Med J Suid-Afr Tydskr Vir Geneeskd. 2009;99: 88–91.

44. Hesseling AC, Marais BJ, Gie RP, Schaaf HS, Fine PEM, Godfrey-Faussett P, et al. The risk of disseminated Bacille Calmette-Guerin (BCG) disease in HIV-infected children. Vaccine. 2007;25: 14–18. doi:10.1016/j.vaccine.2006.07.020

45. Revised BCG vaccination guidelines for infants at risk for HIV infection [Internet]. WHO; Available: http://www.who.int/wer

46. Guidance for national tuberculosis programmes on the management of tuberculosis in children. Geneva: World Health Organization; 2015.

47. National Department of Health, South Africa. National consolidated guidelines for the prevention of mother-to-child transmission of HIV (PMTCT) and the management of HIV in children, adolescents and adults. Pretoria: Department of Health; 2015.

48. National Department of Health, South Africa. Clinical Guidelines: PMTCT (Prevention of Mother-to-Child Transmission), 2010, National Department of Health, South Africa; South African National AIDS Council [Internet]. Available: http://www.fidssa.co.za/Content/Documents/PMTCT_Guidelines.pdf

49. Stinson K, Goemaere E, Coetzee D, van Cutsem G, Hilderbrand K, Osler M, et al. Cohort Profile: The Khayelitsha antiretroviral programme, Cape Town, South Africa. Int J Epidemiol. 2016; dyw057. doi:10.1093/ije/dyw057

50. Whitworth HS, Scott M, Connell DW, Dongés B, Lalvani A. IGRAs – The gateway to T cell based TB diagnosis. Methods. 2013;61: 52–62. doi:10.1016/j.ymeth.2012.12.012

51. Tameris MD, Hatherill M, Landry BS, Scriba TJ, Snowden MA, Lockhart S, et al. Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. The Lancet. 2013;381: 1021–1028. doi:10.1016/S0140-6736(13)60177-4

52. Andrews JR, Nemes E, Tameris M, Landry BS, Mahomed H, McClain JB, et al. Serial QuantiFERON testing and tuberculosis disease risk among young children: an observational cohort study. Lancet Respir Med. 2017;5: 282–290. doi:10.1016/S2213-2600(17)30060-7

53. Nemes E, Rozot V, Geldenhuys H, Bilek N, Mabwe S, Abrahams D, et al. Optimization and Interpretation of Serial QuantiFERON Testing to Measure Acquisition of *Mycobacterium tuberculosis* Infection. Am J Respir Crit Care Med. 2017;196: 638–648. doi:10.1164/rccm.201704-0817OC

PART B: STRUCTURED LITERATURE REVIEW

1. INTRODUCTION

Tuberculosis (TB) remains one of the world's biggest public health threats despite a worldwide decrease in TB incidence and mortality since 2000 [1]. In 2015, the estimated TB incidence worldwide was 10.4 million new active TB cases. TB claimed the lives of an estimated 1.4 million people worldwide in 2015, with an additional 0.4 million deaths in people living with HIV. Mortality rates were high in children with TB, especially those younger than 5 years of age and those co-infected with HIV. South Africa has one of the highest burdens of TB and HIV in the world, with an estimated incidence of 454 000 new TB cases per year and one in five of all HIV-infected people, living in South Africa [1]. TB is the leading cause of death in South Africa, accounting for 7.2% of all deaths [2] with a TB mortality rate in HIV-uninfected people of 44/100 000 population in 2015, more than double the global estimate [1,3], and 133/100 000 population in HIV- infected people [1].

Mycobacterium tuberculosis (M.tb) infection, TB disease and HIV infection are most common in women of reproductive age (15-49 years of age), particularly in South African women [1], and are a major cause of maternal morbidity and mortality in these women [4]. HIV-infected pregnant women with latent TB infection (LTBI) and women in early postpartum are at greater risk of developing TB disease than HIV-uninfected, non-pregnant women [4,5]. Maternal HIV and TB co-infection has serious consequences on infant morbidity and mortality [6]. In particular, the rate of *M.tb* exposure in children born to HIV-infected mothers is high, and they are at a high risk of developing active TB disease following infection, including those who remain HIV-uninfected, in high TB burden settings [7– 9].

Here, the existing literature was examined in order to understand the relationship between socio-demographic and clinical risk factors and *M.tb* infection and TB disease particularly in HIV-infected mothers and their HIV-exposed infants.

2. OBJECTIVES OF THE LITERATURE REVIEW

The focus of this study is to determine the burden of and risk factors for TB in HIV-infected mothers and HIV-exposed infants in the first year of life, specifically in a population enrolled in a TB vaccine trial in TB and HIV endemic areas of South Africa. The literature review aims to illustrate the importance of the study by examining the existing literature on the prevalence and incidence of *M.tb* infection and TB disease specifically in the context of HIV, and the risk factors that determine the transition from *M.tb* exposure to *M.tb* infection and to TB disease in infants and adults [10,11] but specifically in HIV-infected pregnant and postpartum women and the infants in resource-limited settings, particularly in South Africa. The literature review also aims to identify any gaps in the literature.

3. LITERATURE SEARCH STRATEGY

Online searches for review and study-specific articles, global and national guidelines and government policy documents were conducted between August 2013 and February 2018. Majority of the literature reviewed were published between 2009 and 2017, covering the period of the TB vaccine. Peer-reviewed articles preferably published in high impact journals in English and with online access to the full text, were selected. Further sources were identified by searching reference lists at the end of recently published articles selected in the initial searches. Medical subject headings (MeSH) or keywords were used to search the databases of Google and PubMed. The search phrases or keywords included, among others: TB burden, TB prevalence, TB incidence, TB risk factors, HIV-infected in pregnant women, HIV-infected postpartum women, HIV-exposed infants and TB, TB diagnosis, including QuantiFERON or gamma interferon release assays, PMTCT and ART. A literature review matrix (Table 1) outlines the criteria for inclusion in this review.

3. SUMMARY OF LITERATURE

3.1 Burden of HIV Infection in perinatal women

Approximately one fifth of South African women of reproductive age are HIV-infected [12]. Of major concern is the population of young South African females aged 15 – 24 years. This age group of females has the highest incidence of HIV compared to any other group of females or males, accounting for nearly 37% of annual new HIV infections [13]. The HIV status of pregnant women is of critical importance to their health and that of their children: between 2005 and 2010, HIV infection was associated with an estimated 70% of maternal deaths (WHO definition: during pregnancy or between 42 days and 1year post termination thereof [14]) in South Africa [15] and almost 50% of all deaths of children younger than 5 years [16], although these rates dropped significantly by 2015 [2,17]. HIV prevalence in pregnant women who attend public antenatal clinic services in South Africa is approximately 30% [13], which has remained relatively stable since 2004 [18]. The majority of infants and children who are HIV-infected were infected through mother- to-child transmission (MTCT). Of increasing significance to the vertical transmission of HIV is maternal HIV seroconversion during late pregnancy, after antenatal screening, and while breastfeeding [19]. The South African prevention of MTCT (PMTCT) programme has proven to be effective: there is greater than 95% antenatal HIV testing of HIV-infected pregnant women attending antenatal services, >92% uptake of CD4 cell count testing, increased antiretroviral therapy (ART) uptake and increased infant prophylaxis uptake [20] and there has been significant progress in reducing the early MTCT rates (at 6 weeks postpartum) from 3.5% in 2010 to an estimated 1.2% in 2016 [20,21].

3.2 Burden of TB in perinatal, HIV-infected women

As with HIV infection, *M.tb* infection and TB disease is most common during a women's reproductive age of 15 to 29 years and is a major cause of maternal morbidity and mortality, especially in HIV-infected women [4].

Routine screening for *M.tb* infection and TB disease during pregnancy is not standard practice in majority of countries [4,22] and diagnosis is difficult as physiological changes associated with pregnancy may mask early TB symptoms [23]. The prevalence of active TB among pregnant women is estimated to be 0.07%-0.5% in HIV-uninfected women and 0.7%-11% in HIV-infected women in high burden countries [5]. HIV-infected pregnant women with LTBI are more likely to progress to active disease [4]. TB disease is a major cause of maternal morbidity and mortality, especially in HIV-infected women [2], accounting for 15 to 35% of maternal deaths worldwide in 2013 [24] and 26.3% of maternal deaths in South Africa between 2011 and 2013 [25]. The risk of maternal morbidity in pregnant women with active TB was reported to be three times greater than pregnant women who did not have TB [25,26] and these outcomes are worse in pregnant women with TB-HIV co-infection than in those who are only TB or HIV-infected, with an increased risk of maternal and infant mortality of 300% [6]. Pregnancy complications in women with TB include greater odds of antenatal admission and miscarriage, postpartum haemorrhage, difficulties in labour and pre-eclampsia [26,27].

The risk factors affecting maternal outcomes include TB-HIV co-infection, site of disease (pulmonary or extrapulmonary), timing of TB diagnosis and type, timing of initiation and length of TB drug treatment [27,28]. Studies have shown that maternal (and perinatal) outcomes in pregnant women with TB are better when treatment is initiated in the first trimester as opposed to initiation in the second or third trimester [27].

Postpartum women are twice as likely to develop TB as non-pregnant women [29], with reported incidence rates of 5% [30] and 11% [31] with median onset at 3 months post-delivery [30].

3.2.1 Infant mortality and morbidity associated with maternal HIV co-infection

Infants of pregnant women with TB have increased risks of adverse outcomes compared to infants of women without TB. These include an increased risk of perinatal death [6], preterm birth, low birth-weight [6,28] and “small for gestational age” compared to babies born to mothers without TB [27,32]. These perinatal outcomes are higher in women co-infected with HIV and TB than in pregnant women infected with either TB or HIV [5].

3.3 Risk factors of maternal *M.tb* infection and TB disease

Risk of exposure to TB is associated with the prevalence of active pulmonary TB in a given population, is influenced by several risk factors [10,33] and is greater in high TB prevalence regions [34]. Transmission of TB is almost exclusively airborne, from a source with active pulmonary TB disease. The risk of becoming infected depends on;

- Infectivity of the TB case (frequency of coughing, density of bacilli in the sputum and microbial virulence),
- Intensity and duration of the exposure to the TB case
- Susceptibility of that person to infection [10].

The lifetime risk of developing TB disease in healthy *M.tb* infected individuals is 5-15% and is highest in the first two years of infection. Adult TB disease results from either recent infection or reactivation of latent or previously acquired infection. In endemic areas, most transmission of TB to adults appears to occur outside the household [35–37], possibly at places of social gatherings such as informal bars (shebeens), churches, hospitals or clinics and public transport in minibus taxis, or prior imprisonment [37].

HIV co-infection is recognized as the strongest risk factor for TB disease increasing the risk either by facilitating reactivation of a latent infection or by favouring the progression of a recently acquired infection towards active disease in HIV-infected patients [38]. Globally, HIV-infected adults infected with TB are 19 times more likely to develop TB than HIV-uninfected adults, in the absence of preventive therapy [39]. In South Africa 61% of TB cases are HIV-infected [40]. A recent study showed that TB incidence rates were higher in HIV-infected individuals than HIV-uninfected individuals living in the same community, despite ART [41]. Middelkoop *et al* (2015) [37], found that 60% of TB cases among HIV-infected patients, including those receiving ART in a high HIV and TB burden area with high ART coverage, were from recent *M.tb* infection. Lawn *et al* (2009) [42] demonstrated that TB incidence was higher in those with CD4 counts <200 cells/∝l who were on ART for the first 4months, although TB rates remained high at CD4 counts between 200-500 cells/∝l during long-term ART.

Pregnancy affects the course of TB, as a result of immunological changes that can mask symptoms of TB and increase the susceptibility to new infection and reactivation and more rapid progression to TB disease [4,22]. Pregnancy is a risk factor for development of TB disease in HIV-infected women with LTBI in endemic areas [4,43]. Risk factors for TB disease in postpartum HIV-infected women included a CD4 count <200 cells/mm^3, an HIV viral load of > 50 000 copies/ml and *M.tb* infection [30] as well as subclinical TB during pregnancy, nutritional stress due to lactation, lack of sleep, rapid hormonal changes and an altered immune response [30].

Besides contact with a source case, HIV status and pregnancy or postpartum, other risk factors include [28,37,44–46]:

- Low socio-economic status, the main confounder for TB exposure, infection and disease.
- Age, particularly maternal
- Genetic susceptibility (certain host genes may play a role in determining susceptibility)
- Ethnicity
- Diabetes, cancer, silicosis and other immunosuppressive treatment
- Previous TB treatment
- Smoking
- Alcohol misuse and drug abuse as adults may frequent places such as bars that are more likely to have other TB sources, increasing the risk of

developing TB due to impaired immunity and are less likely to have successful TB treatment
- Overcrowding
- Migration from rural areas to urban residences and back.

3.4 Burden of TB in infants

Of the estimated 10.4 million new active cases of tuberculosis globally [1], 1 million (10%) were among children (0-14 years). The mortality rate in children with TB, especially those younger than 5 years of age, and those who are HIV-infected, is very high with an estimated 169 000 (31%) deaths in HIV-uninfected children and 41 000 deaths in HIV-infected children in 2015 [1]. Mortality is significantly higher in children aged 0-4 years than in those aged 5-14 years [47].

The WHO estimated TB incidence in South African children (0-14 years) was 33 000 in children in 2015 [1]. There is limited data on the estimates of TB incidence in children under the age of 5 and infants aged 1 year and younger in South Africa as most estimates report TB in children between the ages of 0 and 14 only. Also, diagnosis of paediatric TB is difficult, as childhood TB is usually paucibacillary, leading to undiagnosed and hence untreated TB disease. South African studies showed a high burden of TB in children younger than 5 years of age in Cape Town and surrounding areas, with the highest incidence in those aged 12-23 months [48]. The TB incidence in HIV-uninfected children younger than 2 years old in 2002 was between 858-866/100 000 person-years [49] and 511/100 000 population in a cohort of HIV-uninfected children younger than 5 years of age in 2009 [50]. The estimated incidence of TB in a population of infants aged 1 and younger between 2004-2006 was 1596/100 000 in HIV-infected infants and 66/100 000 in HIV-uninfected infants [51].

3.5 Burden of TB in HIV-exposed, uninfected infants

Although the number of HIV-infected infants is declining due to expanding PMTCT programmes providing ART during pregnancy, delivery and breastfeeding [52], the number of HIV-exposed, uninfected infants is increasing. These infants have been exposed to HIV *in utero* and in some cases to multiple antiretroviral drugs at birth or throughout the period of breastfeeding [53], for which the effect on their health is uncertain. Several studies have shown that these HIV-exposed, uninfected infants have increased mortality rates, increased infectious morbidity, immune dysfunction and impaired growth compared with HIV-unexposed infants [52,53]. Mortality of HIV-exposed, uninfected infants in a large study done before maternal ART was widely used, was as much as two to four times as high [52,54] as that of HIV-unexposed infants. HIV-exposed, uninfected infant mortality was inversely affected by maternal CD4 count (the lower the maternal CD4 count, the higher the risk of infant mortality), was higher in the first year of life, and was influenced by the time of weaning from breast milk [52]. Infant morbidity and mortality was mainly due to lower respiratory tract and gastrointestinal infections [52,55]. Infants of HIV-infected mothers are at increased risk of exposure to *M.tb* and high risk of developing active TB disease following infection [7], whether they are HIV-infected or not [7–9]. In a South African study, the estimated incidence of TB exposure was 10.03% and a rate of disease of 2% [7]. In another South African study, the incidence of TB in HIV-exposed,

uninfected children younger than 2 years of age was reported to be 4.1% and 12.1% in HIV-infected infants [8]. Up to 50% of HIV-exposed, uninfected infants will develop TB, 30% of whom will have disseminated disease following *M.tb* exposure and infection [56].

3.6 Risk factors of childhood *M.tb* infection and TB disease

Infants or young children with new *M.tb* infection represent recent and ongoing transmission within the community and therefore act as a sentinel for TB transmission [57,58]. Rates of childhood *M.tb* infection are associated with prevalence of adult TB in the same community [59]. In contrast to TB disease progression in adults, childhood TB disease results from recent infection, predominantly from exposure to sputum smear positive adult household contacts, as child-child transmission is not common [57]. In 2015, globally an estimated 1.2 million children under the age of 5 years had a household contact with active TB [1]. Older children interact more with adults than younger children and they can therefore either be infected at home, within close social networks [57] or within the community. The risk of *M.tb* infection and progression to TB disease in children, including those younger than 2 years of age, was greater if household exposure was to a sputum smear-positive source case, due to greater bacillary loads of these contacts, than to a sputum smear-negative source case, such as HIV-infected adults [11,58].

The risk of infection increases with more daily interaction and longer duration of contact with the source case [11]. Progression to disease is usually within 12-23 months of infection [49] with an estimated 50% of infants progressing to TB disease in the first year of life if their mothers have active TB disease, in the absence of Isoniazid Preventive Therapy (IPT) or if Bacillus Calmette-Guérin (BCG)-unvaccinated [60].

Age is an important risk factor in disease progression [11,34]. Infected infants have a 50% lifetime risk of progression to disease following infection [11], whilst children from 1-2 years and 3-5 years of age have a 20-30% and 5% risk, respectively. The risk in those 5-10 years old and older than 10 years have a 2% and 5% risk, respectively [34]. Chronic malnourished children have an increased risk of TB disease, including extrapulmonary disease because of an altered immune response [61].

Vertical transmission of TB (congenital TB) to the foetus *in utero* or intrapartum is considered to be rare in low HIV burden settings and difficult to diagnose, but can occur, particularly if the mother is HIV-infected and has disseminated TB, miliary or meningeal TB [17,28,62]. Studies conducted in South Africa reported congenital TB in 3-16% infants [28,30,46] born to HIV-infected women. Mortality is high at 22-50% [56].

HIV infection is a very significant risk for childhood TB disease. HIV-infected children are at increased risk of progressing to active TB as well as developing serious forms of TB such as disseminated (miliary) TB and TB meningitis [60] and are more likely to have HIV-TB co-infection that increases the child's risk of *M.tb* exposure. In a South African study, HIV-infected infants were at a 24.1 fold higher risk of pulmonary

TB and a 17.1 fold higher risk of disseminated TB compared with HIV-uninfected infants [51].

Other risk factors, besides HIV infection for exposure to and infection with *M.tb* and TB disease in infants and children include (Figure 1):

3.6.1 Socio-demographic and economic factors

- Composition of the household, as risk of TB contact increases with more adults living in one house [34]
- Physical structure of the house i.e how many rooms there are as living areas [63]
- Sleeping arrangements (infants and children may sleep with adults or with other children) [34]
- Presence of tobacco/cigarette smokers in the house [58,64] as smoke exposure is associated with *M.tb* infection in children with adult TB contact [65–67]
- Indoor pollution from biofuels used for heating or cooking may interfere with mucosal integrity and mucosal immunity
- Poor ventilation in the home, where droplet tubercle bacilli stay airborne for longer and survive longer in dark spaces than if exposed to sunlight [34]
- Alcohol abuse [34]

3.6.2 Biological/clinical risk factors

- Helminth (worm) infestation [34]
- Genetic susceptibility and race/ethnicity [10,33]
- Breastfeeding or Formula Feeding (in low resource settings) present risks for *M.tb* infection (and other infections) from mother, although the risk of *M.tb* is negligible if breast feeding is exclusive and mother has been on anti-TB therapy [5,62]
- Lack of BCG vaccination, which can protect against TB, especially disseminated TB and TBM in children [68].

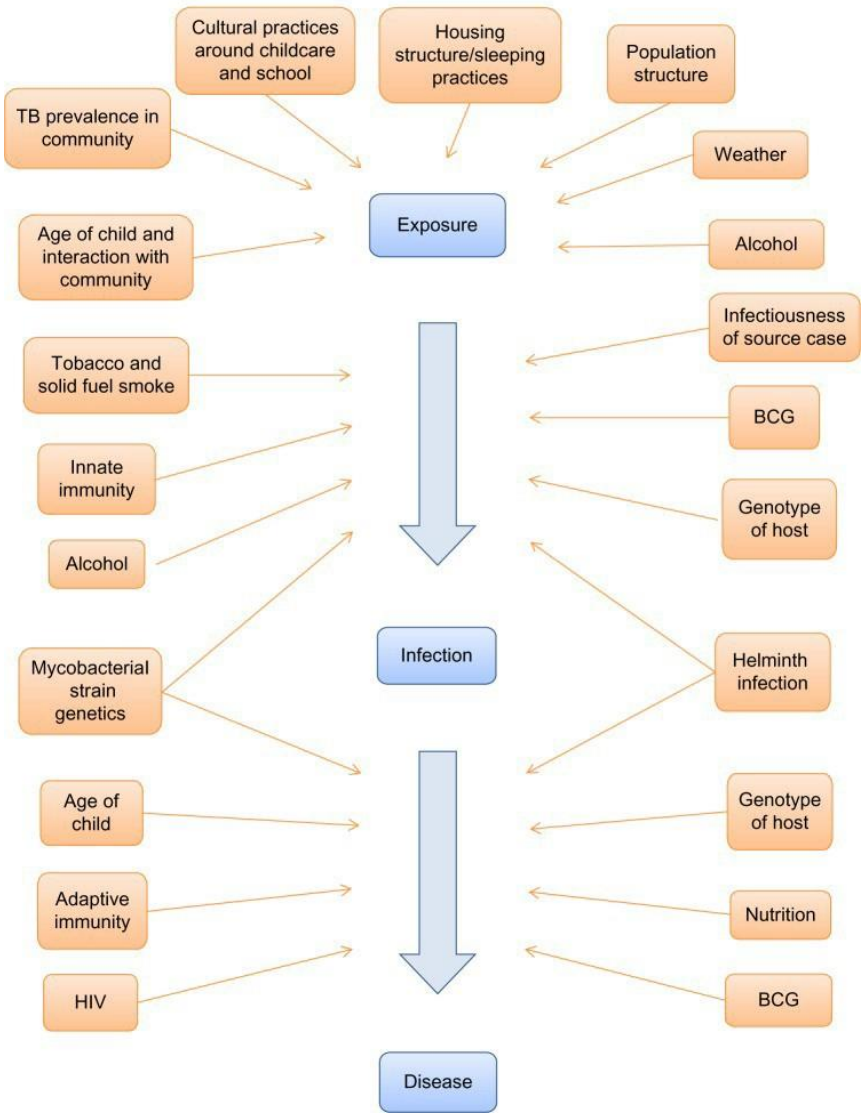


Figure 1: Risk factors for tuberculosis exposure, infection and disease in children. Source: Seddon and Shingadia, 2014 [34]

3.7 Risk factors of *M.tb* infection and TB disease in HIV-exposed, uninfecetd infants

Data on *M.tb* infection and TB disease is limited in HIV-exposed, uninfected infants. In addition to the risk factors described in section 3.6, specifically household contact [7] and adverse environmental and socio-economic conditions, other risk factors include duration of breastfeeding and quality of breast milk, severity of maternal HIV disease [53] and vertical transmission of TB [46]. Immune dysfunction has been reported in HIV- exposed infants and immune activation could lead to increased susceptibility to infection [53]. BCG vaccination is contra-indicated in HIV-infected infants due to the risk of local and disseminated BCG disease and BCG immune constitutional inflammatory syndrome (BCG-IRIS) [68,69]. However, delaying BCG vaccination in HIV-exposed infants until they are confirmed HIV-uninfected puts these infants at risk of being infected with *M.tb* in the first weeks of life in high TB and maternal HIV prevalent settings [69,70].

4. NEED FOR FURTHER RESEARCH

Further research into HIV-infected pregnant and postpartum women is necessary especially with respect to the epidemiology, immunology and pathogenesis of tuberculosis [5].

More population-based estimates of TB incidence in HIV-uninfected and HIV-, infected infants and children younger than 5 years in resource-limited, high HIV and TB disease burden settings are needed and there is a need for longitudinal studies on *M.tb* exposure, infection and TB disease, particularly in the increasing population of HIV-exposed, uninfected infants. Longitudinal studies are required to investigate the immunological and biological mechanisms that result in increased mortality and infectious morbidity and impaired growth in HIV-exposed, uninfected infants [54]. An important public health question to investigate is whether these outcomes are a result of long-term HIV-1 and/or *in utero* ART exposure or whether these outcomes are a result of adverse socio-economic factors associated with being born into an HIV-affected household [55,71]

5. CONCLUSION

HIV-infected pregnant women with LTBI and in early postpartum are at greater risk of *M.tb* infection and TB disease, with increased risk of maternal and infant morbidity and mortality, than HIV-uninfected, non-pregnant women. HIV co-infection is the strongest risk factor for maternal TB disease. Infants of HIV-infected mothers are at an increased risk of exposure to *M.tb* and developing active TB disease following infection, even if they are HIV-uninfected. Household TB contact is one of the greatest risks for infant *M.tb* infection.

Currently, there are limited data on risks for *M.tb* infection and TB disease in pregnant and postpartum women, particularly those who are HIV-infected, and HIV-exposed, uninfected infants. Therefore, determining which of these mother-infants pairs are at the highest risk of becoming infected with *M.tb* and developing TB disease is critical in order to reduce the burden of disease and death caused by TB in this population. Towards this, improved and cost-effective TB screening, particularly antenatal and post-partum, as well infant and household screening, active case finding programmes among pregnant women [4], with heightened clinical awareness of TB diagnosis in pregnancy and postpartum [28], improved maternal care and ART regimens during and after pregnancy [55] and provision of TB interventions such as preventive chemotherapy and new, effective TB vaccine candidates for HIV-exposed, uninfected infants are imperative.

6. REFERENCES

1. World Health Organization. Global tuberculosis report 2016 [Internet]. 2016. Available: www.who.int/tb/publications/global_report/en/

2. Statistics South Africa. Mortality and causes of death in South Africa, 2015 [Internet]. Available: http://www.statssa.gov.za/publications/P03093/P030932015.pdf

3. National Department of Health, South Africa. Joint Review of HIV, TB and PMTCT Programmes in South Africa - April 2014 | Health Systems Trust [Internet]. [cited 29 Jan 2017]. Available: http://www.hst.org.za/publications/joint-review-hiv-tb-and-pmtct-programmes-south-africa-april-2014

4. Bates M, Ahmed Y, Kapata N, Maeurer M, Mwaba P, Zumla A. Perspectives on tuberculosis in pregnancy. Int J Infect Dis. 2015;32: 124–127. doi:10.1016/j.ijid.2014.12.014

5. Mathad JS, Gupta A. Tuberculosis in Pregnant and Postpartum Women: Epidemiology, Management, and Research Gaps. Clin Infect Dis Off Publ Infect Dis Soc Am. 2012;55: 1532–1549. doi:10.1093/cid/cis732

6. World Health Organization. Tuberculosis in Women 2015 [Internet]. Available: www.who.int/tb

7. Cotton MF, Schaaf HS, Lottering G, Weber HL, Coetzee J, Nachman S, et al. Tuberculosis exposure in HIV-exposed infants in a high-prevalence setting. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2008;12: 225–227.

8. Madhi SA, Nachman S, Violari A, Kim S, Cotton MF, Bobat R, et al. Primary isoniazid prophylaxis against tuberculosis in HIV-exposed children. N Engl J Med. 2011;365: 21–31. doi:10.1056/NEJMoa1011214

9. Martinson NA, Moultrie H, van Niekerk R, Barry G, Coovadia A, Cotton M, et al. HAART and risk of tuberculosis in HIV-infected South African children: a multi-site retrospective cohort. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2009;13: 862–867.

10. Lienhardt C. From exposure to disease: the role of environmental factors in susceptibility to and development of tuberculosis. Epidemiol Rev. 2001;23: 288–301.

11. Marais BJ, Rabie H, Cotton MF. TB and HIV in children – advances in prevention and management. Paediatr Respir Rev. 2011;12: 39–45. doi:10.1016/j.prrv.2010.09.002

12. Statistics South Africa. Mid-year population estimates 2015 [Internet]. Available: https://www.statssa.gov.za/publications/P0302/P03022015.pdf

13. National Aids Council, South Africa. National Strategic Plan for HIV, TB and STIs 2017-2022 [Internet]. Available: http://sanac.org.za/2017/09/06/download-the-full-version-of-the-national-strategic-plan-for-hiv-tb-and-stis-2017-2022/

14. World Health Oganization. International Classification of diseases and related health problems [Internet]. 1992. Available: http://apps.who.int/classifications/icd10/browse/2016/en#/XV

15. National Department of Health, South Africa. Saving mothers 2008-2010: Fifth report on the confidential enquiries into Maternal Deaths in South Africa.

16. Bamford L, Stephen C, Patrick M. "Saving Children 2009": Evaluating quality of care through mortality auditing. SAJCH. 2011;5: 28–32.

17. National Department of Health, South Africa. National tuberculosis management guidelines 2014. Pretoria: Department of Health; 2014.

18. National Department of Health, South Africa. The 2013 National Antenatal Sentinel HIV Prevalence Survey South Africa. 2015.

19. Johnson LF, Stinson K, Newell M-L, Bland RM, Moultrie H, Davies M-A, et al. The contribution of maternal HIV seroconversion during late pregnancy and breastfeeding to mother-to-child transmission of HIV. J Acquir Immune Defic Syndr 1999. 2012;59: 417–425. doi:10.1097/QAI.0b013e3182432f27

20. Goga A, Chirinda W, Bhardwaj S, Pillay Y, Sherman G, Ng'oma K, et al. Eliminating mother-to-child transmission of HIV in South Africa, 2002-2016: progress, challenges and the last mile plan. Chapter 13 SAHR 20th Ed. 2016; 137–146.

21. Goga AE, Dinh T, Jackson DJ, Lombard CJ, Puren A, Sherman G, et al. Population–level effectiveness of PMTCT Option A on early mother–to–child (MTCT) transmission of HIV in South Africa: implications for eliminating MTCT. J Glob Health. 2016;6. doi:10.7189/jogh.06.020405

22. Mathad JS, Bhosale R, Sangar V, Mave V, Gupte N, Kanade S, et al. Pregnancy Differentially Impacts Performance of Latent Tuberculosis Diagnostics in a High-Burden Setting. PLOS ONE. 2014;9: e92308. doi:10.1371/journal.pone.0092308

23. Hoffmann CJ, Variava E, Rakgokong M, Masonoke K, van der Watt M, Chaisson RE, et al. High Prevalence of Pulmonary Tuberculosis but Low Sensitivity of Symptom Screening among HIV-Infected Pregnant Women in South Africa. Cattamanchi A, editor. PLoS ONE. 2013;8: e62211. doi:10.1371/journal.pone.0062211

24. Sugarman J, Colvin C, Moran AC, Oxlade O. Tuberculosis in pregnancy: an estimate of the global burden of disease. Lancet Glob Health. 2014;2: e710–e716. doi:10.1016/S2214-109X(14)70330-4

25. National Department of Health, South Africa. Saving Mothers 2011-2013: Sixth report on the confidential enquiries into maternal deaths in South Africa.

26. Nguyen HT, Pandolfini C, Chiodini P, Bonati M. Tuberculosis care for pregnant women: a systematic review. BMC Infect Dis. 2014;14: 617. doi:10.1186/s12879-014-0617-x

27. Sobhy S, Babiker Z, Zamora J, Khan K, Kunst H. Maternal and perinatal mortality and morbidity associated with tuberculosis during pregnancy and the postpartum period: a systematic review and meta-analysis. BJOG Int J Obstet Gynaecol. 2016; n/a-n/a. doi:10.1111/1471-0528.14408

28. Bekker A, Schaaf HS, Draper HR, Kriel M, Hesseling AC. Tuberculosis Disease during Pregnancy and Treatment Outcomes in HIV-Infected and Uninfected Women at a Referral Hospital in Cape Town. PLOS ONE. 2016;11: e0164249. doi:10.1371/journal.pone.0164249

29. Zenner D, Kruijshaar ME, Andrews N, Abubakar I. Risk of tuberculosis in pregnancy: a national, primary care-based cohort and self-controlled case series study. Am J Respir Crit Care Med. 2012;185: 779–784. doi:10.1164/rccm.201106-1083OC

30. Gupta A, Nayak U, Ram M, Bhosale R, Patil S, Basavraj A, et al. Postpartum tuberculosis incidence and mortality among HIV-infected women and their infants in Pune, India, 2002-2005. Clin Infect Dis Off Publ Infect Dis Soc Am. 2007;45: 241–249. doi:10.1086/518974

31. Nachega J, Coetzee J, Adendorff T, Msandiwa R, Gray GE, McIntyre JA, et al. Tuberculosis active case-finding in a mother-to-child HIV transmission prevention programme in Soweto, South Africa. AIDS Lond Engl. 2003;17: 1398–1400. doi:10.1097/01.aids.0000060408.18106.d9

32. Lin H-C, Lin H-C, Chen S-F. Increased risk of low birthweight and small for gestational age infants among women with tuberculosis: Pregnancy and tuberculosis. BJOG Int J Obstet Gynaecol. 2010;117: 585–590. doi:10.1111/j.1471-0528.2010.02504.x

33. Hargreaves JR, Boccia D, Evans CA, Adato M, Petticrew M, Porter JDH. The Social Determinants of Tuberculosis: From Evidence to Action. Am J Public Health. 2011;101: 654–662. doi:10.2105/AJPH.2010.199505

34. Seddon JA, Shingadia D. Epidemiology and disease burden of tuberculosis in children: a global perspective. Infect Drug Resist. 2014;7: 153–165. doi:10.2147/IDR.S45090

35. Verver S, Warren RM, Munch Z, Richardson M, van der Spuy GD, Borgdorff MW, et al. Proportion of tuberculosis transmission that takes place in households in a high-incidence area. The Lancet. 2004;363: 212–214. doi:10.1016/S0140-6736(03)15332-9

36. Crampin AC, Glynn JR, Traore H, Yates MD, Mwaungulu L, Mwenebabu M, et al. Tuberculosis transmission attributable to close contacts and HIV status, Malawi. Emerg Infect Dis. 2006;12: 729–735. doi:10.3201/eid1205.050789

37. Middelkoop K, Mathema B, Myer L, Shashkina E, Whitelaw A, Kaplan G, et al. Transmission of Tuberculosis in a South African Community With a High Prevalence of HIV Infection. J Infect Dis. 2015;211: 53–61. doi:10.1093/infdis/jiu403

38. Santin M, Muñoz L, Rigau D. Interferon-γ Release Assays for the Diagnosis of Tuberculosis and Tuberculosis Infection in HIV-Infected Adults: A Systematic Review and Meta-Analysis. PLOS ONE. 2012;7: e32482. doi:10.1371/journal.pone.0032482

39. World Health Organization. TB/HIV Factsheet 2016 [Internet]. Available: www.who.int/tb/challenges/hiv/factsheets/en/

40. World Health Organization. Global tuberculosis report 2015 [Internet]. Geneva, Switzerland: World Health Organization; 2015. Available: apps.who.int/iris/bitstream/10665/191102/1/9789241565059_eng.pdf

41. Gupta A, Wood R, Kaplan R, Bekker L-G, Lawn SD. Tuberculosis incidence rates during 8 years of follow-up of an antiretroviral treatment cohort in South Africa: comparison with rates in the community. PloS One. 2012;7: e34156. doi:10.1371/journal.pone.0034156

42. Lawn SD, Myer L, Edwards D, Bekker L-G, Wood R. Short-term and long-term risk of tuberculosis associated with CD4 cell recovery during antiretroviral therapy in South Africa: AIDS. 2009;23: 1717–1725. doi:10.1097/QAD.0b013e32832d3b6d

43. Pillay T, Khan M, Moodley J, Adhikari M, Coovadia H. Perinatal tuberculosis and HIV-1: considerations for resource-limited settings. Lancet Infect Dis. 2004;4: 155–165. doi:10.1016/S1473-3099(04)00939-9

44. National Department of Health, South Africa. National consolidated guidelines for the prevention of mother-to-child transmission of HIV (PMTCT) and the management of HIV in children, adolescents and adults. Pretoria: Department of Health; 2015.

45. Pai M, Denkinger CM, Kik SV, Rangaka MX, Zwerling A, Oxlade O, et al. Gamma Interferon Release Assays for Detection of Mycobacterium tuberculosis Infection. Clin Microbiol Rev. 2014;27: 3–20. doi:10.1128/CMR.00034-13

46. Pillay T, Sturm AW, Khan M, Adhikari M, Moodley J, Connolly C, et al. Vertical transmission of Mycobacterium tuberculosis in KwaZulu Natal: impact of HIV-1 co-infection. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2004;8: 59–69.

47. Jenkins HE, Yuen CM, Rodriguez CA, Nathavitharana RR, McLaughlin MM, Donald P, et al. Mortality in children diagnosed with tuberculosis: a systematic review and meta-analysis. Lancet Infect Dis. 2016; doi:10.1016/S1473-3099(16)30474-1

48. Moyo S, Verver S, Mahomed H, Hawkridge A, Kibel M, Hatherill M, et al. Age-related tuberculosis incidence and severity in children under 5 years of age in Cape Town, South Africa. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2010;14: 149–154.

49. Mahomed H, Kibel M, Hawkridge T, Schaaf HS, Hanekom WA, Iloni K, et al. The impact of a change in bacille Calmette-Guérin vaccine policy on tuberculosis incidence in children in Cape Town, South Africa. Pediatr Infect Dis J. 2006;25: 1167–1172. doi:10.1097/01.inf.0000243765.33880.54

50. Wood R, Lawn SD, Caldwell J, Kaplan R, Middelkoop K, Bekker L-G. Burden of New and Recurrent Tuberculosis in a Major South African City Stratified by Age and HIV-Status. Cattamanchi A, editor. PLoS ONE. 2011;6: e25098. doi:10.1371/journal.pone.0025098

51. Hesseling AC, Cotton MF, Jennings T, Whitelaw A, Johnson LF, Eley B, et al. High Incidence of Tuberculosis among HIV-Infected Infants: Evidence from a South African Population- Based Study Highlights the Need for Improved Tuberculosis Control Strategies. Clin Infect Dis. 2009;48: 108–114. doi:10.1086/595012

52. Kourtis AP, Wiener J, Kayira D, Chasela C, Ellington SR, Hyde L, et al. Health outcomes of HIV-exposed uninfected African infants. AIDS Lond Engl. 2013;27: 749–759. doi:10.1097/QAD.0b013e32835ca29f

53. Evans C, Jones CE, Prendergast AJ. HIV-exposed, uninfected infants: new global challenges in the era of paediatric HIV elimination. Lancet Infect Dis. 2016;16: e92–e107. doi:10.1016/S1473-3099(16)00055-4

54. Cotton MF, Slogrove A, Rabie H. Infections in HIV-exposed Uninfected Children With Focus on Sub-Saharan Africa: Pediatr Infect Dis J. 2014;33: 1085–1086. doi:10.1097/INF.0000000000000489

55. Afran L, Garcia Knight M, Nduati E, Urban BC, Heyderman RS, Rowland-Jones SL. HIV-exposed uninfected children: a growing population with a vulnerable immune system? Clin Exp Immunol. 2014;176: 11–22. doi:10.1111/cei.12251

56. Bekker A, Du Preez K, Schaaf HS, Cotton MF, Hesseling AC. High tuberculosis exposure among neonates in a high tuberculosis and human immunodeficiency virus burden setting. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2012;16: 1040–1046. doi:10.5588/ijtld.11.0821

57. Middelkoop K, Bekker L-G, Morrow C, Zwane E, Wood R. Childhood tuberculosis infection and disease: a spatial and temporal transmission analysis in a South African township. South Afr Med J Suid-Afr Tydskr Vir Geneeskd. 2009;99: 738–743.

58. Singh M, Mynak ML, Kumar L, Mathew JL, Jindal SK. Prevalence and risk factors for transmission of infection among children in household contact with adults having pulmonary tuberculosis. Arch Dis Child. 2005;90: 624–628. doi:10.1136/adc.2003.044255

59. Bunyasi EW, Schmidt B-M, Abdullahi LH, Mulenga H, Tameris M, Luabeya A, et al. Prevalence of latent TB infection and TB disease among adolescents in high TB burden countries in Africa: a systematic review protocol. BMJ Open. 2017;7. doi:10.1136/bmjopen-2016-014609

60. National Department of Health, South Africa. Guidelines for the management of Tuberculosis in children, South Africa, 2013, National Department of Health [Internet]. Available: www.kznhealth.gov.za/family/National-Childhood-TB-Guidelines-2013-ZA.pdf

61. Jenum S, Selvam S, Mahelai D, Jesuraj N, Cárdenas V, Kenneth J, et al. Influence of Age and Nutritional Status on the Performance of the Tuberculin

62. Skin Test and QuantiFERON-TB Gold In-Tube in Young Children Evaluated for Tuberculosis in Southern India: Pediatr Infect Dis J. 2014;33: e260–e269. doi:10.1097/INF.0000000000000399

63. Adhikari M, Jeena P, Bobat R, Archary M, Naidoo K, Coutsoudis A, et al. HIV-Associated Tuberculosis in the Newborn and Young Infant. Int J Pediatr. 2011;2011: 354208. doi:10.1155/2011/354208

64. Seddon JA, Hesseling AC, Godfrey-Faussett P, Fielding K, Schaaf HS. Risk factors for infection and disease in child contacts of multidrug-resistant tuberculosis: a cross-sectional study. BMC Infect Dis. 2013;13: 392. doi:10.1186/1471-2334-13-392

65. Lin H-H, Chiang Y-T, Chuang J-H, Yang S-L, Chang H-Y, Ezzati M, et al. Exposure to Secondhand Smoke and Risk of Tuberculosis: Prospective Cohort Study. PLOS ONE. 2013;8: e77333. doi:10.1371/journal.pone.0077333

66. den Boon S, Verver S, Marais BJ, Enarson DA, Lombard CJ, Bateman ED, et al. Association between passive smoking and infection with Mycobacterium tuberculosis in children. Pediatrics. 2007;119: 734–739. doi:10.1542/peds.2006-1796

67. Huang C-C, Tchetgen ET, Becerra MC, Cohen T, Galea J, Calderon R, et al. Cigarette smoking among tuberculosis patients increases risk of transmission to child contacts. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2014;18: 1285–1291. doi:10.5588/ijtld.14.0309

68. Lin H-H, Ezzati M, Murray M. Tobacco Smoke, Indoor Air Pollution and Tuberculosis: A Systematic Review and Meta-Analysis. Novotny TE, editor. PLoS Med. 2007;4: e20. doi:10.1371/journal.pmed.0040020

69. Hesseling AC, Marais BJ, Gie RP, Schaaf HS, Fine PEM, Godfrey-Faussett P, et al. The risk of disseminated Bacille Calmette-Guerin (BCG) disease in HIV-infected children. Vaccine. 2007;25: 14–18. doi:10.1016/j.vaccine.2006.07.020

70. Hesseling AC, Cotton MF, Fordham von Reyn C, Graham SM, Gie RP, Hussey GD. Consensus statement on the revised World Health Organization recommendations for BCG vaccination in HIV-infected infants. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2008;12: 1376–1379.

71. Nemes E, Hesseling AC, Tameris M, Mauff K, Downing K, Mulenga H, et al. Safety and Immunogenicity of Newborn MVA85A Vaccination and Selective, Delayed Bacille Calmette-Guerin (BCG) for Infants of HIV Infected Mothers: A Phase 2 Randomized Controlled Trial. Clin Infect Dis Off Publ Infect Dis Soc Am. 2017; doi:10.1093/cid/cix834

72. Mofenson LM. New Challenges in the Elimination of Pediatric HIV Infection: The Expanding Population of HIV-Affected but Uninfected Children. Clin Infect Dis. 2015;doi:10.1093/cid/civ064

Table 1: Sample of Literature Review Matrix

Research Question: What is the burden of and risk factors for TB in HIV-infected mothers and HIV-exposed infants in the first year of life?						
Author(s), Title, Journal	Year published	Purpose/Objective	Methodology/ Design	Participant characteristics	Analysis and results	Comments
Burden of TB and HIV						
WHO; Global Tuberculosis Report	2014, 2015, 2016	To report on the global TB epidemic for the preceding year of the report	Global TB report on aspects of TB specifically burden of TB (prevalence, incidence & mortality)	NA	Data collected via data collection forms from individual countries, territories, WHO regions and the world	Global TB burden and HIV-TB co-infection stats were included in proposal introduction, literature review and publication. The burden of TB, HIV and MDR-TB stats for the South Africa for the preceding year of the report is provided the country profile found in the in annexes.
National Department of Health, South Africa. The 2013 National Antenatal Sentinel HIV Prevalence Survey South Africa. 2015.	2015	To estimate prevalence of HIV infection and disease at national, provincial and district levels	Survey	33 077 women attending antenatal clinics for the first time	Biological specimens (blood) processed by ELISA at central laboratories. Provincial and National prevalence estimates calculated	Used for assessing the burden of HIV in women of reproductive age (15-29)
Burden of TB in HIV-infected pregnant and postpartum women						
Mathad JS, Gupta A. Tuberculosis in Pregnant and Postpartum Women: Epidemiology, Management, and Research Gaps. Clin Infect Dis Off Publ Infect Dis Soc Am. 2012;55: 1532–1549.	2012	To review *M.tb* infection and TB disease in pregnant and postpartum women including burden thereof, affect of pregnancy on TB, screening, diagnosis and management	Review of available data and studies	NA	Review of available literature	Used for assessing the burden and risks of TB in HIV-infected pregnant and postpartum women. Future research requirements highlighted.
Gupta A, Nayak U, Ram M, Bhosale R, Patil S, Basavraj A, *et al*. Postpartum tuberculosis incidence and mortality among HIV-infected women and their infants in Pune, India, 2002-2005. Clin Infect Dis Off Publ Infect Dis Soc Am. 2007;45: 241–249.	2007	To investigate postpartum TB incidence and mortality in HIV-infected women-infant pairs	Study	715 mothers-infant pairs followed up 1 year post delivery	TST on women at routine clinic visit post delivery. 24 developed TB = incidence of 5%. High incidence of postpartum maternal and infant mortality. Active screening and IPT needed in India	Actual data from a study rather than a review. Incidence, time onset and risk factors for TB disease reported
Burden of TB in HIV-exposed, uninfected infants						
Hesseling AC, Cotton MF, Jennings T, Whitelaw A, Johnson LF, Eley B, et al. High Incidence of Tuberculosis among HIV-Infected Infants: Evidence from a South African Population-Based Study Highlights the Need for Improved Tuberculosis Control Strategies. Clin	2009	Estimate incidence of TB in HIV-infected and HI-uninfected infants in the Western Province, South Africa	Prospective Study	245 culture-confirmed TB cases (infants)	Hospital surveillance data of annual number of culture-confirmed TB cases among infants between 2004-2006 TB Incidence 1596 cases and 65,9 cases per 100,000 population among HIV-infected infants and HIV-uninfected infants respectively Relative risk was 24.2	Indication of the high burden of TB in HIV-infected and HIV-uninfected infants in the Western Cape Province, South Africa

Infect Dis. 2009;48:108114.						
Cotton MF, Schaaf HS, Lottering G, Weber HL, Coetzee J, Nachman S, et al. Tuberculosis exposure in HIV-exposed infants in a high-prevalence setting. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2008;12: 225–227.	2008	To determine incidence of TB in HIV-exposed infants after household exposure	Study	766 HIV-exposed infants (3-4 months old)	Household TB contact in 77 (10.1%) infants	Need for TB screening of mothers at PMTCT clinics to reduce burden of *M.tb* infection and TB disease in HIV-exposed infants
Maternal and infant risk factors for *M.tb* infection and TB disease						
Seddon JA, Shingadia D. Epidemiology and disease burden of tuberculosis in children: a global perspective. Infect Drug Resist. 2014;7: 153–165.	2014	Review epidemiology of TB in children	Review	NA	Analysis of available data and studies	Comprehensive review of the risk factors for M.tb infection and TB disease in children. Good figure used in literature review
Bekker A, Schaaf HS, Draper HR, Kriel M, Hesseling AC. Tuberculosis Disease during Pregnancy and Treatment Outcomes in HIV-Infected and Uninfected Women at a Referral Hospital in Cape Town. PLOS ONE. 2016;11: e0164249.	2016	To investigate TB HIV-infected and uninfected pregnant women and assess treatment outcomes	Prospective cohort Study	HIV-infected and HIV uninfected pregnant women routinely admitted to a large referral hospital in Cape Town	74 women had TB of which 72% were HIV-infected. All maternal and infant deaths occurred in HIV-infected women	Outcomes of TB in HIV-infected pregnant women and their infants
Risk factors for *M.tb* infection and TB disease in HIV-exposed, uninfected infants						
Evans C, Jones CE, Prendergast AJ. HIV-exposed, uninfected infants: new global challenges in the era of paediatric HIV elimination. Lancet Infect Dis. 2016;16: e92–e107	2016	Review effects of HI-exposure on outcomes observed in the increasing population of HIV-exposed, uninfected infants	Review	NA	Increased mortality, morbidity due to infectious disease (including TB), impaired growth and immune dysfuntion in HIV-exposed uninfected infants higher than HIV-uninfected infants	Number of HIV-infected infants declining due to PMTCT but number of HIV-exposed, uninfected infants increasing.
Afran L, Garcia Knight M, Nduati E, Urban BC, Heyderman RS, Rowland-Jones SL. HIV-exposed uninfected children: a growing population with a vulnerable immune system? Clin Exp Immunol. 2014;176: 11–22.	2014	Public health question of effect of HIV and ART in utero in HIV-exposed, uninfected infants and improved maternal	Review	NA	-	Burden of TB in these infants and effect of maternal HIV exposure and ART on these infants

Adapted from Health Sciences Literature Review Made Easy: The Matrix Method *by Judith Garrard*

PART C: JOURNAL READY MANUSCRIPT

The Burden of Perinatal Tuberculosis in HIV-Infected Mothers and Their Infants.

Katrina J Downing

Abstract

Background: HIV-exposed infants are at greater risk of *Mycobacterium tuberculosis* (*M.tb*) infection and tuberculosis disease (TB) compared to HIV-unexposed infants. We aimed to determine the burden of, and risk factors for TB in HIV-infected mothers and their infants.

Methods: Healthy, HIV-exposed South African infants and their mothers were followed for suspected TB from birth to 1 year of age in a vaccine trial. Mothers were tested at baseline for *M.tb* infection using the QuantiFERON-TB Gold (QFT) assay; infants were tested at 1 year. Adjusted Odds ratios (AOR) or coefficients (coef, 95% confidence intervals; CI) are reported for risk of *M.tb* infection and TB.

Results: 248 mother-infant pairs were enrolled. Two infants were HIV-infected (<1%). Prevalence of maternal *M.tb* infection was 42.7% (CI 36.54 – 48.94). Mothers with CD4 count ≥350 cells/mm^3 had increased odds of QFT positivity (OR 1.98, CI 1.12-3.52) and for every 50 cells/mm^3 increase in maternal CD4 count, maternal QFT IFN-γ values (IU/ml) increased by 17% (coef 0.155, CI 0.09-0.22). Incidence rate of maternal TB was 1.36/100 person-years (CI 0.44-4.22). Incidence rate of infant *M.tb* infection and TB was 2.47 (CI 1.03-5.93) and 3.62/100 person-years (CI 1.81-7.23), respectively. Infants with a new household TB contact (HHC) had increased odds of *M.tb* infection (OR 17.71, CI 2.5-123.5), and higher QFT values (coef 4.06, CI 2.68-5.49). Infant odds of TB disease increased with higher QFT values (AOR 1.34, CI 1.08-1.66), HHC (AOR 10.81, CI 1.53-76.42) and maternal smoking (AOR 10.73, CI 1.96-58.89).

Conclusion: Maternal CD4 count affects the QFT result and estimate of *M.tb* infection prevalence. Household exposure is an important driver of *M.tb* transmission to HIV-exposed infants. Maternal and infant improved TB screening and provision of preventive therapy are needed to reduce the burden of childhood TB in this population.

Introduction

South Africa has one of the highest burdens of tuberculosis (TB) and HIV in the world, with an estimated incidence of 454 000 new TB cases per year and one in five of all HIV-infected people, living in South Africa [1].

HIV infection, *M.tb* infection and TB disease are most common during a woman's reproductive age, particularly in South African women [1]. Women aged 15 to 24 years account for nearly 37% of annual new HIV infections [2]. HIV co-infection is recognized as the strongest risk factor for TB disease that either facilitates reactivation of a latent infection or favours the progression of a recently acquired infection towards active disease in HIV-infected individuals. Globally, HIV-infected adults infected with *M.tb* are 19 times more likely to develop TB than HIV-uninfected adults, in the absence of preventive therapy [3], which is similar in South Africa [4]. In South Africa 61% of TB cases are reported to be HIV-infected [5]. HIV-infected pregnant women with latent TB infection (LTBI) are more likely to progress to active disease [6]. The prevalence of active TB among HIV-infected, pregnant women in high TB burden countries is estimated to be 0.7-11%, compared to 0.07-0.5% in HIV-uninfected women [7]. TB disease is a major cause of maternal morbidity and mortality, especially in HIV-infected women [6], accounting for 15 to 35% of maternal deaths worldwide in 2013 [8] and 26.3% of maternal deaths in South Africa between 2011 and 2013 [9]. The risk of maternal morbidity in pregnant women with active TB was reported to be three times greater than pregnant women who did not have TB [9,10] and these outcomes are worse in pregnant women with TB-HIV co-infection than in those who are only TB or HIV-infected [11]. TB in pregnant women also has adverse perinatal and infant outcomes [12–14], which are higher in HIV-TB co-infected women than in pregnant women infected with either TB or HIV [7].

Women in the early postpartum period are twice as likely to develop TB as non-pregnant women [7], within a median of 3 months post-delivery [15], possibly as a result of subclinical active TB during pregnancy, nutritional stress due to lactation, lack of sleep and rapid hormonal changes [15].

Although the number of HIV-infected infants is declining due to expanding Prevention of Mother To Child Transmission (PMTCT) programmes [16], the number of HIV-exposed, uninfected infants is increasing [17]. The rate of *M.tb* exposure in HIV-exposed, uninfected infants is high, and although data on the risk of TB in these infants are limited, they are believed to be at a high risk of developing active TB disease in high TB burden settings following infection, even if they remain HIV-uninfected [18–20].

Here, we report on the socio-demographic and clinical risk factors associated with *M.tb* infection and TB disease in a population of HIV-infected mothers and their HIV-exposed infants enrolled in a Phase II TB vaccine trial conducted in TB and HIV-endemic areas of South Africa.

Methods

Study design and setting

This analysis is based on diagnostic data from a Phase II, double blind, randomized, placebo-controlled, clinical trial to evaluate safety and immunogenicity of a novel TB vaccine, MVA85A and selective, delayed Bacille Calmette-Guerin (BCG) vaccination in infants of HIV-infected mothers. The trial was designed as a proof of concept for a novel tuberculosis vaccination strategy. Written antenatal and postnatal informed consent for infant participation was obtained from mothers. The protocol was approved by the ethics committees of the University of Cape Town (013/2012), Stellenbosch (M12/03/020) and Oxford (02-12), and the South African Medicines Control Council, and registered with Clinicaltrials.gov (NCT01650389). It was conducted between 16 November 2012 and 12 May 2015 at two South African TB vaccine sites in the Western Cape Province; one located in a town 120km outside Cape Town (TB incidence in 2007 of 680/100 000 overall and 1500/100 000 in children younger than 2 years of age), and the other in a partially informal township located in Cape Town (TB incidence in 2009 of 1500/100 000 overall and 3290/100 000 in children 18 months of age). The methodology and main results have been reported previously [21]. Briefly, infants of HIV-infected mothers were enrolled into the trial if the mothers were receiving ART or PMTCT prophylaxis, infant birth weight was >2kg and <4.5kg, and they did not have a household or frequent close contact with TB that had not yet completed TB treatment. Eligible, HIV-exposed infants (n=248) were randomized in a 1:1 ratio to receive either a single dose of MVA85A vaccine or placebo within 96 hours of birth. Since a previous study [22] had shown that MVA85A did not have any efficacy against *M.tb* infection or TB disease, the data from the intervention and placebo arm in this sub-analysis were analyzed together, as was data from the two sites. HIV DNA PCR testing at birth was not routine at the time of the trial. Those infants confirmed to be HIV-uninfected by HIV PCR at 6 weeks of age were given BCG at 8 weeks of age and were retested for HIV at 1 year of age or after cessation of breastfeeding. Infants who were confirmed to be HIV-infected by HIV PCR did not receive BCG and were followed per protocol for safety outcomes.

Study procedure

All participants were followed for one year for safety and immunology endpoints at scheduled visits on Days 7, 28, 42, 56, 63, 84, 112 and 365. Clinical trial staff recorded maternal and infant socio-demographic, clinical, maternal HIV data and active TB surveillance data at these visits.

QuantiFERON-TB Gold In-Tube (QFT, Qiagen) was performed on all mothers at enrolment of their newborns and on infants who successfully completed one year of follow-up. At each visit, data was collected on new household or other close contact of the infant with active TB and maternal and infant symptoms suggestive of TB. Those infants with a close TB contact or who developed a positive QFT or tuberculin skin test (TST) and who were not diagnosed with TB disease were started on isoniazid (IPT), whilst infants who were diagnosed with TB were started on TB drug treatment, both as per SA and WHO guidelines. These infants remained in the trial

[21] and were included in this sub-analysis.

Maternal and Infant *M.tb* infection outcomes

M.tb infection in HIV-infected mothers and HIV-exposed infants is defined as a positive QFT result. The QFT assay results were recorded on the trial database as interferon-gamma (IFN-γ) values in IU/ml and qualitatively as positive, (TB antigen minus nil IFN-γ value $\geq$ 0.35 IU/ml as per the manufacturer's instructions), negative (IFN-γ value < 0.35 IU/ml) or Indeterminate. In this sub-analysis, QFT Indeterminate results for both mothers and their infants were included as QFT negative results.

Maternal and Infant TB disease outcomes

Participants with suspected TB disease were investigated at local hospitals. Maternal incident TB disease was defined as mothers who received TB drug treatment. Incident TB disease was defined in infants based on the clinician's decision to treat for TB, including microbiological (notably TST or QFT), radiological or clinical criteria for diagnosis. This definition included confirmed, unconfirmed and unlikely TB [23].

Statistical analysis

Statistical analysis of the dataset was performed using STATA version 12.0 (DataCorp, USA).

The prevalence of maternal *M.tb* infection was estimated as number of mothers with a positive QFT at the time of enrollment divided by total number of mothers whose infants were enrolled into the study. Infant incidence rate of *M.tb* infection was calculated as person-years of follow-up using number of infants with a positive QFT at Day 365 divided by total number of infants who successfully completed one year of follow-up. The incidence rate of TB disease in infants and mothers was calculated as person-years of follow-up using the number of infants or mothers who were treated for TB during follow-up divided by the total number of infants who successfully completed follow-up.

Univariate associations of socio-demographic and clinical risk factors with maternal and infant QFT positivity and TB disease were assessed by Chi-squared or Fisher's Exact tests and odds ratios (OR) with 95% confidence intervals (95% CI). Those risk factors with a P value <0.05 or that were deemed to be of biological precedence with or without a P value <0.25 were considered for multivariable logistic regression models to explore independent associations between these risk factors and QFT positivity or TB disease in mothers and infants. The association of risk factors with *M.tb* infection (measured by maternal and infant continuous QFT IFN-γ (IU/ml) values) was considered in linear regression models, where non-normally distributed dependent variables were log-transformed and coefficients back-transformed for interpretation. Regression diagnostics were run to check whether the assumptions of linearity, normality of residuals (errors), homogeneity of variance and independence underlying the regression model were justified and identification of outliers and influential points.

Additional ethics approval for this sub-analysis was granted by the University of Cape Town, Faculty of Health Sciences, Human Research Ethics Committee (UCTHREC Reference 659/2017).

Results

Socio-demographic and clinical characteristics of participants

The 248 infants and their HIV-infected mothers enrolled in the trial were included in this sub-analysis.

The median age of mothers was 28.5 years; 193 (80%) of mothers were receiving lifelong ART, whilst 48 (19%) received ART prophylaxis. The median CD4 count was 424 cells/mm^3 (IQR 276-587), where the CD4 count was $\geq$350 cells/mm^3 in 127 (59.6%) mothers; 3 (1.2%) mothers reported that they were currently receiving Isoniazid Preventive Therapy (IPT); 43 (17.3%) reported having previously been treated for TB and 19 (7.7%) smoked.

The median gestational age of infants was 40 weeks; the median birth weight was 3.2kg; 121 (49%) were male and 145 (58%) were breastfed. 217 enrolled infants completed follow-up to Day 365. Two (0.83%) infants were diagnosed as HIV PCR positive: one at 6 weeks of age and was lost to follow-up (time-on-study 111 days) and the other tested HIV PCR positive at one year of age.

Prevalence and demographic and clinical risk factors associated with M.tb infection in HIV-infected pregnant women

Of the 248 HIV infected mothers, 106 mothers had a positive QFT result yielding a prevalence rate of maternal *M.tb* infection of 42.7% (95% CI 36.54 – 48.94). The maternal QFT IFN-γ (IU/ml) values were <0.35 IU/ml (57.3%), $\geq$0.35 - 0.4 IU/ml (3.2%) and $\geq$ 0.4 IU/ml (39.5% (Figure 1). Since the majority of results were <0.35 IU/ml and a large number were $\geq$ 0.4 IU/ml, the results were further stratified as <0.2 IU/ml, 0.2-0.34 IU/ml, 0.35-0.7 IU/ml and >0.7 IU/ml. Data on QFT assay imprecision, including repeatability and reproducibility, has shown that there is a zone of uncertainty (0.2 - 0.7 IU/ml), particularly around the manufacturer's assay cutoff, indicating that a more rigorous definition for QFT-TB conversion is necessary for more definitive detection of *M.tb* infection [24].

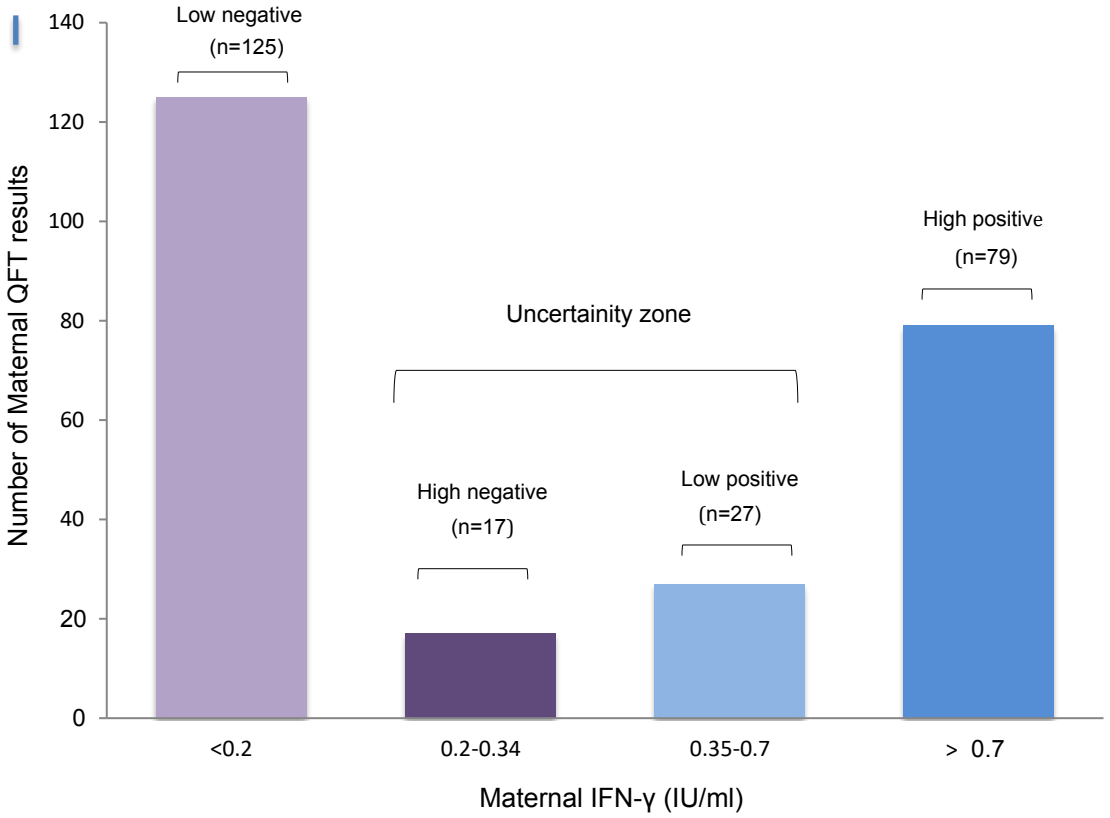


Figure 1: QFT IFN-γ values stratified according to assay's cutoff (0.35 IU/ml) and an uncertainty zone (0.2-0.7 IU/ml) [24]. Purple shaded bars denote low and high negative results and blue shaded bars denote low and high positive results. Maternal QFT IFN-γ results were <0.35 IU/ml (57.3%), ≥0.35 - 0.4 IU/ml (3.2%) and ≥ 0.4IU/ml (39.5%). The number of mothers with QFT IFN-γ <0.2 IU/ml, 0.2-0.34 IU/ml, 0.35-0.7 IU/ml and >0.7 IU/ml are indicated above respective bars.

Mothers with a baseline CD4 count of ≥350 cells/mm^3 had nearly double the odds of having a positive QFT result than mothers with a baseline CD4 count <350 cells/mm^3 (OR 1.98, 95% CI 1.12-3.52) (Table 1). Other risk factors including previous TB treatment, smoking or employment status were not associated with *M.tb* infection in mothers in univariate and multivariable analyses. For every 50 cells/mm^3 increase in maternal CD4 count, maternal QFT IFN-γ IU/ml increased by 17% (coef 0.155, 95% CI 0.09-0.22), on average.

Table 1: Univariate and multivariable models of socio-demographic and clinical risk factors associated with *M.tb* infection (QFT positivity) in HIV-Infected mothers (n=248)

Variable name	QFT + (n=106)	QFT - (n=142)	Univariate analysis		Multivariable analysis	
			OR (95% CI)	P value	OR (95% CI)	P value
Age (Median; IQR)	28.5 (25-33)	28.5 (25-33)	1.01 (0.96-1.07)	0.63		
Number of other adults in household (Median, IQR)	2 (2-3)	2 (2-3)	0.93 (0.74-1.18)	0.56		
Number of other children in household (Median, IQR)	1 (0-2)	1 (0-2)	1.13 (0.93-1.36)	0.21		
Number of Sleeping rooms (Median, IQR)	2 (1-2)	2 (1-2)	1.05 (0.75-1.48)	0.74		
Highest Educational level (n,%)						
Primary (Reference)	11 (10.4%)	11 (7.9%)	-	-	-	-
Junior High	26 (24.5%)	47 (33.6%)	0.55 (0.21-1.45)	0.23		
Senior High	69 (65.1%)	82 (58.6%)	0.84 (0.34-2.06)	0.70		
Employment status: (n,%)						
Employed (=1)	53 (37.3%)	42 (39.6%)	1.10 (0.66-1.85)	0.71		
Unemployed	89 (62.7%)	64 (60.4%)				
Employment type: (n,%)						
Formal (Reference)	22 (52.4%)	26 (49.1%)				
Informal	8 (19.1%)	6 (11.3%)	1.58 (0.47-5.23)	0.46		
Farm	12 (28.6%)	21 (39.6%)	0.68 (0.27-1.68)	0.39		
ART treatment: (n,%)						
PMTCT	24 (22.6%)	25 (17.6%)				
ART (=1)	82 (77.4%)	117 (82.2%)	0.73 (0.39-1.37)	0.33		
Household TB contact (baseline; n,%)	0	5 (3.5%)	-	-		
Previous TB Treatment (n,%)	16 (15.09%)	27 (19%)	0.76 (0.38-1.49)	0.38		
CD4 count (cells/mm^3) (median, IQR)	472.5 (327-618)	362 (254-531)	1.002 (1.00-1.003)	0.002		
CD4 count category (n, %)						
< 350	29 (31.52%)	57 (47.11%)				
≥ 350 (=1)	63 (68.48%)	64 (52.89%)	1.93 (1.10-3.41)	0.022	1.98 (1.12-3.52)	0.018
Smokes (n, %)	9 (8.49%)	10 (7%)	1.22 (0.48-3.13)	0.67		
IPT treatment (n, %)	1 (0.95%)	2 (1.4%)	0.67 (0.06-7.52)	0.75		

* The CD4 count was recorded for 214 mothers; 7 Maternal QFT Indeterminate results included as QFT negative results; Number of observations in *M.tb* infection model = 213, prob>chi2=0.0209

Incidence rate and demographic and clinical risk factors associated with TB disease in HIV-infected mothers

Three cases of TB were diagnosed in 248 mothers, yielding an incidence rate of maternal TB disease of 1.36/100 person-years (95% CI 0.44-4.22). The median time to maternal TB diagnosis was 11.9 months (IQR 2.79-12.06)

Risk factors, including maternal CD4 count ≥350 cells/mm^3, median QFT IFN-γ values >0.4IU/ml or previous TB treatment, were not independently associated with TB disease in mothers in the univariate and multivariable analysis (Table 2).

Table 2: Univariate and multivariable models of demographic and clinical risk factors associated with TB Disease in HIV-Infected mothers (n=248)

Variable name	TB Disease + (n=3)	TB Disease - (n=245)	Univariate analysis		Multivariable analysis	
			OR (95% CI)	P value	AOR (95% CI)	P value
Age (Median; IQR)	28 (26-33)	29 (25-33)	0.999 (0.79-1.26)	0.995		
Number of other adults in household (Median, IQR)	4 (2-4)	2 (2-3)	1.58 (0.79-3.13)	0.19		
Number of other children in household (Median, IQR)	1 (0-2)	1 (0-2)	1.00 (0.43-2.35)	0.997		
Number of Sleeping rooms (Median, IQR)	2 (1-3)	2 (1-2)	1.51 (0.46-4.95)	0.49		
Highest Educational level: (n,%)						
Primary (Reference)	0	22 (9%)	-	-		
Junior High	0	73 (30%)	-	-		
Senior High	3 (100%)	148 (61%)	-	-		
Employment status: (n,%)						
Employed (=1)	2 (66.7%)	93 (38%)	3.27 (0.29-36.55)	0.34		
Unemployed	1 (33.3%)	152 (62%)				
Employment type: (n,%)						
Formal (Reference)	0	48 (51.6%)	-	-		
Informal	0	14 (15%)	-	-		
Farm	2 (100%)	31 (33.3%)	-	-		
ART treatment: (n,%)						
PMTCT	1 (33.3%)	48 (19.6%)				
ART (=1)	2 (66.7%)	197 (80.4%)	0.49 (0.04-5.49)	0.56		
Household TB contact (baseline; n,%)	0	5 (2%)	-	-		
Previous TB Treatment (n,%)	1 (33.3%)	42 (17.1%)	2.42 (0.22-27.27)	0.48	4.86 (0.29-80.87)	0.27
CD4 count (cells/mm^3) (median, IQR)	215.5 (18-413)	427 (276-590)	0.99 (0.98-1.00)	0.144		
CD4 count category (n, %)						
< 350	1 (50%)	85 (40.3%)				
$\geq$ 350 (=1)	1 (50%)	126 (59.7%)	0.68 (0.04-10.93)	0.78	0.72 (0.04-12.23)	0.82
Smokes (n, %)	0	19 (7.8%)	-	-	-	-
IPT treatment (n, %)	0	3 (1.2%)	-	-	-	-
Maternal QFT + (binary; n,%)	2 (66.7%)	104 (43.7%)	2.7 (0.24-30.31)	0.42		
Maternal IFN-γ (IU/ml; median, IQR)	0.46 (-0.01–14.41)	0.17 (0.01–1.36)	1.16 (0.96-1.39)	0.12	0.49 (0.03-7.49)	0.61

* The CD4 count was recorded for 214 mothers. Number of observations in Maternal TB disease multivariable model = 213, prob>chi2=0.5782

Incidence rate and demographic and clinical risk factors associated with M.tb infection in HIV-exposed infants

Of the 207 infants with QFT results at Day 365, 5 were QFT positive. The incidence rate of infant *M.tb* infection was 2.47 per 100 person-years (95% CI 1.03 – 5.93).

Nine (4.6%) infants had new HHCs, 2 (25%) of which were their mothers. Infants who had a new HHC had an 18 fold increased odds of being infected with *M.tb* than those infants who did not (OR 17.71, 95% CI 2.5 -123.5). Other risk factors such as living conditions, birth weight, initial feeding choice, maternal QFT status, maternal smoking or mother's previous TB treatment were not independently associated with

positive *M.tb* infection in HIV-exposed infants (Table 3) in the univariate and multivariable analysis

The majority of infants (n=201, 97.1%) had a QFT IFN-γ result <0.2 IU/ml, 0.48% (n=1) between 0.2 and 0.34 IU/ml and 2.42% (n=5) >0.7 IU/ml. No infants presented with QFT IFN-γ results between 0.35-0.7 IU/ml. For every new HHC, infant QFT IFN-γ values (IU/ml) increased by 58 fold (coef 4.06, 95% CI 2.63-5.49), compared to QFT IFN-γ values of infants who did not have a new HHC.

Incidence rate and demographic and clinical risk factors associated with TB disease in HIV-exposed infants

Of 248 infants, 8 were treated for TB disease. The incidence rate of infant TB disease was 3.62 per 100 person-years (95% CI 1.81-7.23). One infant had a positive *M.tb* culture and 7 were diagnosed based on clinical/radiographic evidence and TB contact history. One of the TB cases was QFT positive, her mother was QFT positive, had previously been treated for TB and the infant had a new HHC that was not her mother. The median time of infant TB disease diagnosis was 8.5 months of follow-up (IQR 4.73 – 12.23). Three of the 8 infants' mothers smoked (37.5%), although none of these mothers had TB disease.

The odds of an HIV-exposed infant having TB disease were 34% greater per 1 unit (IU/ml) increase in their QFT result (AOR 1.34, 95% CI 1.08-1.66), 11 fold greater if they had a new HHC (AOR 10.81, 95% CI 1.53-76.42) compared with an infant who did not and 11 fold greater if their mother smoked (AOR 10.73, 95% CI 1.96-58.89) compared to infants whose mothers did not smoke. The odds were 20 fold greater if their mothers were receiving Izoniazid Preventive Therapy (IPT) (OR 19.83, 95% CI 1.57-249.84) compared to infants whose mothers were not receiving IPT in the univariate model (Table 4). However, this was not included in the multivariable analysis as maternal IPT is a marker of maternal TB risk rather than having any causal relationship with infant TB disease. Maternal QFT status was not independently associated with having infant TB disease, nor were living conditions, initial feeding choice, and maternal CD4 count

Table 3: Univariate and multivariable models of baseline demographic and clinical risk factors associated with *M.tb* Infection (QFT positivity) in HIV-exposed infants (n=207)

Variable name	QFT + (n=5)	QFT - (n=202)	Univariate analysis OR (95% CI)	Univariate analysis P value	Multivariable analysis OR (95% CI)	Multivariable analysis P value
Gestational age (median, IQR)	39 (39-40)	40 (39-40)	0.85 (0.45-1.59)	0.61		
Birth weight (kg; median, IQR)	3.3 (2.8-3.5)	3.2 (2.9-3.4)	1.0 (0.99-1.0)	0.63		
Length (cm; median, IQR)	50 (49-50)	49 (48-52)	0.99 (0.78-1.26)	0.95		
Head circumference (cm; median, IQR)	33 (32.5-35)	33 (32-35)	0.97 (0.58-1.62)	0.91		
Gender, male (n, %)	2 (40%)	100 (49.5%)	0.68 (0.11-4.16)	0.68		
Infant HIV-Infected	0	1 (0.5%)	-	-		
Number of other adults in household (median, IQR)	2 (2-3)	2 (2-3)	0.90 (0.38-2.09)	0.81		
Number of other children in household (median, IQR)	1 (1-1)	1 (0-2)	0.90 (0.44 -1.83)	0.77		
Number of Sleeping rooms (n,%)	2 (1-2)	2 (1-2)	0.76 (0.21-2.78)	0.67		
Educational level (n,%)						
Primary (Reference)	0	18 (8.9%)	-	-		
Junior High	1 (20%)	61 (30.2%)	-			
Senior High	4 (80%)	123 (60.9%)	-			
Employment status (n,%)						
Employed (=1)	2 (40%)	80 (39.6%)	1.02 (0.17-6.22)	0.99		
Unemployed	3 (60%)	122 (60.4%)				
Employment type (n,%)						
Formal (Reference)	2 (100%)	38 (47.6%)	-	-		
Informal	0	13 (16.3%)	-	-		
Farm	0	29 (36.3%)	-	-		
Maternal ART treatment (n,%)						
PMTCT	1 (20%)	40 (19.8%)				
ART(=1)	4 (80.2%)	162 (80.2%)	0.98 (0.11-9.08)	0.99		
Household TB contact (baseline; n,%)	0	5 (2.5%)	-	-		
New household TB contact	2 (40%)	7 (3.6%)	17.71 (2.5-123.49)	0.004	17.71 (2.54-123.49)	0.004
Mother previously on TB treatment (n,%)	2 (40%)	33 (16.3%)	3.41 (0.55-21.23)	0.18		
Mother on IPT (n,%)	0	2 (1%)	-	-		
Maternal CD4 count (cells/mm^3; median, IQR)	367.5 (299–475)	442 (278-597)	0.998 (0.99-1.00)	0.57		
Maternal CD4 count category (n,%)						
<350	2 (50%)	67 (38.7%)				
≥350 (=1)	2 (50%)	106 (61.3%)	0.63 (0.087-4.59)	0.65		
Maternal Smoking (n, %)	0	17 (8.4%)	-	-		
Initial feeding Choice (n,%)						
Breast feeding (=1)	4 (80%)	122 (60,40%)	2.62 (0.29-23.90)	0.392		
Formula Feeding	1 (20%)	80 (39.6%)				
Maternal TB disease (n, %)	0	3 (1.5%)	-	-		
Maternal QFT +ve (binary; n,%)	2 (40%)	91 (45.1%)	0.81 (0.13-4.97)	0.82		
Maternal IFN-γ (IU/ml; median, IQR)	0.27 (0.06-0.46)	0.2 (0.01-1.39)	0.95 (0.60-1.23)	0.99		

* 3 QFT Indeterminate results included as QFT negative results; 41 missing data points removed; Number of observations in Infant TB infection mulitvariable model = 198, prob>chi2=0.

Table 4. Univariate and multivariable models of baseline demographic and clinical risk factors associated with TB Disease in HIV exposed infants (n=248)

Variable name	TB Disease + (n=8)	TB Disease - (n=240)	Univariate analysis		Multivariate analysis	
			OR (95% CI)	P value	AOR (95% CI)	P value
Gestational age (median, IQR)	40 (39-40.5)	40(39-40)	1.17 (0.69-1.95)	0.55		
Birth weight (kg; median, IQR)	3.4 (3.2-3.6)	3.2 (2.9-3.4)	1.00 (0.99-1.00)	0.22		
Length (cm; median, IQR)	51 (50-54.5)	49 (48-52)	1.18 (1.00-1.39)	0.05		
Head circumference (cm; median, IQR)	33 (32-35)	33 (32-35)	0.84 (0.56-1.27)	0.42		
Gender, male (n, %)	4 (50%)	117 (48.8%)	1.05 (0.26-4.30)	0.95		
Infant HIV-Infected	0	2 (0.83%)	-	-		
Number of other adults in household (median, IQR)	3 (2-3)	2 (2-3)	1.22 (0.71-2.08)	0.47		
Number of other children in household (median, IQR)	1 (1)	1 (0-2)	0.80 (0.43-1.48)	0.48		
Sleeping rooms (n, %)	1 (1-2)	2 (1-2)	0.44 (0.13-1.57)	0.28		
Educational level (n,%)						
Primary (Reference)	1 (12.5%)	21 (8.9%)				
Junior High	4 (50%)	67 (28.5%)	1.22 (0.131-1.49)	0.86		
Senior High	3 (37.50%)	149 (62.6%)	0.43 (0.04-4.3)	0.47		
Employment status (n,%)						
Employed (=1)	3 (37.5%)	92 (38.3%)	0.97 (0.23-4.13)	0.96		
Unemployed	5 (62.5%)	148 (61.7%)				
Employment type (n,%)						
Formal (Reference)	1 (33.3%)	46 (51.1%)				
Informal	1 (33.3%)	13 (14.13%)	3.61 (0.21-61.82)	0.38		
Farm	1 (33.3%)	32 (34.78%)	1.47 (0.09-24.35)	0.79		
Maternal ART treatment (n,%)						
PMTCT	2 (25%)	47 (19.6%)				
ART (=1)	6 (75%)	193 (80.4%)	0.73 (0.14-3.7)	0.76		
Baseline household TB contact (n,%)	0	5 (2.1%)	-	-		
New household TB contact	2 (25%)	7 (3.5%)	9.09 (1.55-53.35)	0.014	10.81 (1.52-76.43)	0.017
Mother previously on TB treatment (n,%)	2 (25%)	41 (17.0%)	1.61 (0.32-8.30)	0.56		
Mother on IPT (n,%)	1 (14.3%)	2 (0.8%)	19.8 (1.57-249.85)	0.02		
Maternal CD4 count (median, IQR)	425.5 (320 – 670)	424 (271-587)	1.00 (0.99-1.00)	0.25		
CD4 count category (n,%)						
<350	3 (37.5%)	83 (40.5%)				
≥350 (=1)	5 (62.5%)	122 (59.5%)	1.13 (0.26-4.87)	0.87		
Maternal smoking (n, %)	3 (37.5%)	16 (6.7%)	8.4 (1.83-38.35)	0.006	10.73 (1.96-58.89)	0.006
Initial feeding Choice (n,%)						
Breast feeding (=1)	5 (62.5%)	141 (58.75%)	1.17 (0.27-5.01)	0.83		
Formula Feeding	3 (37.5%)	99 (41.25%)				
Maternal TB disease (n, %)	0	3 (1.3%)	-	-		
Maternal QFT + (binary; n,%)	3 (37.5%)	103 (42.9%)	0.79 (0.19-3.42)	0.76		
Maternal IFN-γ (IU/ml; median,IQR)	0.04 (0-3.89)	0.19 (0.01-1.31)	1.01 (0.83-1.24)	0.90		
Infant QFT + (binary, n,%)	2 (25%)	3 (1.51%)	21.78 (3.05-155.37)	0.002		
Infant IFN-γ (IU/ml; median,IQR)	0.01 (-0.005-0.47)	0 (-0.02-0.02)	1.27 (1.03-1.57)	0.02	1.34 (1.08-1.66)	0.009

* Number of observations in Infant TB disease multivariable model = 198, prob>chi2=0.0022

Discussion

We have shown that in a cohort of HIV-exposed South African infants and their HIV-infected mothers who were actively followed for one year, maternal CD4 count ≥350 cells/mm^3 was strongly associated with QFT positivity that may have affected the estimate of maternal *M.tb* infection. Infant *M.tb* infection was driven by new household TB contact as was infant TB disease in addition to higher QFT values (IU/ml) and maternal smoking.

Although maternal *M.tb* infection (identified as QFT positivity) in HIV-infected mothers was high (42.7%), it was considerably lower than that previously observed in HIV uninfected, young adults in the community (52-60%) [25]. The maternal median CD4 count in this study was relatively high (424 cells/mm^3) due to the inclusion criterion for enrollment of maternal lifelong ART or perinatal ART prophylaxis. The prevalence of QFT positivity was strongly associated with maternal CD4 count ≥ 350 cells/mm^3, possibly suggesting impaired sensitivity of QFT in those with lower CD4 counts. Studies on performance of the QFT assay in HIV-infected individuals have shown that the sensitivity of QFT is lower in HIV-infected individuals, increasing with increasing CD4 cell count [26]. QFT relies on functional CD4 cells and its performance can negatively be influenced by low and impaired CD4 cell counts in HIV-infected individuals. This inclusion criterion may also explain the low (<1%) infant HIV acquisition in this study, which is considerably lower than the perinatal HIV transmission rates in South Africa at the time of the trial (2.7% in 2012) [27].

The incidence rate of maternal TB disease in this study was 1.36 cases/100 person-years. This is similar to TB incidence rates among patients receiving ART, with the highest and lowest rates in patients with CD4 counts <100 and > 500 cells/ mm^3 respectively [28]. In patients with CD4 counts of 401-500 cells/mm^3, the incidence rate was 1.5 cases/100 person years [28]. A recent study showed that the TB rate was greater in HIV-infected persons on ART at 2.70-25.49 cases per person-years, than in HIV-uninfected persons living in the same community [29].

Interestingly, the incidence rate of QFT positivity in HIV-exposed infants at one year of age (2.5%) is lower than that reported for HIV-unexposed infants in this community of 6-7% [22,25], whereas the incidence of TB disease in infants of 3.6% was similar to that reported previously for these communities [22]. In a study in the Western Cape Province, South Africa the potential incidence of TB exposure was 10.03%, with a predicted possible infection rate of 5.01% and a rate of disease of 2.01% [18]. The interpretation of our findings was likely to have been limited by the exclusion criterion for enrolment of mothers with active TB who had not yet completed TB treatment as well as maternal household TB contact(s), which could have reduced the risk of *M.tb* transmission and TB disease in mothers and their infants.

We have shown in this study that a new household contact is independently associated with *M.tb* infection and TB disease in infants, with 2 of the 9 household contacts being the mother. This finding is supported by reports that the majority of children younger than 2 years of age are infected from a household sputum smear-positive source case [30–32], usually the mother or father. The greater the daily interaction and the longer the duration of contact with the source case, the greater

the risk of infection [30]. In particular, HIV-exposed infants are at an increased risk of exposure to *M.tb* and are at a high risk of developing active TB disease following infection [18], whether they are HIV-infected or not [19]. Cotton *et al* (2008) [18] reported that 10.1% HIV-exposed infants in their study had household TB contacts (mothers or fathers) as well as close TB contacts outside of the house.

Andrews *et al* (2017) [33], found that very high IFN-γ values above the highest value of the standard curve of the QFT assay were a reliable estimate of the true IFN-γ values up to about 12.00 IU/ml in young children. They also found that the incidence rate of TB disease was significantly higher in children with very high QFT IFN-γ values (>4.0 IU/ml). These results indicated that QFT IFN-γ values much higher than the manufacturer's recommended cutoff value of 0.35 IU/ml were clinically useful. In our sub-analysis, IFN-γ values for QFT positive infants were above 0.7 IU/ml, and 2 values higher than 12.00 IU/ml were removed from the infant QFT data for the linear regression analysis.

A limited number of studies have shown that tobacco/cigarette smoke exposure is associated with *M.tb* infection in children who are in contact with a patient with TB disease [34–36]. In this analysis, 3 of the 8 infants with TB disease had mothers who smoked, although none of these mothers had TB disease. Clinical diagnosis of infant TB disease in this sub-analysis may have been an overestimation of the true disease rate [21], where clinicians may have diagnosed TB disease in infants with impaired clearance of mucosal secretions or decrease in immune response [37] or reactive airways reactive airways.

At the time of the trial, the South African TB Preventive therapy guidelines (2010) recommended that all HIV-infected people regardless of TST status receive 6 months of IPT [38], which were revised in 2013 to recommend at least 36 months of IPT to TST-positive persons or 6 months of IPT if TST status is unknown [39]. Many studies have shown that treatment for latent TB infection significantly reduces the risk of active TB disease in HIV-infected persons, particularly if taken for 36 months [38,40,41], although this TB prevention benefit declined after cessation of IPT [42]. Despite these recommendations, routine screening for active TB, provision and monitoring of IPT to HIV-infected pregnant women at PMTCT antenatal clinics is suboptimal [39,42]. Notably only 3 (1.21%) of the mothers reported receiving IPT at baseline, 1 of which was QFT positive and did not have TB disease. It is unclear why this was independently associated with infant TB disease but could possibly be due to potential bias as a result of selection of high-risk mothers for IPT.

Interpretation of our findings could be limited by the study population and sample size that was originally selected to assess the safety and immunogenicity of the MVA85A vaccine [21] and not powered to accurately assess *M.tb* incidence rate and TB disease and their risk factors. The small number of incident cases of maternal TB disease, infant QFT positivity and TB disease limited the precision of estimates of these outcomes and of identifying precise estimates of the effect of different risk factors on the outcomes. Further, infants with low birth weight or born preterm, outcomes that could have resulted from mothers with untreated TB during pregnancy [13,14], were excluded from the study. Most children who progress to disease do so

within 12 months of being infected, it being estimated that 50% of infants whose mothers have active TB disease will develop TB disease during the first year of life [43]. Since children from 1-2 years of age have a 20 -30% risk of developing disease [44], the number of cases of *M.tb* infection and TB disease in infants and their mothers may have been higher if the cohort was followed for an additional 12 months.

In conclusion, maternal CD4 count affects the QFT value and may have affected the estimate of maternal *M.tb* infection. Household exposure is an important driver of *M.tb* transmission to HIV-exposed infants with low risk of HIV acquisition. Determining pregnant or postpartum HIV-infected mothers and their infants who are at the greatest risk of *M.tb* exposure, infection and TB disease is critical to reduce the burden of disease and death caused by TB in this population.

References

1. World Health Organization. Global tuberculosis report 2016 [Internet]. 2016. Available: www.who.int/tb/publications/global_report/en/

2. National Aids Council, South Africa. National Strategic Plan for HIV, TB and STIs 2017-2022 [Internet]. Available: http://sanac.org.za/2017/09/06/download-the-full-version-of-the-national-strategic-plan-for-hiv-tb-and-stis-2017-2022/

3. World Health Organization. TB/HIV Factsheet 2016 [Internet]. Available: www.who.int/tb/challenges/hiv/factsheets/en/

4. Wood R, Lawn SD, Caldwell J, Kaplan R, Middelkoop K, Bekker L-G. Burden of New and Recurrent Tuberculosis in a Major South African City Stratified by Age and HIV-Status. Cattamanchi A, editor. PLoS ONE. 2011;6: e25098. doi:10.1371/journal.pone.0025098

5. World Health Organization. Global tuberculosis report 2015 [Internet]. Geneva, Switzerland: World Health Organization; 2015. Available: apps.who.int/iris/bitstream/10665/191102/1/9789241565059_eng.pdf

6. Bates M, Ahmed Y, Kapata N, Maeurer M, Mwaba P, Zumla A. Perspectives on tuberculosis in pregnancy. Int J Infect Dis. 2015;32: 124–127. doi:10.1016/j.ijid.2014.12.014

7. Mathad JS, Gupta A. Tuberculosis in Pregnant and Postpartum Women: Epidemiology, Management, and Research Gaps. Clin Infect Dis Off Publ Infect Dis Soc Am. 2012;55: 1532–1549. doi:10.1093/cid/cis732

8. Sugarman J, Colvin C, Moran AC, Oxlade O. Tuberculosis in pregnancy: an estimate of the global burden of disease. Lancet Glob Health. 2014;2: e710–e716. doi:10.1016/S2214-109X(14)70330-4

9. National Department of Health, South Africa. Saving Mothers 2011-2013: Sixth report on the confidential enquiries into maternal deaths in South Africa.

10. Nguyen HT, Pandolfini C, Chiodini P, Bonati M. Tuberculosis care for pregnant women: a systematic review. BMC Infect Dis. 2014;14: 617. doi:10.1186/s12879-014-0617-x

11. Pillay T, Khan M, Moodley J, Adhikari M, Coovadia H. Perinatal tuberculosis and HIV-1: considerations for resource-limited settings. Lancet Infect Dis. 2004;4: 155–165. doi:10.1016/S1473-3099(04)00939-9

12. WHO Tuberculosis in Women 2015 [Internet]. Available: www.who.int/tb

13. Sobhy S, Babiker Z, Zamora J, Khan K, Kunst H. Maternal and perinatal mortality and morbidity associated with tuberculosis during pregnancy and the postpartum period: a systematic review and meta-analysis. BJOG Int J Obstet Gynaecol. 2016; n/a-n/a. doi:10.1111/1471-0528.14408

14. Lin H-C, Lin H-C, Chen S-F. Increased risk of low birthweight and small for gestational age infants among women with tuberculosis: Pregnancy and tuberculosis. BJOG Int J Obstet Gynaecol. 2010;117: 585–590. doi:10.1111/j.1471-0528.2010.02504.x

15. Gupta A, Nayak U, Ram M, Bhosale R, Patil S, Basavraj A, et al. Postpartum tuberculosis incidence and mortality among HIV-infected women and their infants in Pune, India, 2002-2005. Clin Infect Dis Off Publ Infect Dis Soc Am. 2007;45: 241–249. doi:10.1086/518974

16. Kourtis AP, Wiener J, Kayira D, Chasela C, Ellington SR, Hyde L, et al. Health outcomes of HIV-exposed uninfected African infants. AIDS Lond Engl. 2013;27: 749–759. doi:10.1097/QAD.0b013e32835ca29f

17. Evans C, Jones CE, Prendergast AJ. HIV-exposed, uninfected infants: new global challenges in the era of paediatric HIV elimination. Lancet Infect Dis. 2016;16: e92–e107. doi:10.1016/S1473-3099(16)00055-4

18. Cotton MF, Schaaf HS, Lottering G, Weber HL, Coetzee J, Nachman S, et al. Tuberculosis exposure in HIV-exposed infants in a high-prevalence setting. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2008;12: 225–227.

19. Madhi SA, Nachman S, Violari A, Kim S, Cotton MF, Bobat R, et al. Primary isoniazid prophylaxis against tuberculosis in HIV-exposed children. N Engl J Med. 2011;365: 21–31. doi:10.1056/NEJMoa1011214

20. Martinson NA, Moultrie H, van Niekerk R, Barry G, Coovadia A, Cotton M, et al. HAART and risk of tuberculosis in HIV-infected South African children: a multi-site retrospective cohort. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2009;13: 862–867.

21. Nemes E, Hesseling AC, Tameris M, Mauff K, Downing K, Mulenga H, et al. Safety and Immunogenicity of Newborn MVA85A Vaccination and Selective, Delayed Bacille Calmette-Guerin (BCG) for Infants of HIV Infected Mothers: A Phase 2 Randomized Controlled Trial. Clin Infect Dis Off Publ Infect Dis Soc Am. 2017; doi:10.1093/cid/cix834

22. Tameris MD, Hatherill M, Landry BS, Scriba TJ, Snowden MA, Lockhart S, et al. Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. The Lancet. 2013;381: 1021–1028. doi:10.1016/S0140-6736(13)60177-4

23. Graham SM, Ahmed T, Amanullah F, Browning R, Cardenas V, Casenghi M, et al. Evaluation of tuberculosis diagnostics in children: 1. Proposed clinical case

definitions for classification of intrathoracic tuberculosis disease. Consensus from an expert panel. J Infect Dis. 2012;205 Suppl 2: S199-208. doi:10.1093/infdis/jis008

24. Nemes E, Rozot V, Geldenhuys H, Bilek N, Mabwe S, Abrahams D, et al. Optimization and Interpretation of Serial QuantiFERON Testing to Measure Acquisition of *Mycobacterium tuberculosis* Infection. Am J Respir Crit Care Med. 2017;196: 638–648. doi:10.1164/rccm.201704-0817OC

25. Mahomed H, Hughes EJ, Hawkridge T, Minnies D, Simon E, Little F, et al. Comparison of mantoux skin test with three generations of a whole blood IFN-gamma assay for tuberculosis infection. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2006;10: 310–316.

26. Aabye MG, Ravn P, PrayGod G, Jeremiah K, Mugomela A, Jepsen M, et al. The Impact of HIV Infection and CD4 Cell Count on the Performance of an Interferon Gamma Release Assay in Patients with Pulmonary Tuberculosis. Marais B, editor. PLoS ONE. 2009;4: e4220. doi:10.1371/journal.pone.0004220

27. National Department of Health, South Africa. Joint Review of HIV, TB and PMTCT Programmes in South Africa - April 2014 | Health Systems Trust [Internet]. [cited 29 Jan 2017]. Available: http://www.hst.org.za/publications/joint-review-hiv-tb-and-pmtct-programmes-south-africa-april-2014

28. Lawn SD, Myer L, Edwards D, Bekker L-G, Wood R. Short-term and long-term risk of tuberculosis associated with CD4 cell recovery during antiretroviral therapy in South Africa: AIDS. 2009;23: 1717–1725. doi:10.1097/QAD.0b013e32832d3b6d

29. Gupta A, Wood R, Kaplan R, Bekker L-G, Lawn SD. Tuberculosis incidence rates during 8 years of follow-up of an antiretroviral treatment cohort in South Africa: comparison with rates in the community. PloS One. 2012;7: e34156. doi:10.1371/journal.pone.0034156

30. Marais BJ, Rabie H, Cotton MF. TB and HIV in children – advances in prevention and management. Paediatr Respir Rev. 2011;12: 39–45. doi:10.1016/j.prrv.2010.09.002

31. Middelkoop K, Bekker L-G, Morrow C, Zwane E, Wood R. Childhood tuberculosis infection and disease: a spatial and temporal transmission analysis in a South African township. South Afr Med J Suid-Afr Tydskr Vir Geneeskd. 2009;99: 738–743.

32. Singh M, Mynak ML, Kumar L, Mathew JL, Jindal SK. Prevalence and risk factors for transmission of infection among children in household contact with adults having pulmonary tuberculosis. Arch Dis Child. 2005;90: 624–628. doi:10.1136/adc.2003.044255

33. Andrews JR, Nemes E, Tameris M, Landry BS, Mahomed H, McClain JB, et al. Serial QuantiFERON testing and tuberculosis disease risk among young children: an observational cohort study. Lancet Respir Med. 2017;5: 282–290. doi:10.1016/S2213-2600(17)30060-7

34. den Boon S, Verver S, Marais BJ, Enarson DA, Lombard CJ, Bateman ED, et al. Association between passive smoking and infection with Mycobacterium

tuberculosis in children. Pediatrics. 2007;119: 734–739. doi:10.1542/peds.2006-1796

35. Lin H-H, Ezzati M, Murray M. Tobacco Smoke, Indoor Air Pollution and Tuberculosis: A Systematic Review and Meta-Analysis. Novotny TE, editor. PLoS Med. 2007;4: e20. doi:10.1371/journal.pmed.0040020

36. Huang C-C, Tchetgen ET, Becerra MC, Cohen T, Galea J, Calderon R, et al. Cigarette smoking among tuberculosis patients increases risk of transmission to child contacts. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2014;18: 1285–1291. doi:10.5588/ijtld.14.0309

37. Narasimhan P, Wood J, MacIntyre CR, Mathai D. Risk Factors for Tuberculosis. Pulm Med. 2013;2013. doi:10.1155/2013/828939

38. National Department of Health, South Africa. Guidelines for Tuberculosis Preventive Therapy among HIV Infected individuals in South Africa [Internet]. 2010. Available: www.who.int/hiv/pub/guidelines/south_africa_hiv_tb.pdf

39. Churchyard GJ, Mametja LD, Mvusi L, Ndjeka N, Hesseling AC, Reid A, et al. Tuberculosis control in South Africa: Successes, challenges and recommendations. S Afr Med J. 2014;104: 244. doi:10.7196/SAMJ.7689

40. Rangaka MX, Wilkinson RJ, Boulle A, Glynn JR, Fielding K, van Cutsem G, et al. Isoniazid plus antiretroviral therapy to prevent tuberculosis: a randomised double-blind, placebo-controlled trial. The Lancet. 2014;384: 682–690. doi:10.1016/S0140-6736(14)60162-8

41. Samandari T, Agizew TB, Nyirenda S, Tedla Z, Sibanda T, Shang N, et al. 6-month versus 36-month isoniazid preventive treatment for tuberculosis in adults with HIV infection in Botswana: a randomised, double-blind, placebo-controlled trial. The Lancet. 2011;377: 1588–1598. doi:10.1016/S0140-6736(11)60204-3

42. Uwimana J, Jackson D. Integration of tuberculosis and prevention of mother-to-child transmission of HIV programmes in South Africa. Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis. 2013;17: 1285–1290. doi:10.5588/ijtld.12.0068

43. National Department of Health, South Africa. Guidelines for the management of Tuberculosis in children, South Africa, 2013, National Department of Health [Internet]. Available: www.kznhealth.gov.za/family/National-Childhood-TB-Guidelines-2013-ZA.pdf

44. Seddon JA, Shingadia D. Epidemiology and disease burden of tuberculosis in children: a global perspective. Infect Drug Resist. 2014;7: 153–165. doi:10.2147/IDR.S45090

PART D: APPENDICES

LIST OF APPENDICES

APPENDIX A: ADDITIONAL DISSERTATION ANALYSIS

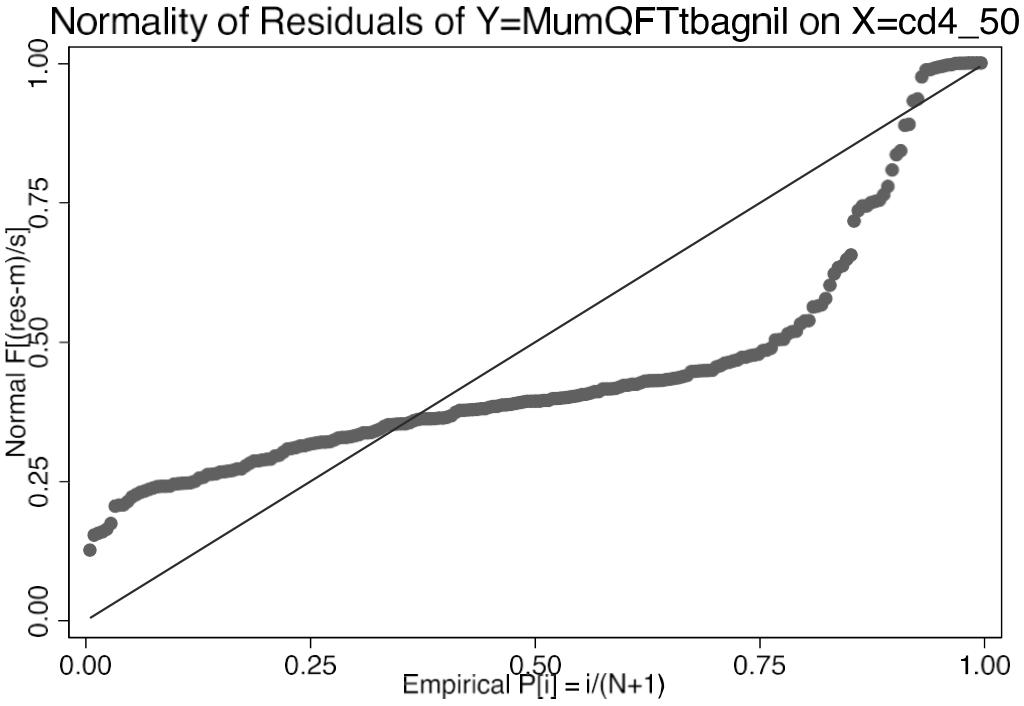
APPENDIX A1: Multivariable linear regression of demographic and clinical risk factors associated with *M.tb* infection in HIV-infected mothers

1. Linear regression model 1: Simple model with just one variable: Maternal QFT IFN-γ (dependent); Maternal CD4 count per 50 cells/mm^3

xi:regress MumQFTtbagnil cd4_50

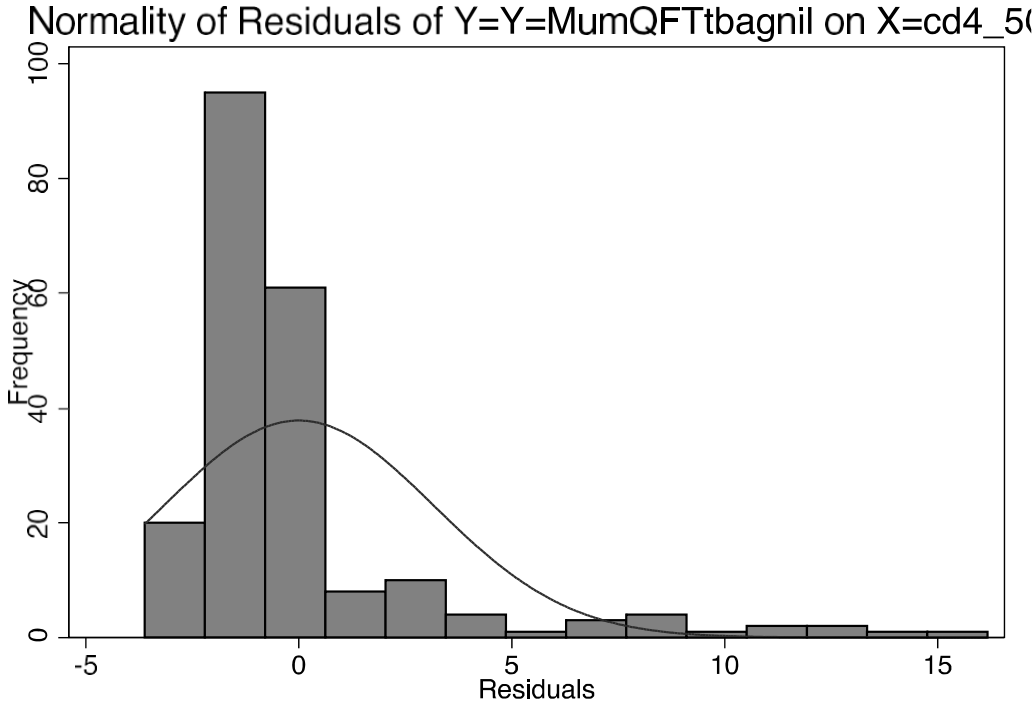
```
      Source |       SS       df       MS              Number of obs =     213
-------------+------------------------------           F(  1,   211) =   14.53
       Model |  147.345951     1  147.345951           Prob > F      =  0.0002
    Residual |  2139.03522   211  10.1376077           R-squared     =  0.0644
-------------+------------------------------           Adj R-squared =  0.0600
       Total |  2286.38117   212  10.7848168           Root MSE      =   3.184

------------------------------------------------------------------------------
MumQFTtbag~l |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
      cd4_50 |   .1887015   .0494964     3.81   0.000     .0911306    .2862723
       _cons |  -.1182334   .4894361    -0.24   0.809    -1.083044    .8465776
------------------------------------------------------------------------------
```



Shapiro-Wilk W test for normal data

Variable	Obs	W	V	z	Prob>z
res	213	0.65193	54.858	9.244	0.00000



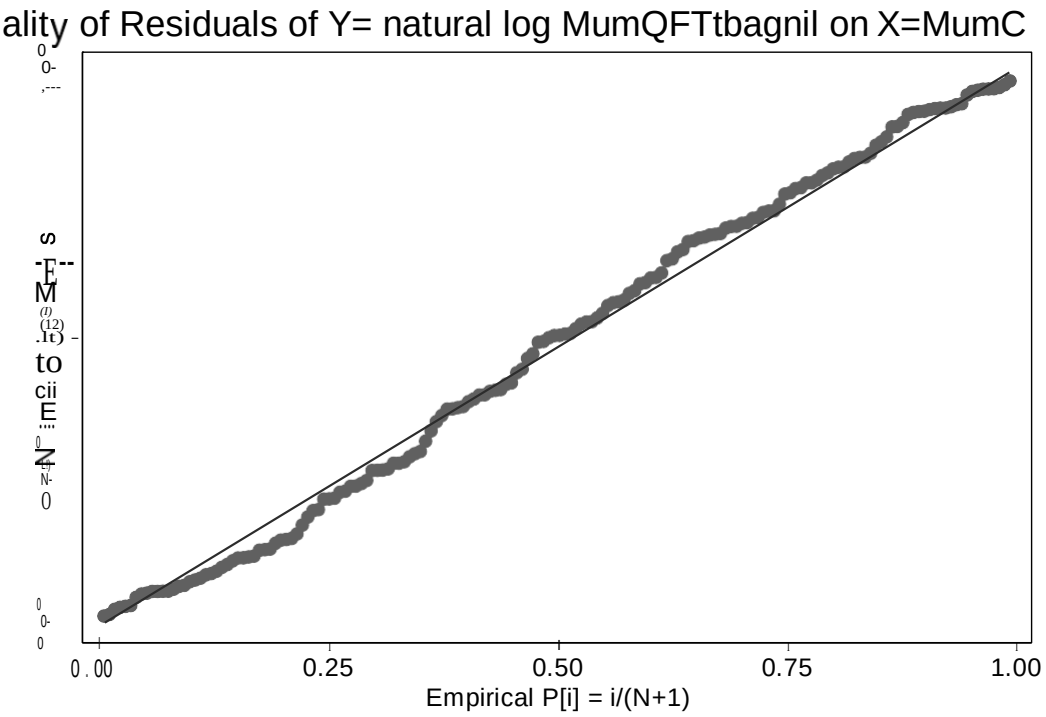
The residuals are not normally distributed as the p value is less than 0.05 therefore don't have enough evidence to reject the null hypothesis which is that variable is normally distributed.

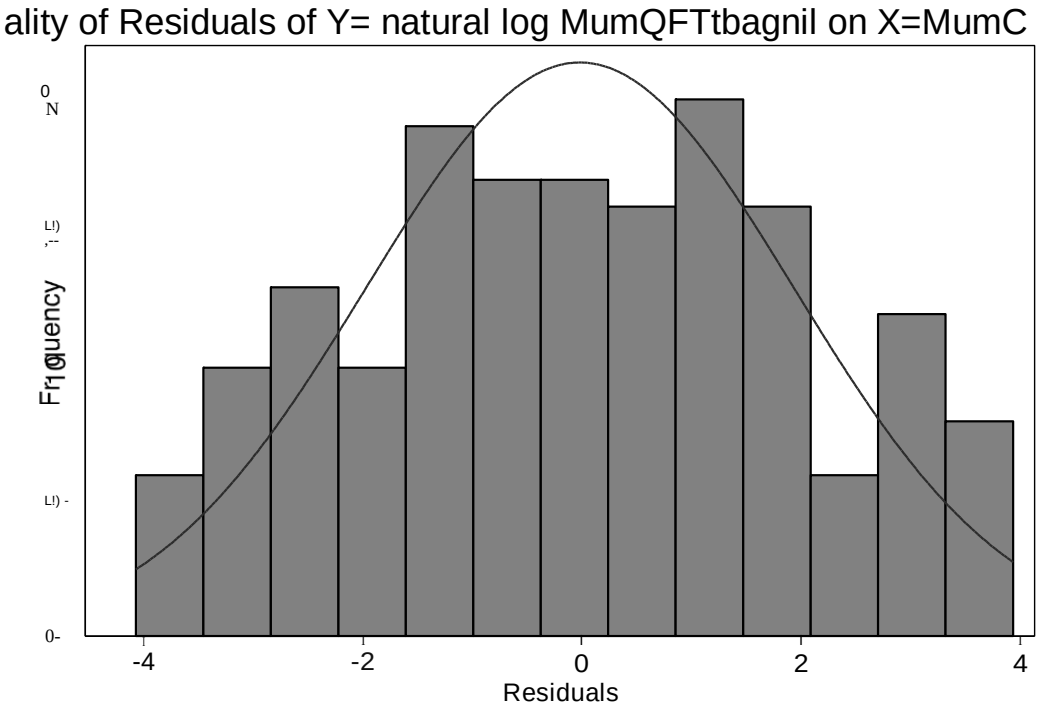
2. Linear regression model 2: Simple model with the dependent variable (Maternal QFT IFN-γ) log transformed (natural log) and Maternal CD4 count

```
      Source |       SS       df       MS              Number of obs =     170
-------------+------------------------------           F(  1,   168) =   14.85
       Model |  57.2200388     1  57.2200388           Prob > F      =  0.0002
    Residual |  647.265005   168  3.85276789           R-squared     =  0.0812
-------------+------------------------------           Adj R-squared =  0.0758
       Total |  704.485044   169  4.16855056           Root MSE      =  1.9628

------------------------------------------------------------------------------
MumQFTtbag~n |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
 MumCD4Count |   .0025877   .0006715     3.85   0.000     .0012621    .0039133
       _cons |  -2.116279   .3371543    -6.28   0.000    -2.781884   -1.450674
------------------------------------------------------------------------------
```

2.1 Checking for normality of residuals





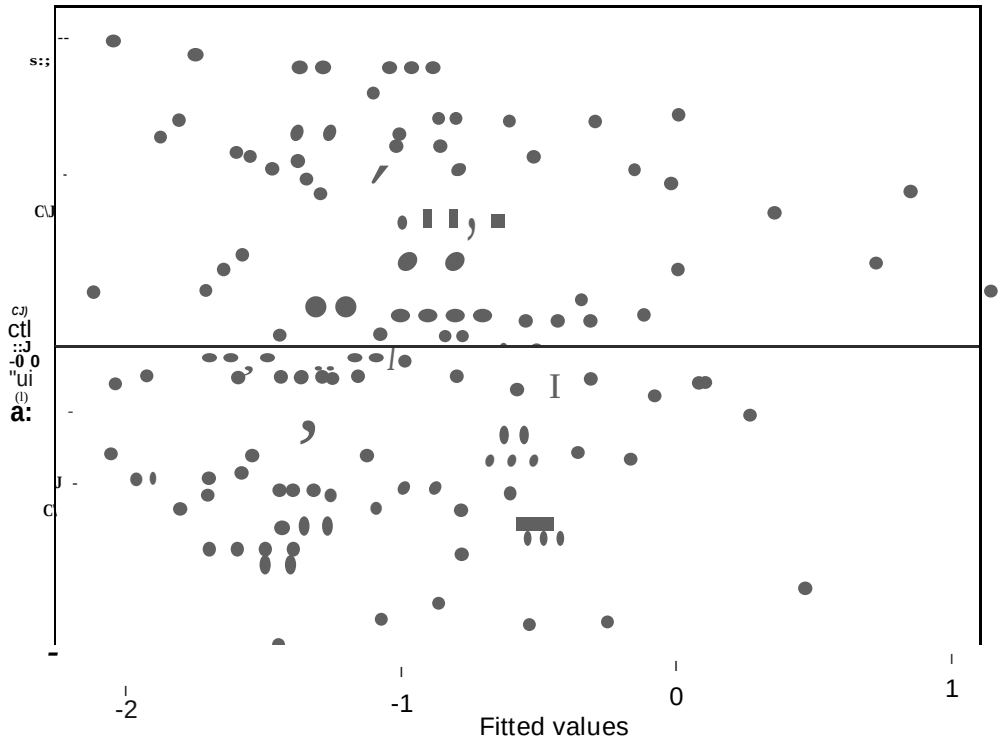
Shapiro-Wilk W test for normal data

Variable	Obs	W	V	z	Prob>z

res13	170	0.98005	2.585	2.167	0.01510

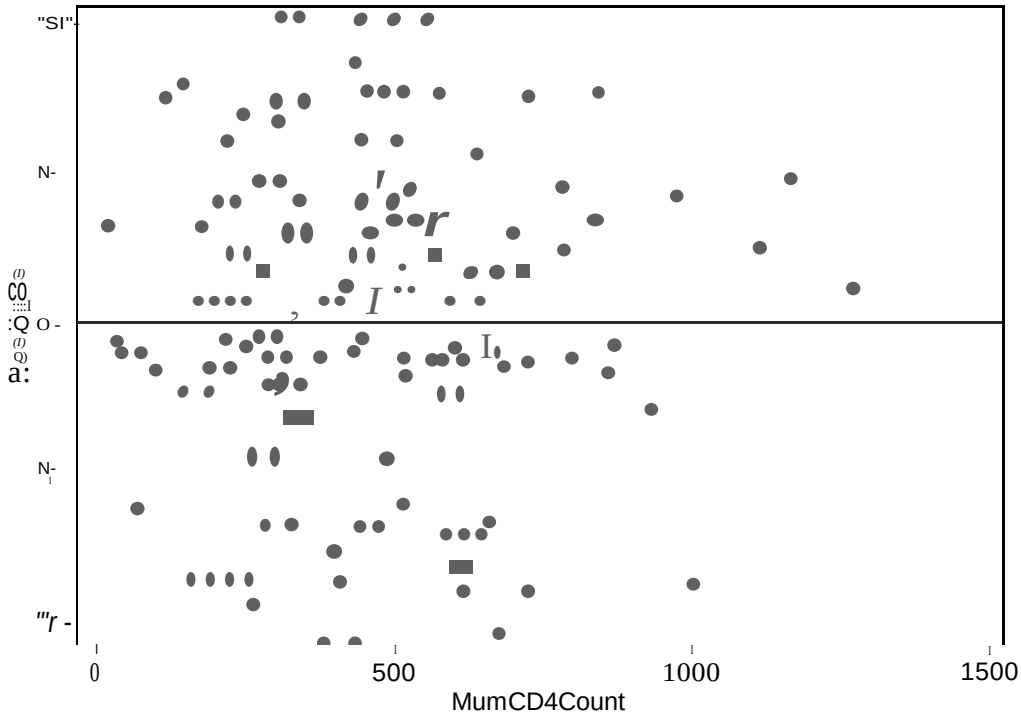
Residuals still not completely normally distributed

2.2 Checking for homogeneity

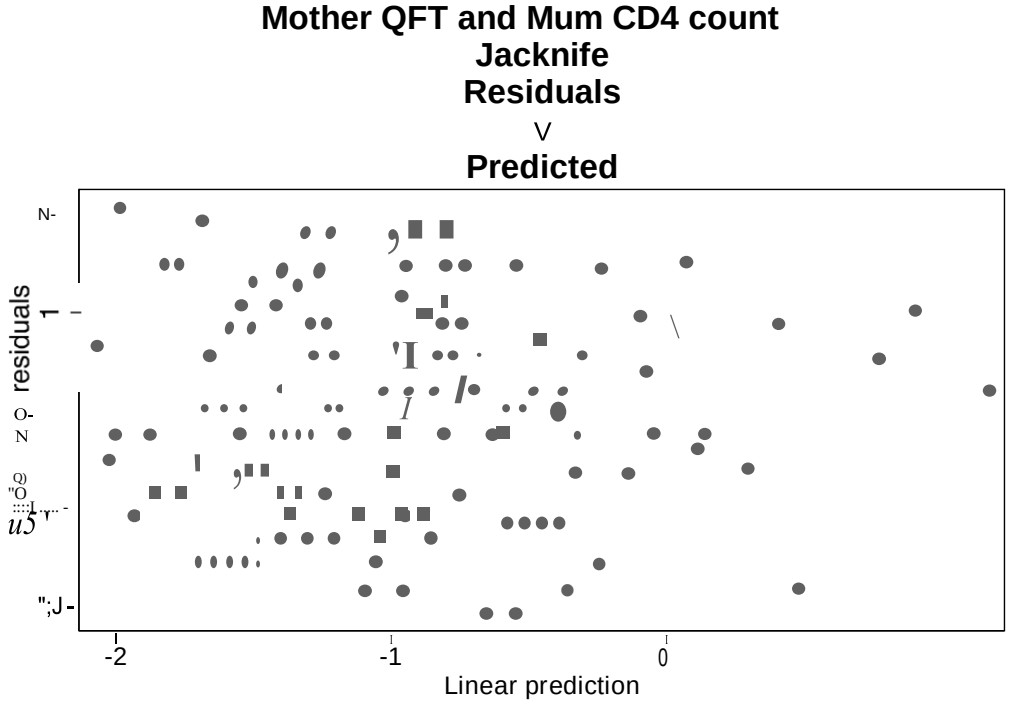


Scatter is random/does not have a pattern and so it is ok.

2.3 Checking for linearity for each of the variables



The residuals are randomly scatter therefore the model is ok.



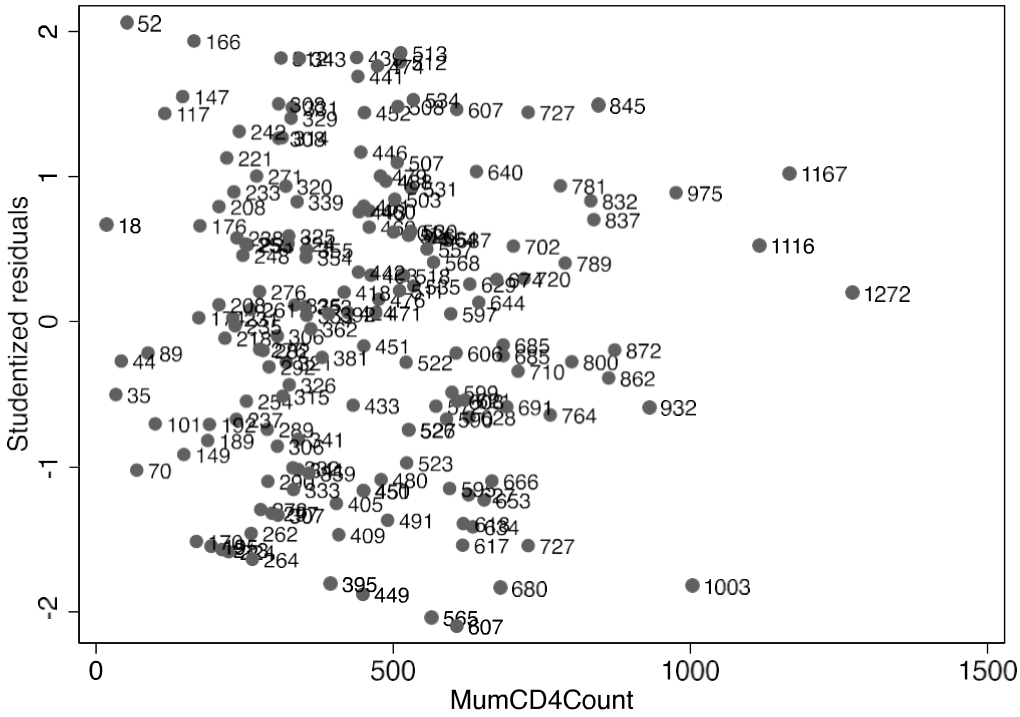
2.4 Checking for collinearity

The mean VIF is 1.00 but don't need to do this as there is only one variable

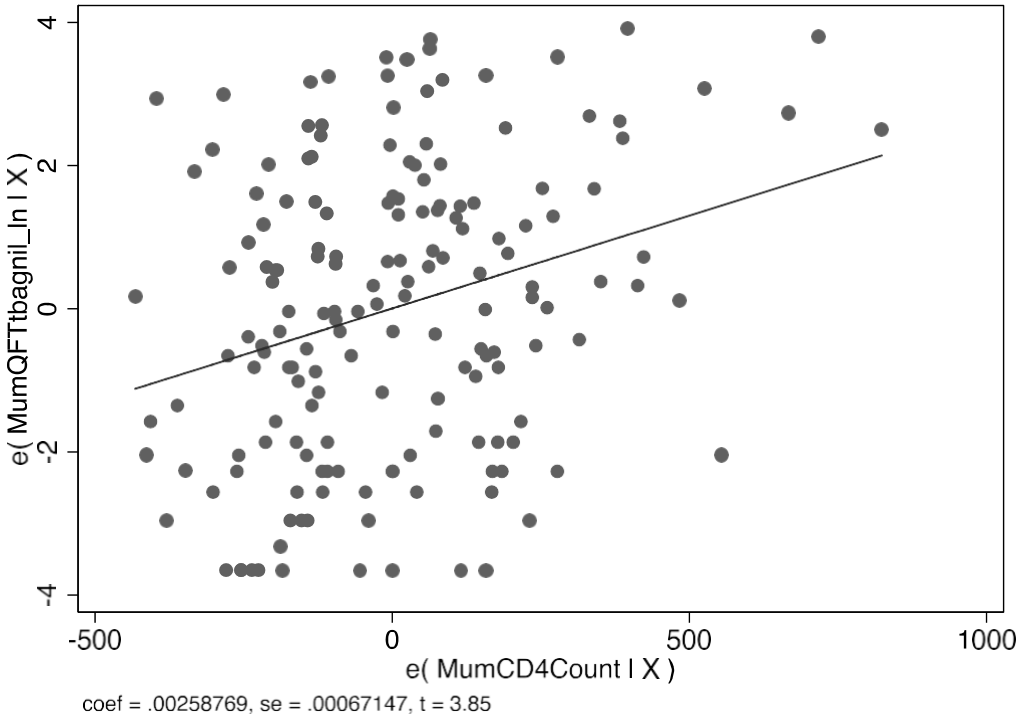
2.5 Checking for outliers

	MumCD4~t	resstud2
1.	413	.
3.	.	.
6.	255	.
7.	391	.
12.	565	-2.039305
14.	359	.
32.	442	.
35.	333	.
39.	242	.
40.	431	.
41.	205	.
48.	242	.
49.	167	.
57.	.	.
59.	.	.
61.	263	.
66.	255	.
71.	616	.
74.	306	.
76.	224	.
80.	52	2.061417
83.	607	-2.098317
84.	341	.
90.	498	.
92.	480	.
95.	424	.
97.	196	.
101.	874	.
103.	427	.
107.	.	.
111.	.	.
114.	175	.
120.	.	.
138.	694	.
139.	.	.
145.	.	.
147.	.	.
149.	.	.
153.	130	.
154.	.	.
155.	865	.
157.	.	.
158.	.	.
160.	200	.
163.	.	.
164.	.	.
165.	852	.
168.	380	.
170.	.	.
171.	380	.
173.	.	.

```
175. |         .           . |
176. |       507           . |
183. |         .           . |
185. |       527           . |
     |-----------------------|
186. |       883           . |
189. |         .           . |
195. |         .           . |
197. |       417           . |
205. |       598           . |
     |-----------------------|
206. |       195           . |
207. |       346           . |
211. |       174           . |
213. |         .           . |
217. |       622           . |
     |-----------------------|
218. |       622           . |
220. |         .           . |
224. |         .           . |
225. |         .           . |
226. |       396           . |
     |-----------------------|
227. |         .           . |
228. |         .           . |
230. |         .           . |
231. |         .           . |
233. |         .           . |
     |-----------------------|
241. |         .           . |
242. |         .           . |
244. |         .           . |
245. |       545           . |
247. |         .           . |
     |-----------------------|
248. |         .           . |
     +-----------------------+
```



Avplot:



2.6 Checking for outliers and influential points

2.6.1 Leverage

There were no leverage values greater than 0.106

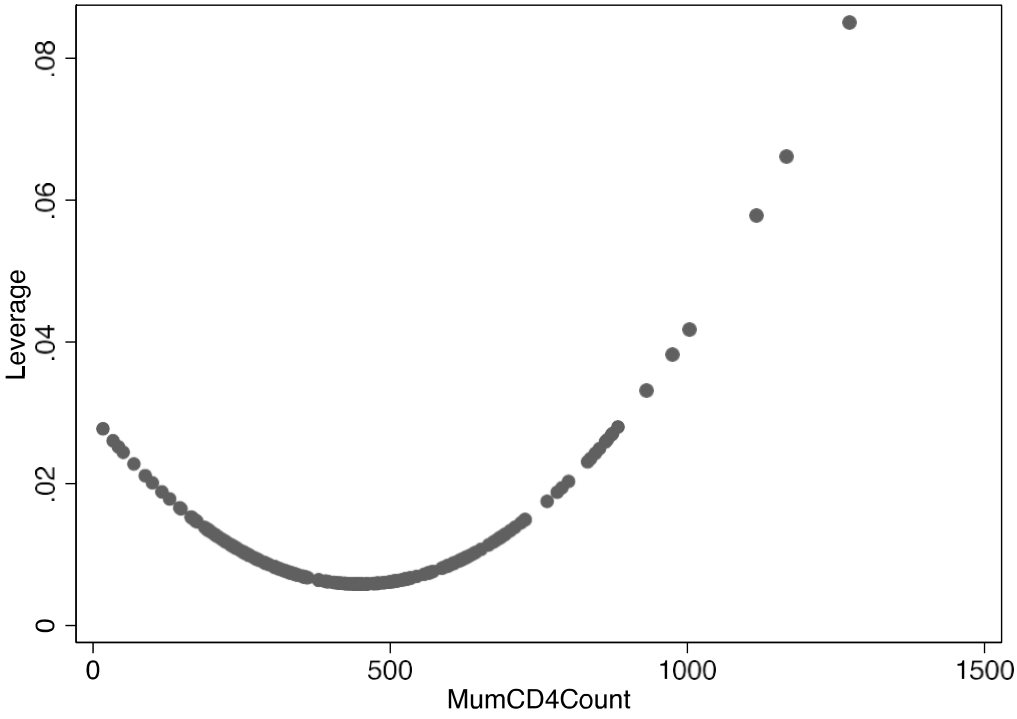
	lev2
1.	.0060364
2.	.0098753
3.	.
4.	.0113565
5.	.0075754
6.	.0102995
7.	.0062799
8.	.0276494
9.	.0123846
10.	.0075196
11.	.0095784
12.	.0074494
13.	.0082848
14.	.0068362
15.	.0146221
16.	.0058836
17.	.0088888
18.	.0331509
19.	.0072545
20.	.0193879
21.	.0058824
22.	.0080878
23.	.0062272
24.	.0096019
25.	.007492
26.	.0200775
27.	.0065012

```
28. | .0147505 |
29. | .0072545 |
30. | .0066638 |
    |----------|
31. | .0123846 |
32. | .0058886 |
33. | .0096201 |
34. | .0102542 |
35. | .0074647 |
    |----------|
36. | .0069672 |
37. | .0058958 |
38. | .0118216 |
39. | .0109104 |
40. | .0059215 |
    |----------|
41. | .0128656 |
42. | .0058904 |
43. | .0164344 |
44. | .0058958 |
45. | .0075317 |
    |----------|
46. | .0059134 |
47. | .0112557 |
48. | .0109104 |
49. | .0152072 |
50. | .0088514 |
    |----------|
51. | .0081019 |
52. | .0063427 |
53. | .0091742 |
54. | .0077191 |
55. | .0060578 |
    |----------|
56. | .0174733 |
57. |        . |
58. | .0138104 |
59. |        . |
60. |  .005887 |
    |----------|
61. | .0099432 |
62. | .0059044 |
63. | .0087565 |
64. | .0165754 |
65. | .0124157 |
    |----------|
66. | .0102995 |
67. | .0074107 |
68. | .0111063 |
69. | .0106235 |
70. | .0121116 |
    |----------|
71. |  .009135 |
72. | .0069226 |
73. | .0085961 |
74. | .0082848 |
75. | .0099869 |
    |----------|
76. | .0118216 |
77. | .0074224 |
78. | .0082182 |
79. | .0059857 |
80. | .0243527 |
    |----------|
81. | .0065183 |
82. | .0061118 |
83. | .0087933 |
84. | .0072545 |
85. | .0085055 |
    |----------|
86. | .0067422 |
87. | .0068362 |
88. |  .006435 |
89. | .0093962 |
90. | .0061601 |
    |----------|
91. | .0149081 |
```

```
 92. | .0059928 |
 93. | .0061954 |
 94. | .0060722 |
 95. | .0059572 |
     |----------|
 96. | .0069226 |
 97. | .0133897 |
 98. | .0076447 |
 99. | .0059572 |
100. | .0093156 |
     |----------|
101. | .0269918 |
102. | .0065892 |
103. | .0059405 |
104. | .0187593 |
105. | .0078081 |
     |----------|
106. |  .009333 |
107. |         . |
108. | .0096621 |
109. | .0082514 |
110. | .0080241 |
     |----------|
111. |         . |
112. | .0066448 |
113. | .0058947 |
114. | .0146862 |
115. | .0058827 |
     |----------|
116. | .0103451 |
117. | .0114582 |
118. | .0127198 |
119. |   .00622 |
120. |         . |
     |----------|
121. | .0058886 |
122. | .0059539 |
123. | .0058827 |
124. | .0202767 |
125. | .0059659 |
     |----------|
126. |  .010739 |
127. | .0188032 |
128. | .0058824 |
129. | .0059928 |
130. | .0111559 |
     |----------|
131. | .0059969 |
132. | .0062722 |
133. |  .023474 |
134. | .0084359 |
135. | .0103909 |
     |----------|
136. | .0088303 |
137. | .0083672 |
138. | .0128906 |
139. |         . |
140. | .0065711 |
     |----------|
141. | .0072402 |
142. | .0149081 |
143. | .0082182 |
144. | .0079925 |
145. |         . |
     |----------|
146. | .0242075 |
147. |         . |
148. | .0126952 |
149. |         . |
150. | .0100309 |
     |----------|
151. | .0134491 |
152. | .0134491 |
153. |  .017812 |
154. |         . |
155. | .0261066 |
     |----------|
```

```
156. | .0063575 |
157. |        . |
158. |        . |
159. | .0058832 |
160. | .0131544 |
     |----------|
161. | .0258158 |
162. | .0210725 |
163. |        . |
164. |        . |
165. | .0248615 |
     |----------|
166. | .0081996 |
167. | .0121422 |
168. | .0064441 |
169. | .0058827 |
170. |        . |
     |----------|
171. | .0064441 |
172. | .0126952 |
173. |        . |
174. | .0093962 |
175. |        . |
     |----------|
176. | .0062722 |
177. | .0059375 |
178. | .0091571 |
179. | .0076899 |
180. | .0062858 |
     |----------|
181. | .0113786 |
182. | .0092135 |
183. |        . |
184. | .0119808 |
185. | .0065892 |
     |----------|
186. | .0278959 |
187. | .0065711 |
188.  | .006086 |
189. |        . |
190. | .0063281 |
     |----------|
191. | .0144588 |
192. | .0138369 |
193. | .0150101 |
194. | .0069448 |
195. |        . |
     |----------|
196. | .0074647 |
197. | .0060043 |
198. | .0109104 |
199. | .0072043 |
200. | .0078383 |
     |----------|
201. | .0073056 |
202. | .0417624 |
203. | .0098997 |
204. | .0101389 |
205. | .0084706 |
     |----------|
206. | .0134491 |
207. | .0071307 |
208. | .0259672 |
209. | .0661637 |
210. | .0067739 |
     |----------|
211. | .0147505 |
212. | .0087933 |
213. |        . |
214. | .0230232 |
215. | .0067222 |
     |----------|
216. | .0136287 |
217. | .0093734 |
218. | .0093734 |
219. | .0579011 |
220. |        . |
```

```
     |----------|
221. | .0117918 |
222. | .0082848 |
223. | .0103193 |
224. |        . |
225. |        . |
     |----------|
226. | .0062146 |
227. |        . |
228. |        . |
229. | .0133563 |
230. |        . |
     |----------|
231. |        . |
232. | .0152734 |
233. |        . |
234. | .0267934 |
235. | .0382254 |
     |----------|
236. | .025104 |
237. | .0087773 |
238. | .0062663 |
239. | .0227169 |
240. | .0850919 |
     |----------|
241. |        . |
242. |        . |
243. | .007661 |
244. |        . |
245. | .0069545 |
     |----------|
246. | .006428 |
247. |        . |
248. |        . |
     +----------+
```



2.6.2 Cook's Distance

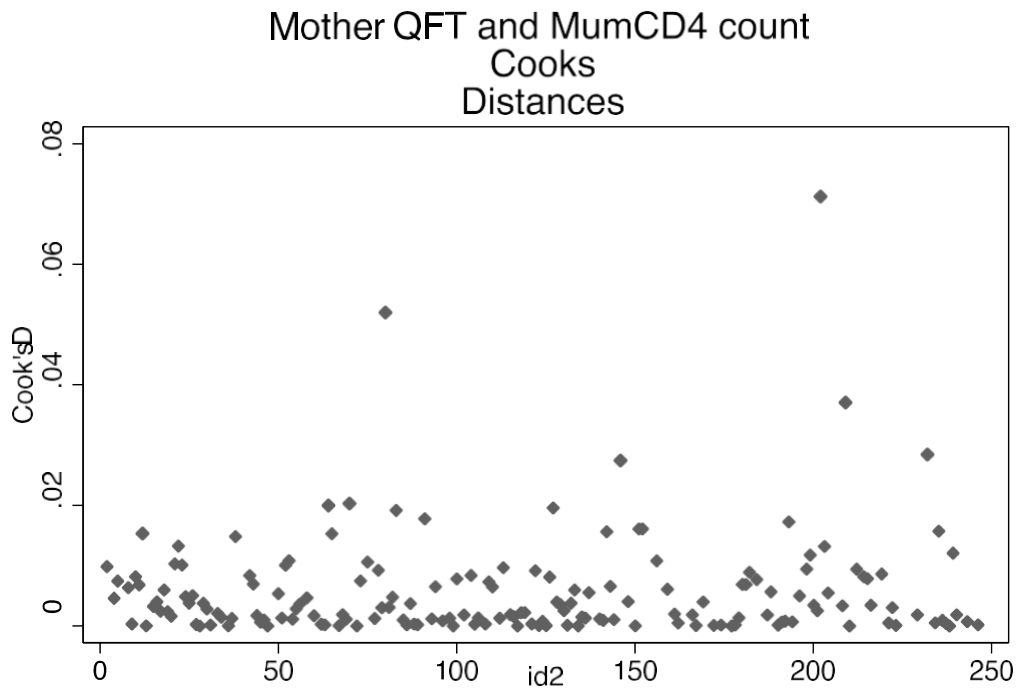
```
     +----------+
     |  cooksd2 |
     |----------|
  1. |        . |
```

```
 2. | .0098374 |
 3. |        . |
 4. | .0045886 |
 5. | .0074421 |
    |----------|
 6. |        . |
 7. |        . |
 8. | .0063467 |
 9. | .0003408 |
10. | .0081672 |
    |----------|
11. | .0068184 |
12. | .0153183 |
13. | .0000393 |
14. |        . |
15. | .0032511 |
    |----------|
16. | .0040208 |
17. |  .002452 |
18. |  .005975 |
19. | .0023833 |
20. | .0016229 |
    |----------|
21. | .0102953 |
22. |   .01323 |
23. | .0100875 |
24. | .0048671 |
25. | .0037984 |
    |----------|
26. |  .005048 |
27. | .0002528 |
28. | 6.31e-06 |
29. |  .003764 |
30. | .0028288 |
    |----------|
31. | .0001586 |
32. |        . |
33. | .0020879 |
34. | .0014646 |
35. |        . |
    |----------|
36. | .0000399 |
37. | .0012618 |
38. | .0147933 |
39. |        . |
40. |        . |
    |----------|
41. |        . |
42. | .0083456 |
43. | .0069527 |
44. | .0017349 |
45. | .0006406 |
    |----------|
46. | .0009819 |
47. | 4.11e-06 |
48. |        . |
49. |        . |
50. | .0053585 |
    |----------|
51. |  .001317 |
52. | .0100521 |
53. | .0108194 |
54. | .0011318 |
55. | .0028539 |
    |----------|
56. | .0036528 |
57. |        . |
58. | .0046686 |
59. |        . |
60. | .0016996 |
    |----------|
61. |        . |
62. | .0003082 |
63. | .0002035 |
64. | .0200559 |
65. | .0152682 |
    |----------|
```

```
 66. |         . |
 67. |  .0000506 |
 68. |  .0018798 |
 69. |  .0011275 |
 70. |  .0203589 |
     |-----------|
 71. |         . |
 72. |  7.21e-06 |
 73. |  .0074633 |
 74. |         . |
 75. |  .0105935 |
     |-----------|
 76. |         . |
 77. |  .0012414 |
 78. |  .0092366 |
 79. |  .0030263 |
 80. |  .0520278 |
     |-----------|
 81. |  .0030821 |
 82. |  .0047738 |
 83. |  .0191422 |
 84. |         . |
 85. |  .0010088 |
     |-----------|
 86. |  .0002073 |
 87. |  .0037123 |
 88. |  .0003305 |
 89. |  .0002067 |
 90. |         . |
     |-----------|
 91. |  .0177508 |
 92. |         . |
 93. |  .0011956 |
 94. |   .006498 |
 95. |         . |
     |-----------|
 96. |  .0008651 |
 97. |         . |
 98. |  .0012997 |
 99. |  .0000127 |
100. |  .0077921 |
     |-----------|
101. |         . |
102. |  .0018337 |
103. |         . |
104. |   .008374 |
105. |  .0003079 |
     |-----------|
106. |  .0013641 |
107. |         . |
108. |  .0003305 |
109. |  .0073043 |
110. |  .0064726 |
     |-----------|
111. |         . |
112. |   .001303 |
113. |  .0096615 |
114. |         . |
115. |  .0018775 |
     |-----------|
116. |  .0015598 |
117. |  3.63e-06 |
118. |  .0022067 |
119. |  .0022304 |
120. |         . |
     |-----------|
121. |  .0003486 |
122. |  .0091558 |
123. |   .000081 |
124. |  .0007808 |
125. |  .0000728 |
     |-----------|
126. |  .0081134 |
127. |  .0195422 |
128. |  .0039701 |
129. |  .0035428 |
130. |  .0025425 |
```

```
     |----------|
131. | .0001259 |
132. | .0037818 |
133. | .0059631 |
134. | .0000133 |
135. | .0015068 |
     |----------|
136. | .0013267 |
137. | .005525  |
138. |        . |
139. |        . |
140. | .0011773 |
     |----------|
141. | .0009201 |
142. | .0156048 |
143. | .0065803 |
144. | .001057  |
145. |        . |
     |----------|
146. | .0274933 |
147. |        . |
148. | .004048  |
149. |        . |
150. | .0000375 |
     |----------|
151. | .0160697 |
152. | .0160697 |
153. |        . |
154. |        . |
155. |        . |
     |----------|
156. | .0107901 |
157. |        . |
158. |        . |
159. | .0060832 |
160. |        . |
     |----------|
161. | .0019814 |
162. | .0004953 |
163. |        . |
164. |        . |
165. |        . |
     |----------|
166. | .0018496 |
167. | .000078  |
168. |        . |
169. | .0039794 |
170. |        . |
     |----------|
171. |        . |
172. | .0000902 |
173. |        . |
174. |   .00017 |
175. |        . |
     |----------|
176. |        . |
177. | .0000114 |
178. | .0001798 |
179. | .0013608 |
180. | .0068814 |
     |----------|
181. | .006873  |
182. | .0088913 |
183. |        . |
184. | .0076861 |
185. |        . |
     |----------|
186. |        . |
187. | .0018221 |
188. | .0056607 |
189. |        . |
190. | .0001488 |
     |----------|
191. | .0006554 |
192. | .0008106 |
193. | .0172225 |
194. | .0006945 |
```

```
195. |        . |
     |----------|
196. | .0049871 |
197. |        . |
198. | .009421  |
199. | .0117343 |
200. | .0034407 |
     |----------|
201. | .0025171 |
202. | .0712725 |
203. | .0131868 |
204. | .0054703 |
205. |        . |
     |----------|
206. |        . |
207. |        . |
208. | .0033423 |
209. | .0371318 |
210. | 7.78e-06 |
     |----------|
211. |        . |
212. | .0094021 |
213. |        . |
214. | .008173  |
215. | .0078325 |
     |----------|
216. | .0034434 |
217. |        . |
218. |        . |
219. | .0086098 |
220. |        . |
     |----------|
221. | .000511  |
222. | .0030536 |
223. | .0000949 |
224. |        . |
225. |        . |
     |----------|
226. |        . |
227. |        . |
228. |        . |
229. | .0018437 |
230. |        . |
     |----------|
231. |        . |
232. | .0284476 |
233. |        . |
234. | .0005188 |
235. | .0156954 |
     |----------|
236. | .0009387 |
237. | .000425  |
238. | 9.54e-06 |
239. | .0120633 |
240. | .0018495 |
     |----------|
241. |        . |
242. |        . |
243. | .0007253 |
244. |        . |
245. |        . |
     |----------|
246. | .0001939 |
247. |        . |
248. |        . |
     +----------+
```



There were no Cooksd values greater than 0.33

2.6.3 dfbeta

	dfMumCD~t
1.	.
3.	.
6.	.
7.	.
14.	.
32.	.
35.	.
39.	.
40.	.
41.	.
48.	.
49.	.
57.	.
59.	.
61.	.
64.	-.1615313
66.	.
70.	-.14573
71.	.
74.	.
76.	.
80.	-.283633
84.	.
90.	.
91.	-.1472014
92.	.
95.	.
97.	.
101.	.
103.	.

```
     |-----------|
107. |         . |
111. |         . |
114. |         . |
120. |         . |
127. | -.1643942 |
     |-----------|
138. |         . |
139. |         . |
142. | .1378987 |
145. |         . |
146. | .2047692 |
     |-----------|
147. |         . |
149. |         . |
153. |         . |
154. |         . |
155. |         . |
     |-----------|
157. |         . |
158. |         . |
160. |         . |
163. |         . |
164. |         . |
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165. |         . |
168. |         . |
170. |         . |
171. |         . |
173. |         . |
     |-----------|
175. |         . |
176. |         . |
183. |         . |
185. |         . |
186. |         . |
     |-----------|
189. |         . |
193. | .1452773 |
195. |         . |
197. |         . |
202. | -.3523567 |
     |-----------|
205. |         . |
206. |         . |
207. |         . |
209. |   .260155 |
211. |         . |
     |-----------|
213. |         . |
217. |         . |
218. |         . |
220. |         . |
224. |         . |
     |-----------|
225. |         . |
226. |         . |
227. |         . |
228. |         . |
230. |         . |
     |-----------|
231. |         . |
232. |  -.188549 |
233. |         . |
235. |  .1628704 |
241. |         . |
     |-----------|
242. |         . |
244. |         . |
245. |         . |
247. |         . |
248. |         . |
     +-----------+
```
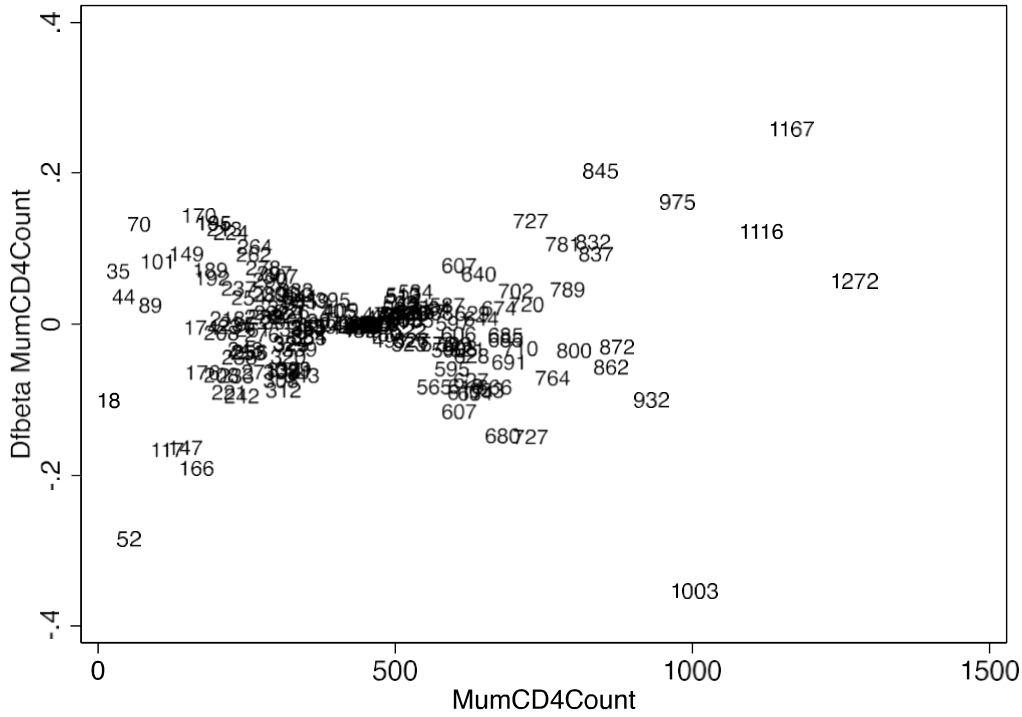



The outliers and influential points seem to be 1003, 1167, 1116 and 1272. These are positions/subjects 202, 209, 219 and 240 respectively. There are also two other outliers/influential points 52 and 18. Will rerun model with points 1003, 52 and 18 removed.

	MumCD4~t	lev2	cooksd2	h2	dfMumCD~t
1.	413	.0060364	.	.0060364	.
2.	634	.0098753	.0098374	.0098753	-.0894529
3.	.	.	.	.	.
4.	233	.0113565	.0045886	.0113565	-.066471
5.	329	.0075754	.0074421	.0075754	-.0578411
6.	255	.0102995	.	.0102995	.
7.	391	.0062799	.	.0062799	.
8.	18	.0276494	.0063467	.0276494	-.0997994
9.	685	.0123846	.0003408	.0123846	-.0188645
10.	331	.0075196	.0081672	.0075196	-.0598439
11.	627	.0095784	.0068184	.0095784	-.0726292
12.	565	.0074494	.0153183	.0074494	-.0810295
13.	306	.0082848	.0000393	.0082848	.0047621
14.	359	.0068362	.	.0068362	.
15.	176	.0146221	.0032511	.0146221	-.0622368
16.	446	.0058836	.0040208	.0058836	-.0013141
17.	289	.0088888	.002452	.0088888	.0406711
18.	932	.0331509	.005975	.0331509	-.0989517
19.	341	.0072545	.0023833	.0072545	.029995
20.	789	.0193879	.0016229	.0193879	.047431
21.	449	.0058824	.0102953	.0058824	.0001821
22.	312	.0080878	.01323	.0080878	-.0855204
23.	395	.0062272	.0100875	.0062272	.0336483
24.	271	.0096019	.0048671	.0096019	-.0614075
25.	332	.007492	.0037984	.007492	.0404011
26.	101	.0200775	.005048	.0200775	.0843592
27.	522	.0065012	.0002528	.0065012	-.0069181

28.	174	.0147505	6.31e-06	.0147505	-.0027471
29.	341	.0072545	.003764	.0072545	.0377373
30.	531	.0066638	.0028288	.0066638	.0257455
31.	685	.0123846	.0001586	.0123846	-.0128663
32.	442	.0058886	.	.0058886	.
33.	628	.0096201	.0020879	.0096201	-.0402106
34.	256	.0102542	.0014646	.0102542	-.0352629
35.	333	.0074647	.	.0074647	.
36.	353	.0069672	.0000399	.0069672	-.0035141
37.	460	.0058958	.0012618	.0058958	.0023946
38.	224	.0118216	.0147933	.0118216	.1224611
39.	242	.0109104	.	.0109104	.
40.	431	.0059215	.	.0059215	.
41.	205	.0128656	.	.0128656	.
42.	441	.0058904	.0083456	.0058904	-.0047955
43.	149	.0164344	.0069527	.0164344	.094442
44.	460	.0058958	.0017349	.0058958	.0028092
45.	568	.0075317	.0006406	.0075317	.0167088
46.	433	.0059134	.0009819	.0059134	.0032034
47.	235	.0112557	4.11e-06	.0112557	.0019761
48.	242	.0109104	.	.0109104	.
49.	167	.0152072	.	.0152072	.
50.	290	.0088514	.0053585	.0088514	.0599927
51.	587	.0081019	.001317	.0081019	.0268082
52.	512	.0063427	.0100521	.0063427	.0384457
53.	617	.0091742	.0108194	.0091742	-.0884702
54.	324	.0077191	.0011318	.0077191	-.0231593
55.	488	.0060578	.0028539	.0060578	.0128541
56.	764	.0174733	.0036528	.0174733	-.0694918
57.	.	.	.	.	.
58.	189	.0138104	.0046686	.0138104	.0731402
59.	.	.	.	.	.
60.	443	.005887	.0016996	.005887	-.001631
61.	263	.0099432	.	.0099432	.
62.	463	.0059044	.0003082	.0059044	.0015123
63.	606	.0087565	.0002035	.0087565	-.0115246
64.	147	.0165754	.0200559	.0165754	-.1615313
65.	213	.0124157	.0152682	.0124157	.1273087
66.	255	.0102995	.	.0102995	.
67.	335	.0074107	.0000506	.0074107	-.0045566
68.	238	.0111063	.0018798	.0111063	-.041969
69.	248	.0106235	.0011275	.0106235	-.0316486
70.	680	.0121116	.0203589	.0121116	-.14573
71.	616	.009135	.	.009135	.
72.	355	.0069226	7.21e-06	.0069226	-.001468
73.	297	.0085961	.0074633	.0085961	.0687951
74.	306	.0082848	.	.0082848	.
75.	262	.0099869	.0105935	.0099869	.0936245
76.	224	.0118216	.	.0118216	.
77.	564	.0074224	.0012414	.0074224	.022652
78.	308	.0082182	.0092366	.0082182	-.0727296
79.	479	.0059857	.0030263	.0059857	.0102228
80.	52	.0243527	.0520278	.0243527	-.283633
81.	523	.0065183	.0030821	.0065183	-.0245189
82.	405	.0061118	.0047738	.0061118	.0189649
83.	607	.0087933	.0191422	.0087933	-.1137123
84.	341	.0072545	.	.0072545	.
85.	599	.0085055	.0010088	.0085055	-.0248882
86.	535	.0067422	.0002073	.0067422	.0072502
87.	359	.0068362	.0037123	.0068362	.0321936
88.	518	.006435	.0003305	.006435	.0075138
89.	276	.0093962	.0002067	.0093962	-.0123976
90.	498	.0061601	.	.0061601	.
91.	727	.0149081	.0177508	.0149081	-.1472014

92.	480	.0059928	.	.0059928	.
93.	501	.0061954	.0011956	.0061954	.0109711
94.	409	.0060722	.006498	.0060722	.020228
95.	424	.0059572	.	.0059572	.
96.	355	.0069226	.0008651	.0069226	-.0160882
97.	196	.0133897	.	.0133897	.
98.	572	.0076447	.0012997	.0076447	-.0244313
99.	424	.0059572	.0000127	.0059572	-.0005635
100.	278	.0093156	.0077921	.0093156	.0759354
101.	874	.0269918	.	.0269918	.
102.	527	.0065892	.0018337	.0065892	-.0198079
103.	427	.0059405	.	.0059405	.
104.	781	.0187593	.008374	.0187593	.107181
105.	321	.0078081	.0003079	.0078081	.0122907
106.	621	.009333	.0013641	.009333	-.0316923
107.	.	.	.	.	.
108.	629	.0096621	.0003305	.0096621	.0160353
109.	307	.0082514	.0073043	.0082514	.0649103
110.	314	.0080241	.0064726	.0080241	-.0588871
111.	.	.	.	.	.
112.	530	.0066448	.001303	.0066448	.0172605
113.	439	.0058947	.0096615	.0058947	-.006412
114.	175	.0146862	.	.0146862	.
115.	451	.0058827	.0018775	.0058827	.0004689
116.	254	.0103451	.0015598	.0103451	.0366077
117.	231	.0114582	3.63e-06	.0114582	-.0018742
118.	691	.0127198	.0022067	.0127198	-.0486114
119.	503	.00622	.0022304	.00622	.0155487
120.	.	.	.	.	.
121.	442	.0058886	.0003486	.0058886	-.0008549
122.	474	.0059539	.0091558	.0059539	.0149211
123.	451	.0058827	.000081	.0058827	-.0000972
124.	800	.0202767	.0007808	.0202767	-.0332045
125.	476	.0059659	.0000728	.0059659	.0014233
126.	653	.010739	.0081134	.010739	-.085792
127.	117	.0188032	.0195422	.0188032	-.1643942
128.	450	.0058824	.0039701	.0058824	-.0002855
129.	480	.0059928	.0035428	.0059928	-.0114322
130.	237	.0111559	.0025425	.0111559	.0489478
131.	418	.0059969	.0001259	.0059969	-.0021863
132.	507	.0062722	.0037818	.0062722	.021695
133.	837	.023474	.0059631	.023474	.0943963
134.	597	.0084359	.0000133	.0084359	.0028318
135.	253	.0103909	.0015068	.0103909	-.0360835
136.	608	.0088303	.0013267	.0088303	-.0297008
137.	595	.0083672	.005525	.0083672	-.0573382
138.	694	.0128906	.	.0128906	.
139.	.	.	.	.	.
140.	526	.0065711	.0011773	.0065711	.0156799
141.	557	.0072402	.0009201	.0072402	.0185362
142.	727	.0149081	.0156048	.0149081	.1378987
143.	308	.0082182	.0065803	.0082182	-.061269
144.	315	.0079925	.001057	.0079925	.0235732
145.	.	.	.	.	.
146.	845	.0242075	.0274933	.0242075	.2047692
147.	.	.	.	.	.
148.	208	.0126952	.004048	.0126952	-.0658413
149.	.	.	.	.	.
150.	261	.0100309	.0000375	.0100309	-.0055497
151.	195	.0134491	.0160697	.0134491	.1350202
152.	195	.0134491	.0160697	.0134491	.1350202
153.	130	.017812	.	.017812	.
154.	.	.	.	.	.
155.	865	.0261066	.	.0261066	.

156.	513	.0063575	.0107901	.0063575	.0404474
157.	.	.	.	.	.
158.	.	.	.	.	.
159.	452	.0058832	.0060832	.0058832	.0013412
160.	200	.0131544	.	.0131544	.
161.	862	.0258158	.0019814	.0258158	-.0551761
162.	89	.0210725	.0004953	.0210725	.0266463
163.	.	.	.	.	.
164.	.	.	.	.	.
165.	852	.0248615	.	.0248615	.
166.	590	.0081996	.0018496	.0081996	-.0322794
167.	218	.0121422	.000078	.0121422	.008942
168.	380	.0064441	.	.0064441	.
169.	451	.0058827	.0039794	.0058827	-.0006842
170.	.	.	.	.	.
171.	380	.0064441	.	.0064441	.
172.	208	.0126952	.0000902	.0126952	-.0098083
173.	.	.	.	.	.
174.	276	.0093962	.00017	.0093962	.0112439
175.	.	.	.	.	.
176.	507	.0062722	.	.0062722	.
177.	471	.0059375	.0000114	.0059375	.0004592
178.	282	.0091571	.0001798	.0091571	.0113076
179.	325	.0076899	.0013608	.0076899	-.0252441
180.	508	.0062858	.0068814	.0062858	.0298273
181.	666	.0113786	.006873	.0113786	-.0815322
182.	618	.0092135	.0088913	.0092135	-.0804031
183.	.	.	.	.	.
184.	221	.0119808	.0076861	.0119808	-.0885284
185.	527	.0065892	.	.0065892	.
186.	883	.0278959	.	.0278959	.
187.	526	.0065711	.0018221	.0065711	-.0195177
188.	491	.006086	.0056607	.006086	-.019514
189.	.	.	.	.	.
190.	511	.0063281	.0001488	.0063281	.0045648
191.	720	.0144588	.0006554	.0144588	.0278079
192.	710	.0138369	.0008106	.0138369	-.0304488
193.	170	.0150101	.0172225	.0150101	.1452773
194.	354	.0069448	.0006945	.0069448	-.0145424
195.	.	.	.	.	.
196.	333	.0074647	.0049871	.0074647	.0460266
197.	417	.0060043	.	.0060043	.
198.	242	.0109104	.009421	.0109104	-.0933824
199.	343	.0072043	.0117343	.0072043	-.0660652
200.	320	.0078383	.0034407	.0078383	-.0414224
201.	339	.0073056	.0025171	.0073056	-.0312876
202.	1003	.0417624	.0712725	.0417624	-.3523567
203.	264	.0098997	.0131868	.0098997	.1039645
204.	640	.0101389	.0054703	.0101389	.0677865
205.	598	.0084706	.	.0084706	.
206.	195	.0134491	.	.0134491	.
207.	346	.0071307	.	.0071307	.
208.	35	.0259672	.0033423	.0259672	.0717438
209.	1167	.0661637	.0371318	.0661637	.260155
210.	362	.0067739	7.78e-06	.0067739	.0014267
211.	174	.0147505	.	.0147505	.
212.	607	.0087933	.0094021	.0087933	.0791646
213.	.	.	.	.	.
214.	832	.0230232	.008173	.0230232	.110215
215.	534	.0067222	.0078325	.0067222	.0444157
216.	192	.0136287	.0034434	.0136287	.0624712
217.	622	.0093734	.	.0093734	.
218.	622	.0093734	.	.0093734	.
219.	1116	.0579011	.0086098	.0579011	.1241121
220.	.	.	.	.	.

```
     |-----------------------------------------------------|
221. |      674   .0117918    .000511   .0117918     .022569 |
222. |      306   .0082848   .0030536   .0082848    .0420493 |
223. |      644   .0103193   .0000949   .0103193    .0090081 |
224. |        .          .          .          .           . |
225. |        .          .          .          .           . |
     |-----------------------------------------------------|
226. |      396   .0062146          .   .0062146           . |
227. |        .          .          .          .           . |
228. |        .          .          .          .           . |
229. |      702   .0133563   .0018437   .0133563    .0453256 |
230. |        .          .          .          .           . |
     |-----------------------------------------------------|
231. |        .          .          .          .           . |
232. |      166   .0152734   .0284476   .0152734    -.188549 |
233. |        .          .          .          .           . |
234. |      872   .0267934   .0005188   .0267934   -.0283747 |
235. |      975   .0382254   .0156954   .0382254    .1628704 |
     |-----------------------------------------------------|
236. |       44    .025104   .0009387    .025104      .03781 |
237. |      292   .0087773    .000425   .0087773    .0166978 |
238. |      392   .0062663   9.54e-06   .0062663   -.0010783 |
239. |       70   .0227169   .0120633   .0227169    .1337285 |
240. |     1272   .0850919   .0018495   .0850919    .0585122 |
     |-----------------------------------------------------|
241. |        .          .          .          .           . |
242. |        .          .          .          .           . |
243. |      326    .007661   .0007253    .007661    .0183069 |
244. |        .          .          .          .           . |
245. |      545   .0069545          .   .0069545           . |
     |-----------------------------------------------------|
246. |      381    .006428   .0001939    .006428    .0057219 |
247. |        .          .          .          .           . |
248. |        .          .          .          .           . |
     +-----------------------------------------------------+
```

3. Linear regression model 3: Simple model with the dependent variable (Maternal QFT IFN-γ) log transformed (natural log) and Maternal CD4 count per 50 cells/mm3 for more meaningful interpretation. The outliers/influential points were not removed.

```
    Source |       SS       df       MS              Number of obs =     170
-------------+------------------------------           F(  1,   168) =   14.85
       Model |  57.2200372     1  57.2200372           Prob > F      =  0.0002
    Residual |  647.265007   168   3.8527679           R-squared     =  0.0812
-------------+------------------------------           Adj R-squared =  0.0758
       Total |  704.485044   169  4.16855056           Root MSE      =  1.9628

------------------------------------------------------------------------------
MumQFTtbag~n |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
      cd4_50 |   .1293844   .0335733     3.85   0.000     .0631045    .1956644
       _cons |  -2.116279   .3371543    -6.28   0.000    -2.781884   -1.450674
------------------------------------------------------------------------------
```

4. Linear regression model 4: Simple model with the dependent variable (Maternal QFT IFN-γ) log transformed (natural log) and Maternal CD4 count per 50 cells/mm3 for more meaningful interpretation. The outliers/influential points were removed.

```
    Source |       SS       df       MS              Number of obs =     167
-------------+------------------------------           F(  1,   165) =   20.35
       Model |  75.9457144     1  75.9457144           Prob > F      =  0.0000
    Residual |  615.743144   165  3.73177663           R-squared     =  0.1098
-------------+------------------------------           Adj R-squared =  0.1044
       Total |  691.688858   166  4.16680035           Root MSE      =  1.9318

------------------------------------------------------------------------------
MumQFTtbag~n |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
```

```
      modcd4 |   .1550828   .0343772     4.51   0.000      .087207    .2229586
       _cons |  -2.358681   .3441891    -6.85   0.000    -3.038264   -1.679099
------------------------------------------------------------------------------
```

Interpretation of this model:

The F test = 20.35 and is statistically significant at all levels ($p<0.0001$), hence the model explains a significant amount of variation in maternal QFT. $R^2 = 0.1098$ so 10.98% of the variability in the maternal QFT is explained by the model. The linear regression equation is: predicted natural log maternal QFT-γ= -2.359 + 0.155*maternal CD4 count (cells/mm^3/50).

Back transforming the log-transformed maternal QFT-γ: ($e^{0.155}$ – 1)*100 = (1.16-1)*100 = 0.167*100 =16.76%

For every 50 unit (cells/mm^3) increase in maternal CD4 count, maternal QFT IFN -γ IU/ml will increase by 17% (1.17 times), on average.

APPENDIX A: ADDITIONAL DISSERTATION ANALYSIS

APPENDIX A2: Linear regression of demographic and clinical risk factors associated with *M.tb* infection in HIV exposed infants

1. Full dataset without removal of missing QFT results and the two values over 12 IU/ml (13.15 and 14.28 IU/ml)

2. Linear regression model 1: Infant IFN-γ (IU/ml) with outliers removed and new HHC as this model with only one independent variable was the best model

xi:regress BabyQFTtbagnil_mod12 i.InfNewHHC

```
      Source |       SS       df       MS              Number of obs =     196
-------------+------------------------------           F(  1,   194) =    9.98
       Model |  1.30206578     1  1.30206578            Prob > F      =  0.0018
    Residual |  25.3140512   194    .1304848            R-squared     =  0.0489
-------------+------------------------------           Adj R-squared =  0.0440
       Total |   26.616117   195  .136492908            Root MSE      =  .36123

------------------------------------------------------------------------------
BabyQ~l_mod12 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
--------------+---------------------------------------------------------------
_IInfNewHHC_2 |   .3894058   .1232724     3.16   0.002     .1462796     .632532
        _cons |   .0261497   .0264155     0.99   0.323    -.0259487    .0782482
------------------------------------------------------------------------------
```

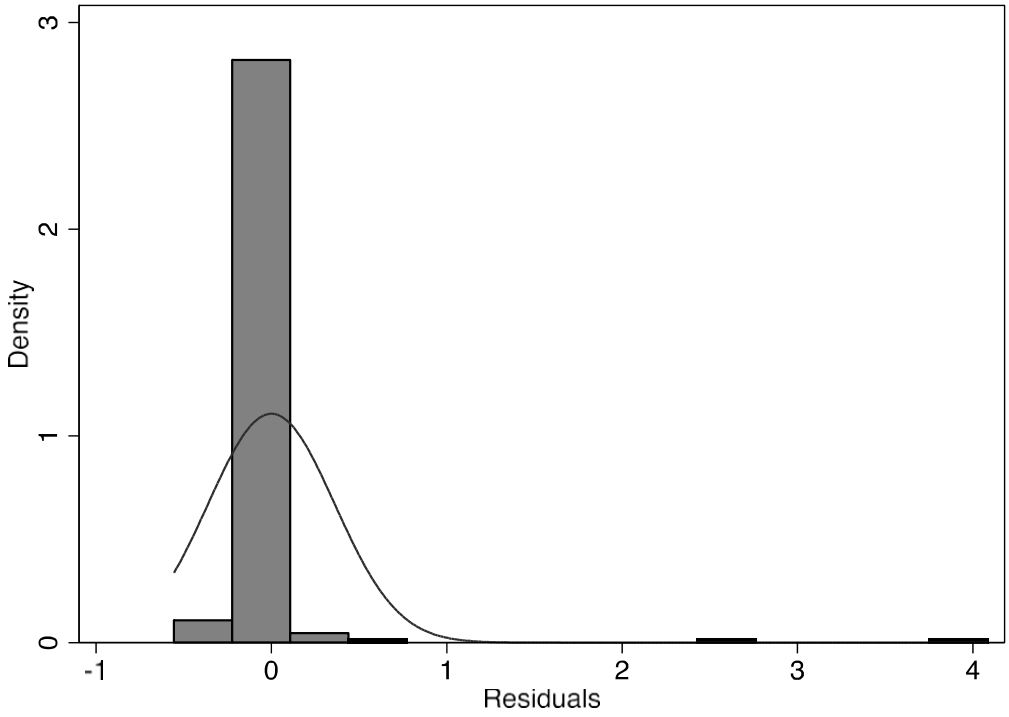
2.1 Checking assumptions.

Dependent variable is not normally distributed.

2.1.1 Checking for normality of residuals

Shapiro-Wilk W test for normal data

Variable	Obs	W	V	z	Prob>z
res1	196	0.21794	114.639	10.899	0.00000

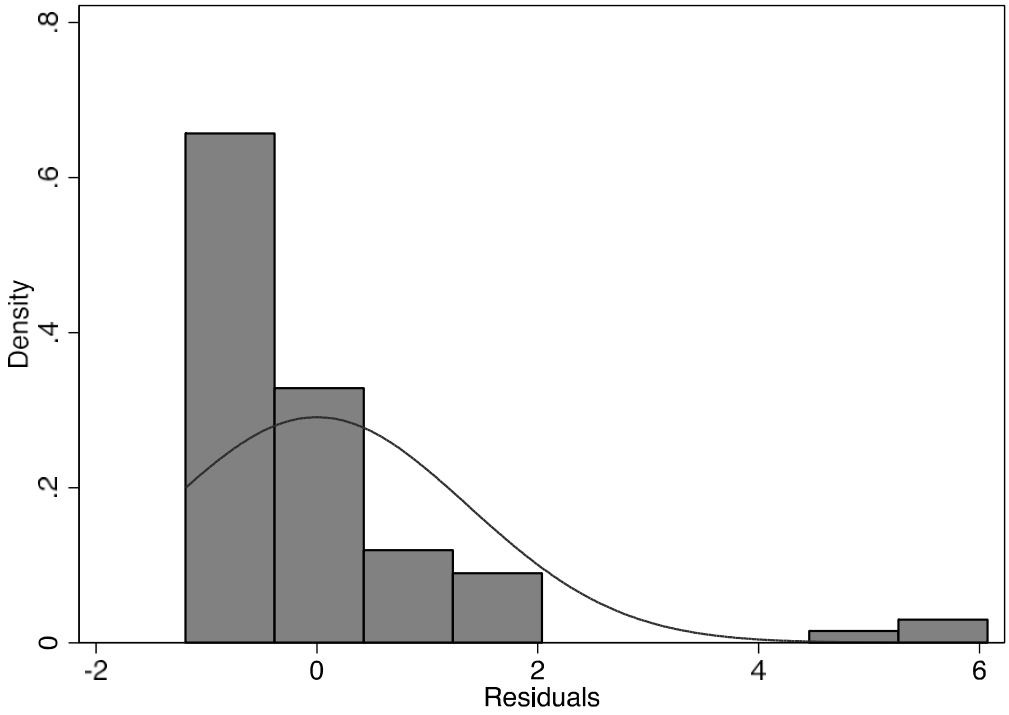


3. Linear regression model 2: Log-transformed (natural log) Infant IFN-γ (IU/ml) with outliers removed and new HHC

3.1 Checking for normality of variables of this model

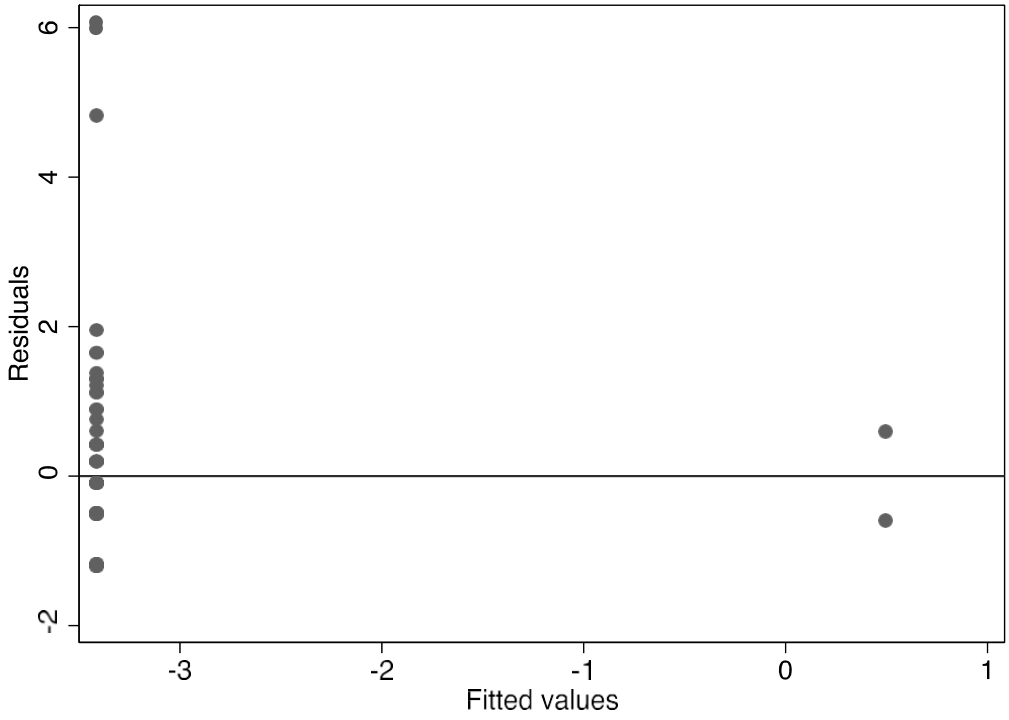
Shapiro-Wilk W test for normal data

Variable	Obs	W	V	z	Prob>z
res3	83	0.75137	17.590	6.296	0.00000



Still not normally distributed

3.2 Checking for homogeneity



No further regression diagnostics run

```
      Source |       SS       df       MS              Number of obs =      81
-------------+------------------------------           F(  1,    79) =   31.85
       Model |  32.1656679     1  32.1656679           Prob > F      =  0.0000
    Residual |   79.785432    79  1.00994218           R-squared     =  0.2873
-------------+------------------------------           Adj R-squared =  0.2783
       Total |    111.9511    80  1.39938875           Root MSE      =   1.005

------------------------------------------------------------------------------
BabyQFTt~2_ln |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
--------------+---------------------------------------------------------------
_IInfNewHHC_2 |   4.060787   .7195521     5.64   0.000     2.628555     5.49302
        _cons |  -3.567506   .1130667   -31.55   0.000     -3.79256   -3.342452
------------------------------------------------------------------------------
```

The linear regression established that a new household TB contact could statistically significantly predict the infant QFT IFN- γ value (IU/ml) with the F test (1,79) = 31.85, p=0.0000). A new household contact accounted for 28.73% (R^2=0.2873) of the explained variability in infant QFT IFN- γ value (IU/ml).
The linear regression equation is: predicted natural log Infant QFT IFN- γ = -3.57 + 4.06*Infant new HHC.
Back transforming the dependent variable: ($e^{4.06}$ -1)*100 = (57.9-1)*100 = 56.9*100 = 569% OR 57.9 fold

Infants who had a new household contact have QFT IFN- γ values (IU/ml) 58 fold higher than those infants without a new household contact, on average This was statistically significant at all levels (p<0.0001).

Appendix B: Letter of approval from UCT Research Ethics Committee

UNIVERSITY OF CAPE TOWN
Faculty of Health Science■
Human Research Ethics Committee

Room ESJ-48 Old Main Bulldlng
Groottl SChuur Hoaplt■I
ObN rv■tory 7925
Telephone (021] 406 6492
!mall: sumayah.arlc:f'dle□@uct.ac.za
Web■lte : www.health.yct.ac.zaffhs/research/humaoetblcsiforms

08 September 2017

HREC RI!F:659/2017

Prof M Hatherlll
Department of Pathology
SATVI-Room S2.01
IDM-FHS

Dear Prof Hatherill

PROJECT TITLE: THE BURDEN OF PERINATAL TUBERCULOSIS IN HIV INFECTED MOTHERS AND THI!IR INFANTS (Masters-candidate-Dr K Downing) sub-study llnked to 013/2012

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee (HREC) for review.

It Is a pleasure to Inform you that the HREC has **formally approved** the above-mentioned study.

Approval la granted for one year untll the 30 September 2018.

Please submit a progress form, using the ·standardised Annual Report Form If the study continues beyond the approval period. Please submit a Standard Closure form if the study Is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethlcs/forms)

We *acknowledge that* the *•tudent: ..or K Downing will also* be *Involved In this study.*

Please quote the HREC REF In all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responslbllity of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **J!IIIII** obtain appropriate Institutional approval, where necessary, before the research may occur.

Yours sincerely

re u '>

PROFESSOR M &LOCKMAN
CHAIRPERSON. FHSHUMAN RESEARCH ETHICS COMMnTI!E

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

This serves to confirm that the University of cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug In patients, based on the Mecllcal Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Cllnical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH 2006), based on the Association of the British Pharmaceutical Industry Guldellnes (ABPI),and Declaration of Helslnkl (2013) guldellnes.

The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part SO, 56 and 312.

Appendix C: Instructions for authors for PLoS ONE Journal

Submission Guidelines

Related information for authors

- Submission system
- Journal scope and publication criteria
- Getting started guide
- Guidelines for revisions
- Publication fees
- Chinese translation of PLOS policies: PLOS编辑与出版规定

Style and Format

File format	Manuscript files can be in the following formats: DOC, DOCX, or RTF. Microsoft Word documents should not be locked or protected. LaTeX manuscripts must be submitted as PDFs. Read the LaTeX guidelines.
Length	Manuscripts can be any length. There are no restrictions on word count, number of figures, or amount of supporting information. We encourage you to present and discuss your findings concisely.
Font	Use a standard font size and any standard font, except for the font named “Symbol”. To add symbols to the manuscript, use the Insert → Symbol function in your word processor or paste in the appropriate Unicode character.
Headings	Limit manuscript sections and sub-sections to 3 heading levels. Make sure heading levels are clearly indicated in the manuscript text.
Layout and spacing	Manuscript text should be double-spaced. Do not format text in multiple columns.
Page and line numbers	Include page numbers and line numbers in the manuscript file. Use continuous line numbers (do not restart the numbering on each page).
Footnotes	Footnotes are not permitted. If your manuscript contains footnotes, move the information into the main text or the reference list, depending on the content.
Language	Manuscripts must be submitted in English. You may submit translations of the manuscript or abstract as supporting information. Read the supporting information guidelines.
Abbreviations	Define abbreviations upon first appearance in the text. Do not use non-standard abbreviations unless they appear at least three times in the text. Keep abbreviations to a minimum.
Reference style	PLOS uses “Vancouver” style, as outlined in the ICMJE sample references. See reference formatting examples and additional instructions below.
Equations	We recommend using MathType for display and inline equations, as it will provide the most reliable outcome. If this is not possible, Equation Editor or Microsoft's Insert→Equation function is acceptable.

Avoid using MathType, Equation Editor, or the Insert→Equation function to insert single variables (e.g., “$a^2 + b^2 = c^2$”), Greek or other symbols (e.g., β, Δ, or ′ [prime]), or mathematical operators (e.g., x, ≥, or ±) in running text. Wherever possible, insert single symbols as normal text with the correct Unicode (hex) values.

Do not use MathType, Equation Editor, or the Insert→Equation function for only a portion of an equation. Rather, ensure that the entire equation is included. Equations should not contain a mix of different equation tools. Avoid “hybrid” inline or display equations, in which part is text and part is MathType, or part is MathType and part is Equation Editor.

Nomenclature

Use correct and established nomenclature wherever possible.

Units of measurement	Use SI units. If you do not use these exclusively, provide the SI value in parentheses after each value. Read more about SI units.
Drugs	Provide the Recommended International Non-Proprietary Name (rINN).
Species names	Write in italics (e.g., *Homo sapiens*). Write out in full the genus and species, both in the title of the manuscript and at the first mention of an organism in a paper. After first mention, the first letter of the genus name followed by the full species name may be used (e.g., *H. sapiens*).
Genes, mutations, genotypes, and alleles	Write in italics. Use the recommended name by consulting the appropriate genetic nomenclature database (e.g., HUGO for human genes). It is sometimes advisable to indicate the synonyms for the gene the first time it appears in the text. Gene prefixes such as those used for oncogenes or cellular localization should be shown in roman typeface (e.g., v-fes, c-MYC).
Allergens	The systematic allergen nomenclature of the World Health Organization/International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee should be used for manuscripts that include the description or use of allergenic proteins. For manuscripts describing new allergens, the systematic name of the allergen should be approved by the WHO/IUIS Allergen Nomenclature Sub-Committee prior to manuscript publication. Examples of the systematic allergen nomenclature can be found at the WHO/IUIS Allergen Nomenclature site.

Copyediting manuscripts

Prior to submission, authors who believe their manuscripts would benefit from professional editing are encouraged to use language-editing and copyediting services. Obtaining this service is the responsibility of the author, and should be done before initial submission. These services can be found on the web using search terms like “scientific editing service” or “manuscript editing service.”

Submissions are not copyedited before publication.

Submissions that do not meet the *PLOS ONE* publication criterion for language standards may be rejected.

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Manuscripts should be organized as follows. Instructions for each element appear below the list.

Beginning section	*The following elements are required, in order:* ! Title page: List title, authors, and affiliations as first page of manuscript ! Abstract ! Introduction
Middle section	*The following elements can be renamed as needed and presented in any order:* ! Materials and Methods ! Results ! Discussion ! Conclusions (optional)

Ending section	*The following elements are required, in order:* ! Acknowledgments ! References ! Supporting information captions (if applicable)
Other elements	! Figure captions are inserted immediately after the first paragraph in which the figure is cited. Figure files are uploaded separately. ! Tables are inserted immediately after the first paragraph in which they are cited. ! Supporting information files are uploaded separately.

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The compiled submission PDF includes low-resolution preview images of the figures after the reference list. The function of these previews is to allow you to download the entire submission as quickly as possible. Click the link at the top of each preview page to download a high-resolution version of each figure. Links to download Supporting Information files are also available after the reference list.

Parts of a Submission

Title

Include a full title and a short title for the manuscript.

Title	Length	Guidelines	Examples
Full title	250 characters	Specific, descriptive, concise, and comprehensible to readers outside the field	Impact of cigarette smoke exposure on innate immunity: A *Caenorhabditis elegans* model Solar drinking water disinfection (SODIS) to reduce childhood diarrhoea in rural Bolivia: A cluster-randomized, controlled trial
Short title	100 characters	State the topic of the study	Cigarette smoke exposure and innate immunity SODIS and childhood diarrhoea

Titles should be written in sentence case (only the first word of the text, proper nouns, and genus names are capitalized). Avoid specialist abbreviations if possible. For clinical trials, systematic reviews, or meta-analyses, the subtitle should include the study design.

Author list

Authorship requirements

All authors must meet the criteria for authorship as outlined in the authorship policy. Those who contributed to the work but do not meet the criteria for authorship can be mentioned in the Acknowledgments. Read more about Acknowledgments.

The corresponding author must provide an ORCID iD at the time of submission by entering it in the user profile in the submission system. Read more about ORCID.

Author names and affiliations

Enter author names on the title page of the manuscript and in the online submission system.

On the title page, write author names in the following order:

- First name (or initials, if used)
- Middle name (or initials, if used)
- Last name (surname, family name)

Each author on the list must have an affiliation. The affiliation includes department, university, or organizational affiliation and its location, including city, state/province (if applicable), and country. Authors have the option to include a current address in addition to the address of their affiliation at the time of the study. The current address should be listed in the byline and clearly labeled “current address.” At a minimum, the address must include the author’s current institution, city, and country.

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Corresponding author

The submitting author is automatically designated as the corresponding author in the submission system. The corresponding author is the primary contact for the journal office and the only author able to view or change the manuscript while it is under editorial consideration.

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- ○ acting as a reviewer
- ○ considering whether to submit
- ○ preparing my submission
- ○ Other

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If a manuscript is submitted on behalf of a consortium or group, include the consortium or group name in the author list, and provide the full list of consortium or group members in the Acknowledgments section. The consortium or group name should be listed in the manuscript file only, and not included in the online submission form. Please be aware that as of October 2016, the National Library of Medicine’s (NLM) policy has changed and PubMed will only index individuals and the names of consortia or group authors listed in the author byline itself. Individual consortium or group author members need to be listed in the author byline in order to be indexed, and if included in the byline, must qualify for authorship according to our criteria.

$ Read about the group authorship policy.

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- Describe any prior interactions with PLOS regarding the submitted manuscript
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- Explain how the study was done, including any model organisms used, without methodological detail
- Summarize the most important results and their significance
- Not exceed 300 words

Abstracts should not include:

- Citations
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Introduction

The introduction should:

- Provide background that puts the manuscript into context and allows readers outside the field to understand the purpose and significance of the study
- Define the problem addressed and why it is important
- Include a brief review of the key literature
- Note any relevant controversies or disagreements in the field
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Materials and Methods

The Materials and Methods section should provide enough detail to allow suitably skilled investigators to fully replicate your study. Specific information and/or protocols for new methods should be included in detail. If materials, methods, and protocols are well established, authors may cite articles where those protocols are described in detail, but the submission should include sufficient information to be understood independent of these references.

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Methods sections describing research using human or animal subjects and/or tissue or field sampling must include required ethics statements. See the reporting guidelines for human research, clinical trials, animal research, and observational and field studies for more information.

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PLOS journals require authors to make all data underlying the findings described in their manuscript fully available without restriction, with rare exception.

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For more information on how best to provide data, read our policy on data availability. PLOS does not accept references to “data not shown.”

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Methods sections describing research using cell lines must state the origin of the cell lines used. See the reporting guidelines for cell line research for more information.

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To enhance the reproducibility of your results, we recommend and encourage you to deposit laboratory protocols in protocols.io, where protocols can be assigned their own persistent digital object identifiers (DOIs).

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Methods sections of manuscripts adding new taxon names to the literature must follow the reporting guidelines below for a new zoological taxon, botanical taxon, or fungal taxon.

Results, Discussion, Conclusions

These sections may all be separate, or may be combined to create a mixed Results/Discussion section (commonly labeled “Results and Discussion”) or a mixed Discussion/Conclusions section (commonly labeled “Discussion”). These sections may be further divided into subsections, each with a concise subheading, as appropriate. These sections have no word limit, but the language should be clear and concise.

Together, these sections should describe the results of the experiments, the interpretation of these results, and the conclusions that can be drawn.

Authors should explain how the results relate to the hypothesis presented as the basis of the study and provide a succinct explanation of the implications of the findings, particularly in relation to previous related studies and potential future directions for research.

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Acknowledgments

Those who contributed to the work but do not meet our authorship criteria should be listed in the Acknowledgments with a description of the contribution.

Authors are responsible for ensuring that anyone named in the Publication Info section agrees to be named.

Do not include funding sources in the Publication Info or anywhere else in the manuscript file. Funding information should only be entered in the financial disclosure section of the submission system.

References

Any and all available works can be cited in the reference list. Acceptable sources include:

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- Unavailable and unpublished work, including manuscripts that have been submitted but not yet accepted (e.g., "unpublished work," "data not shown"). Instead, include those data as supplementary material or deposit the data in a publicly available database.
- Personal communications (these should be supported by a letter from the relevant authors but not included in the reference list)

References are listed at the end of the manuscript and numbered in the order that they appear in the text. In the text, cite the reference number in square brackets (e.g., "We used the techniques developed by our colleagues [19] to analyze the data"). PLOS uses the numbered citation (citation-sequence) method and first six authors, et al.

Do not include citations in abstracts or author summaries.

Make sure the parts of the manuscript are in the correct order *before* ordering the citations.

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Because all references will be linked electronically as much as possible to the papers they cite, proper formatting of the references is crucial.

PLOS uses the reference style outlined by the International Committee of Medical Journal Editors (ICMJE), also referred to as the "Vancouver" style. Example formats are listed below. Additional examples are in the ICMJE sample references.

Areference management tool, EndNote, offers a current style file that can assist you with the formatting of your references. If you have problems with any reference management program, please contact the source company's technical support.

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Source	Format
Published articles	Hou WR, Hou YL, Wu GF, Song Y, Su XL, Sun B, et al. cDNA, genomic sequence cloning and overexpression of ribosomal protein gene L9 (rpL9) of the giant panda (*Ailuropoda melanoleuca*). Genet Mol Res. 2011;10: 1576-1588. Devaraju P, Gulati R, Antony PT, Mithun CB, Negi VS. Susceptibility to SLE in South Indian Tamils may be influenced by genetic selection pressure on TLR2 and TLR9 genes. Mol Immunol. 2014 Nov 22. pii: S0161-5890(14)00313-7. doi: 10.1016/j.molimm.2014.11.005. *Note: A DOI number for the full-text article is acceptable as an alternative to or in addition to traditional volume and page numbers. When providing a DOI, adhere to the format in the example above with both the label and full DOI included at the end of the reference (doi: 10.1016/j.molimm.2014.11.005). Do not provide a shortened DOI or the URL.*
Accepted, unpublished articles	Same as published articles, but substitute "Forthcoming" for page numbers or DOI.
Online articles	Huynen MMTE, Martens P, Hilderlink HBM. The health impacts of globalisation: a conceptual framework. Global Health. 2005;1: 14. Available from: http://www.globalizationandhealth.com/content/1/1/14
Books	Bates B. Bargaining for life: A social history of tuberculosis. 1st ed. Philadelphia: University of Pennsylvania Press; 1992.

Book chapters	Hansen B. New York City epidemics and history for the public. In: Harden VA, Risse GB, editors. AIDS and the historian. Bethesda: National Institutes of Health; 1991. pp. 21-28.
Deposited articles (preprints, e-prints, or arXiv)	Krick T, Shub DA, Verstraete N, Ferreiro DU, Alonso LG, Shub M, et al. Amino acid metabolism conflicts with protein diversity; 1991. Preprint. Available from: arXiv:1403.3301v1. Cited 17 March 2014.
Published media (print or online newspapers and magazine articles)	Fountain H. For Already Vulnerable Penguins, Study Finds Climate Change Is Another Danger. The New York Times. 29 Jan 2014. Available from: http://www.nytimes.com/2014/01/30/science/earth/climate-change-taking-toll-on-penguins-study-finds.html Cited 17 March 2014.
New media (blogs, web sites, or other written works)	Allen L. Announcing PLOS Blogs. 2010 Sep 1 [cited 17 March 2014]. In: PLOS Blogs [Internet]. San Francisco: PLOS 2006 - . [about 2 screens]. Available from: http://blogs.plos.org/plos/2010/09/announcing-plos-blogs/.
Masters' theses or doctoral dissertations	Wells A. Exploring the development of the independent, electronic, scholarly journal. M.Sc. Thesis, The University of Sheffield. 1999. Available from: http://cumincad.scix.net/cgi-bin/works/Show?2e09
Databases and repositories (Figshare, arXiv)	Roberts SB. QPX Genome Browser Feature Tracks; 2013 [cited 2013 Oct 5]. Database: figshare [Internet]. Available from: http://figshare.com/articles/QPX_Genome_Browser_Feature_Tracks/701214
Multimedia (videos, movies, or TV shows)	Hitchcock A, producer and director. Rear Window [Film]; 1954. Los Angeles: MGM.

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Example caption

S1 Text. Title is strongly recommended. Legend is optional.

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$ See our list of recommended repositories.

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All appropriate data sets, images, and information should be deposited in an appropriate public repository. See our list of recommended repositories.

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- Ensembl
- Entrez Gene
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- Online Mendelian Inheritance in Man (OMIM)
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This information should not be in your manuscript file; you will provide it via our submission system.

This information will be published with the final manuscript, if accepted, so please make sure that this is accurate and as detailed as possible. You should not include this information in your manuscript file, but it is important to gather it prior to submission, because your financial disclosure statement cannot be changed after initial submission**.**

Your statement should include relevant grant numbers and the URL of any funder's web site. Please also state whether any individuals employed or contracted by the funders (other than the named authors) played any role in: study design, data collection and analysis, decision to publish, or preparation of the manuscript. If so, please name the individual and describe their role.

$ Read our policy on disclosure of funding sources.

Competing Interests

This information should not be in your manuscript file; you will provide it via our submission system.

All potential competing interests must be declared in full. If the submission is related to any patents, patent applications, or products in development or for market, these details, including patent numbers and titles, must be disclosed in full.

$ Read our policy on competing interests.

Manuscripts disputing published work

For manuscripts disputing previously published work, it is *PLOS ONE* policy to invite a signed review by the disputed author during the peer review process. This procedure is aimed at ensuring a thorough, transparent, and productive review process.

If the disputed author chooses to submit a review, it must be returned in a timely fashion and contain a full declaration of all competing interests. The Academic Editor will consider any such reviews in light of the competing interest.

Authors submitting manuscripts disputing previous work should explain the relationship between the manuscripts in their cover letter, and will be required to confirm that they accept the conditions of this review policy before the manuscript is considered further.

Related manuscripts

Upon submission, authors must confirm that the manuscript, or any related manuscript, is not currently under consideration or accepted elsewhere. If related work has been submitted to *PLOS ONE* or elsewhere, authors must include a copy with the submitted article. Reviewers will be asked to comment on the overlap between related submissions.

We strongly discourage the unnecessary division of related work into separate manuscripts, and we will not consider manuscripts that are divided into “parts.” Each submission to *PLOS ONE* must be written as an independent unit and should not rely on any work that has not already been accepted for publication. If related manuscripts are submitted to *PLOS ONE*, the authors may be advised to combine them into a single manuscript at the editor's discretion.

PLOS does support authors who wish to share their work early and receive feedback before formal peer review. Deposition of manuscripts with preprint servers does not impact consideration of the manuscript at any PLOS journal.

Authors choosing bioRxiv may now concurrently submit directly to select PLOS journals through bioRxiv’s direct transfer to journal service.

$ Read our policies on related manuscripts and preprint servers.

Guidelines for Specific Study Types

Human subjects research

All research involving human participants must have been approved by the authors’ Institutional Review Board (IRB) or by equivalent ethics committee(s), and must have been conducted according to the principles expressed in the Declaration of Helsinki. Authors should be able to submit, upon request, a statement from the IRB or ethics committee indicating approval of the research. We reserve the right to reject work that we believe has not been conducted to a high ethical standard, even when formal approval has been obtained.

Subjects must have been properly instructed and have indicated that they consent to participate by signing the appropriate informed consent paperwork. Authors may be asked to submit a blank, sample copy of a subject consent form. If consent was verbal instead of written, or if consent could not be obtained, the authors must explain the reason in the manuscript, and the use of verbal consent or the lack of consent must have been approved by the IRB or ethics committee.

All efforts should be made to protect patient privacy and anonymity. Identifying information, including photos, should not be included in the manuscript unless the information is crucial and the individual has provided written consent by completing the Consent Form for Publication in a PLOS Journal (PDF). Download additional translations of the form from the Downloads and Translations page. More information about patient privacy, anonymity, and informed consent can be found in the International Committee of Medical Journal Editors (ICMJE) Privacy and Confidentiality guidelines.

Manuscripts should conform to the following reporting guidelines:

- Studies of diagnostic accuracy: STARD
- Observational studies: STROBE
- Microarray experiments: MIAME
- Other types of health-related research: Consult the EQUATOR web site for appropriate reporting guidelines

Methods sections of papers on research using human subjects or samples must include ethics statements that specify:

- **The name of the approving institutional review board or equivalent committee(s)**. If approval was not obtained, the authors must provide a detailed statement explaining why it was not needed
- **Whether informed consent was written or oral**. If informed consent was oral, it must be stated in the manuscript:
  - Why written consent could not be obtained
  - That the Institutional Review Board (IRB) approved use of oral consent
  - How oral consent was documented

For studies involving humans categorized by race/ethnicity, age, disease/disabilities, religion, sex/gender, sexual orientation, or other socially constructed groupings, authors should:

- Explicitly describe their methods of categorizing human populations
- Define categories in as much detail as the study protocol allows
- Justify their choices of definitions and categories, including for example whether any rules of human categorization were required by their funding agency
- Explain whether (and if so, how) they controlled for confounding variables such as socioeconomic status, nutrition, environmental exposures, or similar factors in their analysis

In addition, outmoded terms and potentially stigmatizing labels should be changed to more current, acceptable terminology. Examples: “Caucasian” should be changed to “white” or “of [Western] European descent” (as appropriate); “cancer victims” should be changed to “patients with cancer.”

For papers that include identifying, or potentially identifying, information, authors must download the Consent Form for Publication in a PLOS Journal, which the individual, parent, or guardian must sign once they have read the paper and been informed about the terms of PLOS open-access license. The signed consent form should not be submitted with the manuscript, but authors should securely file it in the individual's case notes and the methods section of the manuscript should explicitly state that consent authorization for publication is on file, using wording like:

The individual in this manuscript has given written informed consent (as outlined in PLOS consent form) to publish these case details.

For more information about *PLOS ONE* policies regarding human subjects research, see the Publication Criteria and Editorial Policies.

Clinical trials

Clinical trials are subject to all policies regarding human research. *PLOS ONE* follows the World Health Organization's (WHO) definition of a clinical trial:

> *A clinical trial is any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes [...] Interventions include but are not restricted to drugs, cells and other biological products, surgical procedures, radiologic procedures, devices, behavioural treatments, process-of-care changes, preventive care, etc.*

All clinical trials must be registered in one of the publicly-accessible registries approved by the WHO or ICMJE (International Committee of Medical Journal Editors). Authors must provide the trial registration number. Prior disclosure of results on a clinical trial registry site will not affect consideration for publication. We reserve the right to inform authors' institutions or ethics committees, and to reject the manuscript, if we become aware of unregistered trials.

PLOS ONE supports prospective trial registration (i.e. before participant recruitment has begun) as recommended by the ICMJE's clinical trial registration policy. **Where trials were not publicly registered before participant recruitment began**, authors must:

- Register all related clinical trials and confirm they have done so in the Methods section
- Explain in the Methods the reason for failing to register before participant recruitment

Clinical trials must be reported according to the relevant reporting guidelines, i.e. CONSORT for randomized controlled trials, TREND for non-randomized trials, and other specialized guidelines as appropriate. The intervention should be described according to the requirements of the TIDieR checklist and guide. Submissions must also include the study protocol as supporting information, which will be published with the manuscript if accepted.

Authors of manuscripts describing the results of clinical trials must adhere to the CONSORT reporting guidelines appropriate to their trial design, available on the CONSORT Statement web site. Before the paper can enter peer review, authors must:

- Provide the registry name and number in the methods section of the manuscript
- Provide a copy of the trial protocol as approved by the ethics committee and a completed CONSORT checklist as supporting information (which will be published alongside the paper, if accepted). This should be named S1 CONSORT Checklist.
- Include the CONSORT flow diagram as the manuscript's “Fig 1”

Any deviation from the trial protocol must be explained in the paper. Authors must explicitly discuss informed consent in their paper, and we reserve the right to ask for a copy of the patient consent form.

The methods section must include the name of the registry, the registry number, and the URL of your trial in the registry database for each location in which the trial is registered.

Animal research

All research involving vertebrates or cephalopods must have approval from the authors' Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee(s), and must have been conducted according to applicable national and international guidelines. Approval must be received prior to beginning research.

Manuscripts reporting animal research must state in the Methods section:

- The full name of the relevant ethics committee that approved the work, and the associated permit number(s).
- Where ethical approval is not required, the manuscript should include a clear statement of this and the reason why. Provide any relevant regulations under which the study is exempt from the requirement for approval.
- Relevant details of steps taken to ameliorate animal suffering.

Example ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Minnesota (Protocol Number: 27-2956). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Authors should always state the organism(s) studied in the Abstract. Where the study may be confused as pertaining to clinical research, authors should also state the animal model in the title.

To maximize reproducibility and potential for re-use of data, we encourage authors to follow the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines for all submissions describing laboratory-based animal research and to upload a completed ARRIVE Guidelines Checklist to be published as supporting information.

Non-human primates

Manuscripts describing research involving non-human primates must report details of husbandry and animal welfare in accordance with the recommendations of the Weatherall report, *The use of non-human primates in research* (PDF), including:

- Information about housing, feeding, and environmental enrichment.
- Steps taken to minimize suffering, including use of anesthesia and method of sacrifice, if appropriate.

Random source animals

Manuscripts describing studies that use random source (e.g. Class B dealer-sourced in the USA), shelter, or stray animals will be subject to additional scrutiny and may be rejected if sufficient ethical and scientific justification for the study design is lacking.

Unacceptable euthanasia methods and anesthetic agents

Manuscripts reporting use of a euthanasia method(s) classified as unacceptable by the American Veterinary Medical Association or use of an anesthesia method(s) that is widely prohibited (e.g., chloral hydrate, ether, chloroform) must include at the time of initial submission, scientific justification for use in the specific study design, as well as confirmation of approval for specific use from their animal research ethics committee. These manuscripts may be subject to additional ethics considerations prior to publication.

Humane endpoints

Manuscripts reporting studies in which death of a regulated animal (vertebrate, cephalopod) is a likely outcome or a planned experimental endpoint, must comprehensively report details of study design, rationale for the approach, and methodology, including consideration of humane endpoints. This applies to research that involves, for instance, assessment of survival, toxicity, longevity, terminal disease, or high rates of incidental mortality.

Definition of a humane endpoint

A humane endpoint is a predefined experimental endpoint at which animals are euthanized when they display early markers associated with death or poor prognosis of quality of life, or specific signs of severe suffering or distress. Humane endpoints are used as an alternative to allowing such conditions to continue or progress to death following the experimental intervention (“death as an endpoint”), or only euthanizing animals at the end of an experiment. Before a study begins, researchers define the practical observations or measurements that will be used during the study to recognize a humane endpoint, based on anticipated clinical, physiological, and behavioral signs. Please see the NC3Rs guidelines for more information. Additional discussion of humane endpoints can be found in this article: Nuno H. Franco, Margarida Correia-Neves, I. Anna S. Olsson (2012) How “Humane” Is Your Endpoint? — Refining the Science-Driven Approach for Termination of Animal Studies of Chronic Infection. PLoS Pathog 8(1): e1002399 doi.org/10.1371/journal.ppat.1002399.

Full details of humane endpoints use must be reported for a study to be reproducible and for the results to be accurately interpreted.

For studies in which death of an animal is an outcome or a planned experimental endpoint, authors should include the following information in the Methods section of the manuscript:

- The specific criteria (i.e. humane endpoints) used to determine when animals should be euthanized.
- The duration of the experiment.
- The numbers of animals used, euthanized, and found dead (if any); the cause of death for all animals.
- How frequently animal health and behavior were monitored.
- All animal welfare considerations taken, including efforts to minimize suffering and distress, use of analgesics or anaesthetics, or special housing conditions.

If humane endpoints were not used, the manuscript should report:

- A scientific justification for the study design, including the reasons why humane endpoints could not be used, and discussion of alternatives that were considered.
- Whether the institutional animal ethics committee specifically reviewed and approved the anticipated mortality in the study design.

Observational and field studies

Methods sections for submissions reporting on any type of field study must include ethics statements that specify:

- Permits and approvals obtained for the work, including the full name of the authority that approved the study; if none were required, authors should explain why
- Whether the land accessed is privately owned or protected
- Whether any protected species were sampled
- Full details of animal husbandry, experimentation, and care/welfare, where relevant

Paleontology and archaeology research

Manuscripts reporting paleontology and archaeology research must include descriptions of methods and specimens in sufficient detail to allow the work to be reproduced. Data sets supporting statistical and phylogenetic analyses should be provided, preferably in a format that allows easy re-use. Read the policy.

Specimen numbers and complete repository information, including museum name and geographic location, are required for publication. Locality information should be provided in the manuscript as legally allowable, or a statement should be included giving details of the availability of such information to qualified researchers.

If permits were required for any aspect of the work, details should be given of all permits that were obtained, including the full name of the issuing authority. This should be accompanied by the following statement:

All necessary permits were obtained for the described study, which complied with all relevant regulations.

If no permits were required, please include the following statement:

No permits were required for the described study, which complied with all relevant regulations.

Manuscripts describing paleontology and archaeology research are subject to the following policies:

- **Sharing of data and materials.** Any specimen that is erected as a new species, described, or figured must be deposited in an accessible, permanent repository (i.e., public museum or similar institution). If study conclusions depend on specimens that do not fit these criteria, the article will be rejected under *PLOS ONE*'s data availability criterion.
- **Ethics.** *PLOS ONE* will not publish research on specimens that were obtained without necessary permission or were illegally exported.

Systematic reviews and meta-analyses

A systematic review paper, as defined by The Cochrane Collaboration, is a review of a clearly formulated question that uses explicit, systematic methods to identify, select, and critically appraise relevant research, and to collect and analyze data from the studies that are included in the review. These reviews differ substantially from narrative-based reviews or synthesis articles. Statistical methods (meta-analysis) may or may not be used to analyze and summarize the results of the included studies.

Reports of systematic reviews and meta-analyses must include a completed PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) checklist and flow diagram to accompany the main text. Blank templates are available here:

- Checklist: PDF or Word document

- Flow diagram: PDF or Word document

Authors must also state in their “Methods” section whether a protocol exists for their systematic review, and if so, provide a copy of the protocol as supporting information and provide the registry number in the abstract.

If your article is a systematic review or a meta-analysis you should:

- State this in your cover letter
- Select “Research Article” as your article type when submitting
- Include the PRISMA flow diagram as Fig 1 (required where applicable)
- Include the PRISMA checklist as supporting information

Meta-analysis of genetic association studies

Manuscripts reporting a meta-analysis of genetic association studies must report results of value to the field and should be reported according to the guidelines presented in *Systematic Reviews of Genetic Association Studies* by Sagoo *et al.*

On submission, authors will be asked to justify the rationale for the meta-analysis and how it contributes to the base of scientific knowledge in the light of previously published results. Authors will also be asked to complete a checklist (DOCX) outlining information about the justification for the study and the methodology employed. Meta-analyses that replicate published studies will be rejected if the authors do not provide adequate justification.

Personal data from third-party sources

For all studies using personal data from internet-based and other third-party sources (e.g., social media, blogs, other internet sources, mobile phone companies), data must be collected and used according to company/website Terms and Conditions, with appropriate permissions. All data sources must be acknowledged clearly in the Materials and Methods section.

$ Read our policy on data availability.

In the Ethics Statement, authors should declare any potential risks to individuals or individual privacy, or affirm that in their assessment, the study posed no such risks. In addition, the following Ethics and Data Protection requirements must be met.

For interventional studies, which impact participants’ experiences or data, the study design must have been prospectively approved by an Ethics Committee, and informed consent is required. The Ethics Committee may waive the requirement for approval and/or consent.

For observational studies in which personal experiences and accounts are not manipulated, consultation with an Ethics or Data Protection Committee is recommended. Additional requirements apply in the following circumstances:

- If information used could threaten personal privacy or damage the reputation of individuals whose data are used, an Ethics Committee should be consulted and informed consent obtained or specifically addressed.
- If authors accessed any personal identifying information, an Ethics or Data Protection Committee should oversee data anonymization. If data were anonymized and/or aggregated before access and analysis, informed consent is generally not required.

Note that Terms of Use contracts do not qualify as informed consent, even if they address the use of personal data for research.

$ See our reporting guidelines for human subjects research.

Cell lines

Authors reporting research using cell lines should state when and where they obtained the cells, giving the date and the name of the researcher, cell line repository, or commercial source (company) who provided the cells, as appropriate.

Authors must also include the following information for each cell line:

For *de novo* (new) cell lines, including those given to the researchers as a gift, authors must follow our

policies for human subjects research or animal research, as appropriate. The ethics statement must include:

- Details of institutional review board or ethics committee approval; AND
- For human cells, confirmation of written informed consent from the donor, guardian, or next of kin

For established cell lines, the Methods section should include:

- A reference to the published article that first described the cell line; AND/OR
- The cell line repository or company the cell line was obtained from, the catalogue number, and whether the cell line was obtained directly from the repository/company or from another laboratory

Authors should check established cell lines using the ICLAC Database of Cross-contaminated or Misidentified Cell Lines to confirm they are not misidentified or contaminated. Cell line authentication is recommended – e.g., by karyotyping, isozyme analysis, or short tandem repeats (STR) analysis – and may be required during peer review or after publication.

Blots and gels

Manuscripts reporting results from blots (including Western blots) and electrophoretic gels should follow these guidelines:

- In accordance with our policy on image manipulation, the image should not be adjusted in any way that could affect the scientific information displayed, e.g. by modifying the background or contrast.
- All blots and gels that support results reported in the manuscript should be provided.
- Original uncropped and unadjusted blots and gels, including molecular size markers, should be provided in either the figures or the supplementary files.
- Lanes should not be overcropped around the bands; the image should show most or all of the blot or gel. Any non-specific bands should be shown and an explanation of their nature should be given.
- The image should include all relevant controls, and controls should be run on the same blot or gel as the samples.
- A figure panel should not include composite images of bands originating from different blots or gels. If the figure shows non-adjacent bands from the same blot or gel, this should be clearly denoted by vertical black lines and the figure legend should provide details of how the figure was made.

Antibodies

Manuscripts reporting experiments using antibodies should include the following information:

- The name of each antibody, a description of whether it is monoclonal or polyclonal, and the host species.
- The commercial supplier or source laboratory.
- The catalogue or clone number and, if known, the batch number.
- The antigen(s) used to raise the antibody.
- For established antibodies, a stable public identifier from the Antibody Registry.

The manuscript should also report the following experimental details:

- The final antibody concentration or dilution.
- A reference to the validation study if the antibody was previously validated. If not, provide details of how the authors validated the antibody for the applications and species used.

We encourage authors to consider adding information on new validations to a publicly available database such as Antibodypedia or CiteAb.

Small and macromolecule crystal data

Manuscripts reporting new and unpublished three-dimensional structures must include sufficient supporting data and detailed descriptions of the methodologies used to allow the reproduction and validation of the structures. All novel structures must have been deposited in a community endorsed database prior to submission (please see our list of recommended repositories).

Small molecule single crystal data

Authors reporting X-Ray crystallographic structures of small organic, metal-organic, and inorganic molecules must deposit their data with the Cambridge Crystallographic Data Centre (CCDC), the Inorganic Crystal Structure Database (ICSD), or similar community databases providing a recognized validation functionality. Authors are also required to include the relevant structure reference numbers within the main text (e.g. the CCDC ID number), as well as the crystallographic information files (.cif format) as Supplementary Information, along with the checkCIF validation reports that can be obtained via the International Union of Crystallography (IUCr).

Macromolecular structures

Authors reporting novel macromolecular structures must have deposited their data prior to submission with the Worldwide Protein Data Bank (wwPDB), the Biological Magnetic Resonance Data Bank (BMRB), the Electron Microscopy Data Bank (EMDB), or other community databases providing a recognized validation

functionality. Authors must include the structure reference numbers within the main text and submit as Supplementary Information the official validation reports from these databases.

Methods, software, databases, and tools

PLOS ONE will consider submissions that present new methods, software, or databases as the primary focus of the manuscript if they meet the following criteria:

Utility
The tool must be of use to the community and must present a proven advantage over existing alternatives, where applicable. Recapitulation of existing methods, software, or databases is not useful and will not be considered for publication. Combining data and/or functionalities from other sources may be acceptable, but simpler instances (i.e. presenting a subset of an already existing database) may not be considered. For software, databases, and online tools, the long-term utility should also be discussed, as relevant. This discussion may include maintenance, the potential for future growth, and the stability of the hosting, as applicable.

Validation
Submissions presenting methods, software, databases, or tools must demonstrate that the new tool achieves its intended purpose. If similar options already exist, the submitted manuscript must demonstrate that the new tool is an improvement over existing options in some way. This requirement may be met by including a proof-of-principle experiment or analysis; if this is not possible, a discussion of the possible applications and some preliminary analysis may be sufficient.

Availability
If the manuscript's primary purpose is the description of new software or a new software package, this software must be open source, deposited in an appropriate archive, and conform to the Open Source Definition. If the manuscript mainly describes a databse, this database must be open-access and hosted somewhere publicly accessible, and any software used to generate a database should also be open source. If relevant, databases should be open for appropriate deposition of additional data. Dependency on commercial software such as Mathematica and MATLAB does not preclude a paper from consideration, although complete open source solutions are preferred. In these cases, authors should provide a direct link to the deposited software or the database hosting site from within the paper.

Software submissions

Manuscripts whose primary purpose is the description of new software must provide full details of the algorithms designed. Describe any dependencies on commercial products or operating system. Include details of the supplied test data and explain how to install and run the software. A brief description of enhancements made in the major releases of the software may also be given. Authors should provide a direct link to the deposited software from within the paper.

Database submissions

For descriptions of databases, provide details about how the data were curated, as well as plans for long-term database maintenance, growth, and stability. Authors should provide a direct link to the database hosting site from within the paper.

$ Read the PLOS policy on sharing materials and software.

New taxon names

Zoological names

When publishing papers that describe a new zoological taxon name, PLOS aims to comply with the requirements of the International Commission on Zoological Nomenclature (ICZN). Effective 1 January 2012, the ICZN considers an online-only publication to be legitimate if it meets the criteria of archiving and is registered in ZooBank, the ICZN's official registry.

For proper registration of a new zoological taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

Anochetus boltoni *Fisher* ***sp. nov.*** *urn:lsid:zoobank.org:act:B6C072CF-1CA6-40C7-8396-534E91EF7FBB*

You will need to contact Zoobank to obtain a GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper.

Please also insert the following text into the **Methods** section, in a sub-section to be called "Nomenclatural Acts":

The electronic edition of this article conforms to the requirements of the amended International Code of Zoological

Nomenclature, and hence the new names contained herein are available under that Code from the electronic edition of this article. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix “http://zoobank.org/”. The LSID for this publication is: urn:lsid:zoobank.org:pub: XXXXXXX. The electronic edition of this work was published in a journal with an ISSN, and has been archived and is available from the following digital repositories: PubMed Central, LOCKSS [author to insert any additional repositories].

All PLOS articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Botanical names

When publishing papers that describe a new botanical taxon, PLOS aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). The following guidelines for publication in an online-only journal have been agreed such that any scientific botanical name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature, and apply only to seed plants, ferns, and lycophytes.

Effective January 2012, the description or diagnosis of a new taxon can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.

Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

Additional information describing recent changes to the Code can be found here.

For proper registration of the new taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

> **Solanum aspersum** S.Knapp, sp. nov. [urn:lsid:ipni.org:names:77103633-1] Type: Colombia. Putumayo: vertiente oriental de la Cordillera, entre Sachamates y San Francisco de Sibundoy, 1600-1750 m, 30 Dec 1940, J. Cuatrecasas 11471 (holotype, COL; isotypes, F [F-1335119], US [US-1799731]).

Journal staff will contact IPNI to obtain the GUID (LSID) after your manuscript is accepted for publication, and this information will then be added to the manuscript during the production phase

In the **Methods** section, include a sub-section called “Nomenclature” using the following wording:

The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

In addition, new names contained in this work have been submitted to IPNI, from where they will be made available to the Global Names Index. The IPNI LSIDs can be resolved and the associated information viewed through any standard web browser by appending the LSID contained in this publication to the prefix http://ipni.org/. The online version of this work is archived and available from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (PubMed Central, LOCKSS etc)].

All PLOS articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Fungal names

When publishing papers that describe a new botanical taxon, PLOS aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). The following guidelines for publication in an online-only journal have been agreed such that any scientific botanical name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature.

Effective January 2012, the description or diagnosis of a new taxon can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.

Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

Additional information describing recent changes to the Code can be found here.

For proper registration of the new taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

> ***Hymenogaster huthii.*** Stielow et al. 2010, sp. nov. [urn:lsid:indexfungorum.org:names:518624]

You will need to contact either Mycobank or Index Fungorum to obtain the GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper. Effective January 2013, all papers describing new fungal species must reference the identifier issued by a recognized repository in the protologue in order to be considered effectively published.

In the **Methods** section, include a sub-section called “Nomenclature” using the following wording (this example is for taxon names submitted to MycoBank; please substitute appropriately if you have submitted to Index Fungorum):

> The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.
>
> In addition, new names contained in this work have been submitted to MycoBank from where they will be made available to the Global Names Index. The unique MycoBank number can be resolved and the associated information viewed through any standard web browser by appending the MycoBank number contained in this publication to the prefix http://www.mycobank.org/MB/. The online version of this work is archived and available from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (PubMed Central, LOCKSS etc)].

All PLOS articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Qualitative research

Qualitative research studies use non-quantitative methods to address a defined research question that may not be accessible by quantitative methods, such as people's interpretations, experiences, and perspectives. The analysis methods are explicit, systematic, and reproducible, but the results do not involve numerical values or use statistics. Examples of qualitative data sources include, but are not limited to, interviews, text documents, audio/video recordings, and free-form answers to questionnaires and surveys.

Qualitative research studies should be reported in accordance to the Consolidated criteria for reporting qualitative research (COREQ) checklist. Further reporting guidelines can be found in the Equator Network's Guidelines for reporting qualitative research.

Give Feedback

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