The association between the magnitude of T-cell interferon-gamma responses to *Mycobacterium tuberculosis* specific antigens and risk of progression to tuberculosis in household contacts tested with QuantiFERON-TB Gold In-Tube Assay.

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PhD Thesis

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I, Kwame Shanaube, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Kwame Shanaube

Structure of the Thesis

Chapter 1 is the introductory chapter of the thesis. It describes the natural history and epidemiology of tuberculosis and the role of cytokines. It also describes tools for diagnosing *Mycobacterium tuberculosis (Mtb)* infection with a particular focus on Interferon-gamma release assays (IGRAs) such as Quantiferon-TB Gold In-Tube (QFT) as an emerging alternative to the Tuberculin skin test (TST). This chapter also describes the aims and objectives as well as the conceptual framework of the study.

Chapter 2 provides a literature review of the studies related to the objectives of this thesis. In this chapter I collate and review the most relevant analytical studies on the association between the magnitude of T-cell Interferon-γ responses to *Mtb* specific antigens and risk of progression to disease. I critically review this evidence with regard to whether IGRAs are better than the TST at predicting individuals who have a high risk of progression to active TB and whether *Mtb* infected individuals with strongly elevated IFN-γ levels are most likely to progress to active disease. I also review studies examining concordance between IGRAs and TST results and the effect of HIV on IGRAs.

Chapter 3 describes the study methods. This chapter first describes the background study, (ZAMSTAR trial) within which the cohort study under discussion in this thesis (QFT cohort study) is nested. The rest of the chapter describes details of the QFT-GIT cohort study with regard to the following: sample size, study duration, study population, measurement tools, baseline and follow-up activities, data collection and statistical analyses.

In **Chapter 4** the results of four small studies examining the performance and operational characteristics of QFT-GIT in a field setting are presented. The first study addresses the sensitivity and test-retest reproducibility of QFT-GIT as well as the effect of CD4+ T-lymphocyte counts and delayed incubation of blood samples on IFN-γ responses. The second study addresses the effect of power outages during incubation on IFN-γ responses. The third study addresses the effect of increased storage temperature on IFN-γ responses. Lastly, the fourth study addresses the financial cost of diagnosing *Mtb* infection using QFT-GIT.

In **Chapter 5** the baseline results of the study are presented. These include a description of the socio-demographic and clinical characteristics of the study population, the prevalence of tuberculous infection as measured by QFT-GIT and TST as well as risk factors associated with positive QFT-GIT and TST results at baseline. Lastly, this chapter presents results on the level of agreement between QFT-GIT and TST results.

In **Chapter 6**, I present survival analysis results whose main aim is to determine incidence rates of TB in HHCs with positive and negative QFT-GIT results; also stratified by HIV status. A similar analysis for TST is presented as a comparison. Results on risk factors associated with incident TB and a sensitivity analysis are also presented in chapter 6.

In **Chapter 7**, I answer the main research question of the thesis: whether individuals with high (≥ 10 IU/ml) levels of IFN-γ in response to *Mtb* specific antigens in the QFT-GIT assay (ESAT-6,

CFP-10 or TB 7.7) are at higher risk of developing TB compared to those with low levels (≥ 0.35<10 IU/ml).

In **Chapter 8** I present results of other exploratory cut-offs points in a sensitivity analysis, in addition to using a cut-off point of ≥ 10 IU/ml.

In **Chapter 9** the main study findings are discussed. A discussion of the main question is presented in addition to a discussion addressing each of the specific objectives of the result chapters outlined above. Objectives are discussed in view of previous research. The study limitations, potential biases and conclusions are also described here.

Lastly in **Chapter 10** a summary of the evolving IGRAs research is presented including other biomarkers for disease progression. In addition, the potential role and use of IGRAs as diagnostic tests in high TB/HIV endemic resource constrained settings is described as well suggestions for future research.

Abstract

Background

Household contacts (HHCs) of pulmonary TB patients are at high risk of Mycobacterium *tuberculosis (Mtb)* infection and early disease development. Tuberculin skin test (TST) has been traditionally used to identify infected individuals; however, its use is limited by low specificity in populations with high levels of BCG vaccination or significant exposure to non-tuberculosis mycobacteria (NTM), and reduced sensitivity in immunocompromised individuals. Interferon-gamma release assays (IGRAs) such as QuantiFERON-TB Gold In-Tube (QFT-GIT) using *Mtb* specific antigens provide an alternative to TST for infection detection. IGRAs are now widely used for the detection of *Mtb* infection and are included in the guidelines of many countries with a low incidence of TB. Despite a growing body of literature on IGRAs, the relationship between the magnitude of T-cell Interferon-γ responses to *Mtb* specific antigens and risk of progression to disease has not been studied.

Objective

The main objective of this study was to determine whether HHCs with high (≥10 IU/ml) levels of IFN-γ in response to *Mtb* specific antigens (ESAT-6, CFP-10 or TB 7.7) in the QFT-GIT assay are at higher risk of developing TB compared to those with low (> 0.35-<10 IU/ml) levels. Other secondary objectives included to determine the following: the performance and operational characteristics of QFT-GIT in a field setting; risk factors associated with positive QFT-GIT results; concordance between the two tests; incidence rates of TB in HHCs with positive and negative QFT-GIT and TST results at baseline as well as positive and negative predictive values.

Method

This study was nested within a large community randomized trial called ZAMSTAR implemented in 16 communities in Zambia and 8 communities in the Western Cape Province of South Africa. A cohort of HIV-positive and HIV-negative adult (≥ 15 years) HHCs were prospectively followed for 2-4 years. Consenting HHCs had blood drawn for HIV antibodies. QFT-GIT test was performed according to the manufacturer's instructions. TST were performed according to the standard IUATLD protocol. A standardized questionnaire was used to collect information on risk factors for TB and TB treatment information (for those with TB).

Results

The feasibility studies showed three main findings. Firstly, the sensitivity of QFT-GIT was greater than that of TST overall, at all the standard TST cut-offs and when stratified by HIV status. The sensitivity of QFT-GIT was 85.6% (95%CI: 77.0-91.9) (indeterminate results excluded) compared to that of TST at 51.6% (95% CI: 40.9-62.2) at a cut-off of ≥ 10 mm. Secondly, test-retest reproducibility of QFT-GIT was high at 91.74% (ICC: 0.90; 95% CI 0.82-0.97). Thirdly, in this setting, some biological and operational factors that affected the performance of QFT-GIT were identified such as HIV positivity, low CD4+ T-lymphocytes, delayed incubation of blood samples and power outages.

For the main study, the study population at baseline consisted of 1,789 HHCs who were predominantly women (71%); median age was 28 years (IQR: 21-43); HIV positivity rate was 27.9%. Prevalence of tuberculous infection was 63.7% as measured by QFT-GIT and 39.6% by TST. There was a low level of agreement between the tests regardless of TST cut-off point (% agreement=59.7%; kappa=0.24). QFT+/TST- discordance (575/719; 80%) was more frequent than QFT-/TST+ discordance (144/719; 20%) at TST ≥10 mm.

Risk factors associated with QFT-GIT positivity were identified at baseline. In multivariable analysis adjusted for sex, age, and community, HIV status was negatively associated with QFT-GIT positivity (aOR: 0.48; 95% CI: 0.37–0.63; p<0.001) whereas residing in an urban area (aOR: 2.37; 95% CI: 1.10–5.13; p<0.03), smear status of index (OR: 1.26; 95% CI: 0.91-1.76; p=0.15) and country (aOR: 1.93; 95% CI: 1.48–2.51; p<0.001) were positively associated with QFT-GIT positivity. Similar results were obtained for TST.

From a total of 1789 HHCs seen at baseline, 1113 (62.2%) HHCs entered follow-up and were included in the main analysis. The overall incidence rate of TB was 20.96/1000 pyrs (95% CI: 15.93-27.58). TB incidence rate was higher among test positive HHCs compared to those who were negative (IRR for QFT-GIT: 1.65; 95% CI: 0.86-3.37; p=0.06) and for TST (IRR: 1.88; 95%CI: 1.04-3.41; p=0.01). Results were similar in univariable analysis (QFT-GIT: 1.66 (95%CI: 0.88-3.11; p=0.11) and TST: 1.89 (95%CI: 1.09-3.28; p=0.02)) and multivariable analysis adjusted for sex, age and HIV (QFT-GIT: 2.20 (95%CI: 1.14-4.25; p=0.02) and TST: 2.19 (95%CI: 1.24-3.86; p=0.007)). Overall, PPV for QFT-GIT was 5.38% (95%CI: 3.84-7.31), compared to TST, 6.57% (95% CI: 4.41- 9.36).

Overall for QFT-GIT, the IRR was higher among HIV negative HHCs (IRR: 3.85; 95%CI: 0.90-34.51; p=0.07) compared to HIV positives (IRR; 1.93; 95%CI: 0.88-4.57; p=0.04). Overall for TST, the IRR for HIV negatives (IRR: 2.21; 95%CI: 0.78-6.72; p=0.05) was similar to that among HIV positives (IRR: 2.32; 95%CI: 1.09-5.00; p=0.009). Univariable analysis showed similar results for both tests. In multivariable analyses adjusted for age, sex and country as an effect modifier, the HR for developing TB was 4.72 (95%CI: 1.35-16.46; p=0.01) in HIV positive QFT-GIT positives compared to 2.13 (95%CI: 0.81-5.60; p=0.12) in HIV positives TST positive HHCs.

Risk factors for TB were identified. In multivariable analyses, adjusted for age, sex , HIV status and country there was strong evidence that occasional smoking, (HR: 4.07; 95%CI:1.31-12.63), HIV positivity (HR: 4.60; 95%CI:2.48-8.56), smear positivity of the index (HR: 2.00 ; 95%CI:1.04-3.87) and country (HR: 1.79 ; 95%1.02-3.15; p=0.04)) were associated with incidence of TB.

Out of the 1,113 HHCs who entered follow-up, 406 HHCs had IFN-γ levels <0.35 IU/ml and were excluded leaving 707 HHCs in analysis for the primary objective. Out of these 536 (75.8%) had IFN-gamma levels ≥ 0.35 and <10 IU/ml (low IFN-γ levels) while 171 (24.2%) HHCs had ≥ 10 IU/ml (high IFN-γ levels).

Out of the 707 HHCs that entered follow-up, 38 (5.4%) HHCs developed active TB over 1558.0 person-years (pyrs) of follow-up, giving an incidence rate of 24.39/1000 pyrs (95% CI: 17.75-33.52).TB incidence rates were 24.51/1000 pyrs (9 cases/367.2 pyrs) in HHCs with high levels

and 24.35 (29 cases/1190.7 pyrs) among those with low levels of IFN-γ, giving an IRR of 1.0 (95% CI: 0.42-2.18; p=0.48).

Overall, unadjusted HR in HHCs with high IFN-γ levels was 1.02 (95%CI: 0.48-2.15; p=0.96) while in multivariable analysis adjusted for age, sex, country and HIV as an effect modifier, HR was 1.74 (95%CI: 0.63-4.79; p=0.29).

TB incidence rates in HIV positives was 51.94/1000 pyrs (3 cases/57.8 pyrs) in HHCs with high levels and 65.29/1000 pyrs (19 cases/291.0 pyrs) among those with low levels of IFN-γ, giving an IRR of 0.79 (95%CI: 0.15-2.70; p=0.38).TB incidence rates in HIV negatives were 19.56/1000 pyrs (6 cases/306.7 pyrs) in HHCs with high levels and 11.47 (10 cases/871.7 pyrs) among those with low levels of IFN-γ, giving an IRR of 1.70 (95%CI: 0.51-5.18, p=0.16).

Unadjusted HR among HIV negative HHCs was 1.73 (95%CI: 0.63-4.77; p=0.29) and 0.75 (95%0.22-2.55; p=0.65) among HIV positive ones respectively. In multivariable analysis adjusted for age, sex and country, the HR remained similar as unadjusted analysis for both HIV negatives and positives.

For all the groups used for sensitivity analysis of the primary question, HHCs with the highest IFN-γ levels had increased IRRs ranging from 1.5 to 2 compared to the reference sub-group. For HIV negatives, HHCs with the highest IFN-γ levels had the highest IRRs in all groups apart from one group. HIV negative HHCs with the highest IFN-γ levels had increased IRRs ranging from 4 to 5-fold compared to the reference sub-group. In comparison, HIV positive HHCs with the highest IFN-γ levels had increased IRRs ranging from 1.6 to 2.6 compared to the reference sub-group.

Conclusions

The principal finding in this study is that there was no difference in incidence rates between HHCs with low and high levels (overall IRR: 1.0 (95% CI: 0.42-2.18)).

Another principal finding was that there was strong evidence of a five-fold increased risk of TB in HIV positive QFT-GIT positive HHCs compared to HIV positive QFT-GIT negative ones (aHR : 4.72; 95%CI: 1.35-16.46; P=0.01). For all the groups used in the sensitivity analysis of the primary question, HHCs with the highest IFN-γ levels had increased IRRs ranging from 1.5 to 2 compared to the reference sub-group. The feasibility studies emphasized the need for stringent sample collection and processing techniques to ensure the accuracy of QFT-GIT results.

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Glossary of terms and abbreviations

ART	Antiretroviral therapy
AFB	Acid-Fast Bacilli
AIDS	Acquired Immune Deficiency Syndrome
BCG	Bacille Calmette-Guérin
CFP-10	Culture Filtrate Protein 10
CI	Confidence interval
DTH	Delayed-type Hypersensitivity
ELISA	Enzyme linked Immunosorbent assay
ELISPOT	Enzyme linked immunospot assay
ESAT-6	Early secretory antigenic target 6
HHCs	Household contacts
HIV	Human Immunodeficiency Virus
HR	Hazard ratio
IGRAs	Interferon-gamma release assays
IFN-γ	Interferon-gamma
IPT	Isoniazid Preventive therapy
IP-10	IFN-γ-inducible protein 10
IRR	Incidence rate ratio
IQR	Interquartile range
LTBI	Latent TB infection (Used as quoted by other authors although not preferred)
Mitogen-nil	Interferon-gamma response to phytohaemagglutinin (a mitogen)
Mtb	*Mycobacterium tuberculosis*
MDR TB	Multidrug-resistant tuberculosis
NPV	Negative predictive value
NTM	Non-Tuberculous mycobacteria

Glossary of terms and abbreviations continued…

PHA	Phytohaemagglutinin
PLWH	People living with HIV
PPD	Purified Protein derivative
PPV	Positive Predictive value
PYRS	Person-years
QFT-G	QuantiFERON®-TB Gold
QFT -GIT	QuantiFERON®-TB Gold In-tube
RR	Rate ratio
OR	Odds ratio
SA	South Africa
SOCS	Secondary outcome cohorts
RD1	Region of Difference-1
TB	Tuberculosis
TBI	Tuberculous Infection (used by this author)
TB antigen-nil	Interferon- gamma response to TB antigens (ESAT-6/CFP-10/TB7.7)
T_H1	T helper 1 cells
T-SPOT	T-SPOT®.TB
TST	Tuberculin skin test
TST-/QFT-	Negative results on both tests
TST+/TST+	Positive results on both tests
TST+/QFT-	TST Positive but QFT-GIT negative result
TST-/QFT+	TST Negative but QFT-GIT positive result
ZAMSTAR	Zambia and South Africa TB and AIDS Reduction trial

Definitions

Biomarker: a biological characteristic that is objectively measured and evaluated as an indicator of physiological or pathological processes, for example as a response to a therapeutic (e.g. drug) or preventive (e.g. vaccine) measure (1).

Cytokines: a varied group of small secreted proteins that mediate cellular interactions in immune and inflammatory responses, cell proliferation and differentiation, and various other processes.

Disease (Tuberculosis): AFB smear-positive or positive culture for *Mycobacterium tuberculosis.*

Exposed: An exposure, leading with a reasonably measurable risk to acquisition of infection with *M.tuberculosis.*

***Mtb* infection**: Is demonstrated by immune responsiveness of the host to *Mtb* antigens (using the tuberculin skin test or interferon-γ release assays).

Positive predictive value: the proportion of individuals with a positive test result who actually have the disease. It reflects the probability that a positive test reflects the underlying condition being tested for.

Negative predictive value: is the proportion of individuals with a negative test result who do not have disease

Sensitivity: the proportion of individuals with disease who will have a positive result

Specificity: the proportion of individuals without the disease who will have a negative result

Reproducibility of a test is an assessment of the extent to which the same tester achieves the same results on repeated testing of the same samples, or the extent to which different testers achieve the same results on the same samples.

Test-retest reproducibility: the ability of the test to produce consistent results when performed under similar circumstances.

Incidence: the number of new cases of disease that develop in a population during a specified period of time.

Prevalence: the proportion of a given population with an infection at a given time.

Performance: How well or badly a test works.

1.0 Introduction

The aim of this chapter is to describe the study objectives, provide a justification and a detailed description of the TB epidemiology and describe the conceptual framework from which the study hypotheses originate.

The specific objectives of this chapter are:

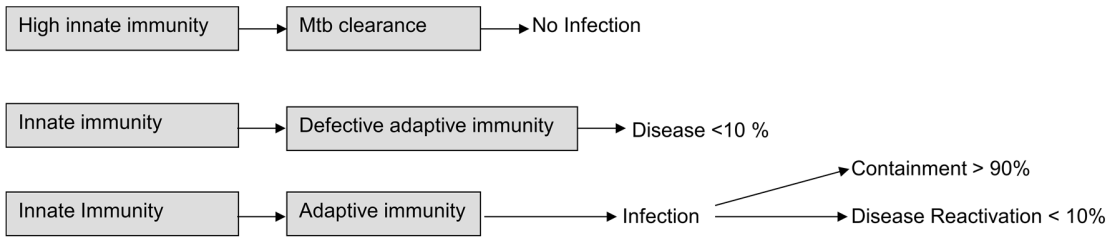
1. To discuss the traditional and current theoretical paradigms of TB immune responses.
2. To give a brief description of the epidemiology of TB with regard to risk factors for exposure, infection and disease.
3. To describe the TB and HIV epidemics in Zambia and SA.
4. To describe tools for diagnosing *Mtb* infection with a particular focus on Interferon-gamma release assays (IGRAs) as an emerging alternative to the TST.
5. To outline the specific primary and secondary objectives for this study.

1.1 Tuberculosis: Infection and Disease

Tuberculosis (TB) is transmitted when a person with infectious TB coughs or sneezes, releasing droplets containing *Mycobacterium tuberculosis (Mtb)* into the air. A susceptible individual can become infected with *Mtb* when breathing in these droplets. Much of the available evidence on infection and progression to disease comes from the pre-HIV era. The most important source of infection is a person with smear-positive pulmonary TB even though those with smear negative TB can transmit infection as well. Left untreated, a smear positive patient on average infects approximately 10-15 individuals per year, for an average duration of infectiousness of 2 years, before becoming noninfectious (due to spontaneous cure or death) (2).

A smear-positive patient on average infects approximately 20 people during their lifetime and creates two new cases of TB, at least one of which will be infectious (3). As long as at least one new case of TB is created by each existing case the cycle of transmission within a community is maintained. Traditionally, three possible outcomes of *Mtb* exposure have been well recognised **(Figure 1.1)**.

Figure 1.1: Innate Response to Mycobacterium Tuberculosis.



Modified from (4)

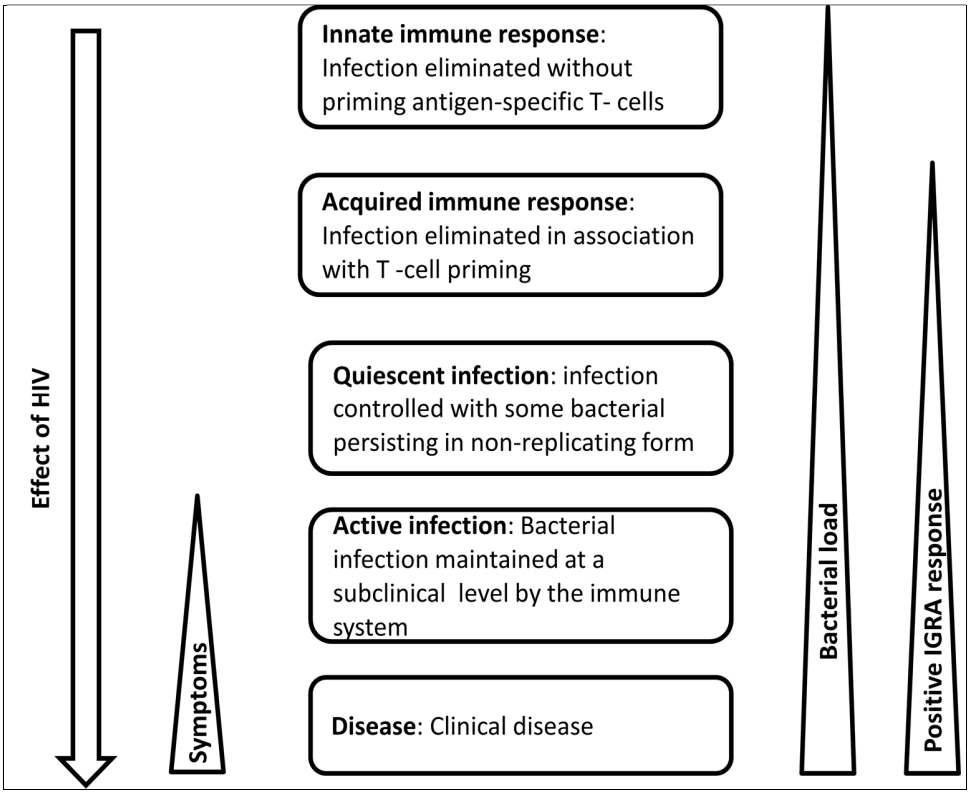
In some individuals, *Mtb* is eliminated by the host immediately upon inhalation (spontaneous healing). In a small number of infected individuals, adaptive immunity fails and they develop primary TB. Approximately 5–10% of recently exposed individuals develop clinically active TB within the first two years after exposure and another 5% in later life as a result of exogenous re-infection or reactivation of a previously acquired latent infection (3). Reactivation of latent infection can occur due to factors such as aging or the immune compromised status of the host.

In the third and the largest group of individuals, infection is contained as a result of successful granuloma formation, a function of strong innate and adaptive immune response by the host, which results in latent infection. In the absence of HIV, most people (around 90%) who become infected with *Mtb* do not develop disease either because their immune system has persistently controlled the mycobacteria or they are no longer infected with *Mtb* (5).

1.1.1 Theoretical Paradigms of TB Immune Responses

There is growing evidence showing that the nature of the relationship between *Mtb* and the human host represents a spectrum of immune responses, mycobacterial metabolic activity, and organism load (6). Traditionally, the outcome of infection by *Mtb* was generally represented as a bimodal distribution between active disease and latent TB on the basis of the presence or absence of clinical symptoms. However, this traditional view is now being challenged. An alternative paradigm has been suggested; it involves viewing *Mtb* infection as a continuous spectrum extending from sterilizing immunity to active infection and clinical disease (7, 8). In three reviews, Young, Barry, Lawn and colleagues (6-8) have divided this spectrum into five different states that represent a dynamic spectrum ranging from immunity to disease **(Figure 1.2)**

Figure 1.2: A Theoretical framework of *Mtb* infection as a Spectrum of Immune responses.



Modified from (6-8)

Below is a brief description of how these suggested immune responses are likely to correlate with Interferon-gamma release assays (IGRAs) results. It should be remembered that IGRAs just like TST are designed to identify an adaptive immune response against *Mtb*. A positive result is primarily a measure of an immunologic response to stimulation by mycobacterial antigens that should not be equated with the presence of live *Mtb* in the human host (6-9).

a) Innate immune response.

Following exposure to *Mtb*, infection may either be eliminated by the innate immune response without the need for T-cell priming. For such individuals IGRA results are negative i.e. there is no immunodiagnostic evidence of T-cell priming (6-8).

b) Acquired immune response

Alternatively, infection may also be eliminated following development of an acquired immune response. The adaptive immune response involves effector memory T-cells (which may be transiently present in the blood if bacteria are cleared) and central memory T-cells (which may remain for life but may not provide protection in all individuals). For such individuals there is T-cell priming and IGRA results are often positive although they may be negative.

c) Quiescent infection

Among those who are unable to eliminate infection following exposure, a majority establishes and maintains immune control. Such individuals typically have evidence of T-cell priming and maintain low bacillary numbers (non-replicating) which may persist for life (6-8). In this state, IGRA results are often positive but may be negative.

d) Active infection

A subsequent shift in the host-pathogen response for an individual with quiescent infection may permit active mycobacterial replication, leading to subclinical active infection. The relationship between "quiescent" and "active" infection is likely to be a dynamic one over time with bidirectional shifts between the two (6-8).

e) Disease

Loss of immune control and escalating mycobacterial load, however, may subsequently lead to the development of symptoms and overt clinical disease. For individuals with TB (disease), IGRA response is often positive but may be negative; approximately 30% of TB patients have a negative IGRA result probably due to immune-suppression. Therefore, there is a poor correlation between disease phenotype and immunodiagnostic test results (10).

The impact of HIV on the proposed host-pathogen relationship has also been reviewed. It has been suggested that "rather than increasing the risk of transition between compartmentalized disease states, HIV co-infection has a fundamental impact on the spectrum of the host-pathogen relationship with a general shift towards poor immune control, high bacillary numbers, and subsequent development of active infection and symptomatic disease". It has been further suggested that recurrent exogenous re-exposure to *Mtb* in high TB prevalence settings is also likely to play an important role, further increasing bacillary numbers and increasing the likelihood of progression to disease .

1.1.2 Cytokines and *Mtb* infection

T-cell mediated immune responses are important in the host control of *Mtb* infection (11). *Mtb* infection results in the induction of a large number of cytokines, and a subset of these have been demonstrated to be essential for control of the infection. Immunologic control of *Mtb* infection is based on a Th-1-type immune response which induces cytokines such as interferon-gamma (IFN-γ), tumour necrosis factor alpha (TNFα), interleukin (IL) -2 and IL-12. Besides, CD4+ T cells, other cytokines may be necessary in TB immunity like CD8+ and natural killer T (NKT) cells (12). The source and biological function of some cytokines involved in *Mtb* control are summarized in **Table 1.1** below:

Table 1.1: Cytokines Involved in *Mtb* infection control (11, 12)

Biomarker	Cell source	Biological activity
IL2	Activated Th1 cells (T cells), CD4+,CD25+, FoxP3+	T- Cell growth factor (T-cell proliferation). Central role in regulating Th1 T cell production of IFN-γ
IFN-γ	Activated Th1 cells, natural killer cells	Activates macrophages for mycobacterial killing, stasis & granuloma formation.
TNFα	Activated Th1 cells and macrophages, NK cells	Involved in localization of infection Enhances the proliferation of T-cells Activates macrophages for mycobacterial killing and granuloma formation.
IL4↑	Th2 cells and B cells (mast cells)	B- cell growth factor
IL6	T cell and macrophages	T & B cell growth & differentiation
IL10↑	T regulatory cells (Tr1/Th3), macrophages and B cells	B-cell differentiation Inhibit macrophage activation Inhibit secretion of inflammatory cytokines (decreases IFN-γ production) Reduces collateral damage
IL-17	CD4 memory cells	Induce cytokine production by epithelia, endothelia & fibroblasts.
CXCL10/ IP-10	Activated T cells, monocytes	Immunostimulant; promotes Th1immunity

All are cytokines except CXCL10 which is a chemokine.

1.1.3 The role of IFN-γ in protective immunity and as a marker of disease.

IFN-γ is a key T helper (Th) type 1 pro-inflammatory cytokine produced primarily by natural killer cells and T cells (Th1-type CD4 T cells; CD8 T cells). It is important in restricting the replication of *Mtb* in the macrophages and a determinant of susceptibility to mycobacterial infections in humans (13). Its main functions include macrophage activation (which in turn kill or inhibit growth of the pathogen), increased expression of MHC molecules and antigen processing components, immunoglobulin class switching and suppression of T_H2 (14).

The ability of CD4+T cells to produce IFN-γ is central in protection against disease (15-17) and the frequency of IFN-γ producing cells has been widely used as a correlate of protection against *Mtb*. This is evident from the increased risk of TB in individuals with deficiencies in their IFN-γ and interleukin-12 (18). Individuals defective in genes for IFN-γ or IFN-γ receptors are susceptible to serious mycobacterial infections, including *Mtb* (19). For instance, although very rare, a number of individuals with mutations in the IFN-γ R1 or IFN-γ R2 chains of the IFN-γ receptor have been identified, and these individuals are more susceptible to infection with mycobacterial (19, 20). In mice, IFN-γ knockout strains are the most susceptible to virulent *Mtb* infection(16, 17). Further evidence is given by the association between CD4+T- cell depletion and elevated susceptibility to active TB in HIV-infected individuals (11).

In addition, TB patients have been shown to demonstrate depressed IFN-γ producing CD4+ T-cell responses (62) with increased expression of IFN-γ after successful completion of therapy (21). The increased IFN-γ expression in healthy, latently infected individuals suggest that it might be a promising biomarker of protective immunity (22). In practice, though, it has proved disappointing (23). Therefore, although IFN-γ is key to protection against TB, its value as a correlate of protection is not sufficient (20). Its detection in isolation is not a sufficient indicator of a protective immune phenotype as those with LTBI and a positive IFN-γ release assay status can progress to active disease and IFN-γ secretion can also be detected in samples from patients with active disease (24). The complexity of the immune response against TB makes it more than likely that additional biomarkers are required for a reliable correlate of protection (25).

Although IFN-γ may be required for protection, it is a marker of disease as well as immunity (20). *Mtb* has developed mechanisms to limit the activation of macrophages by IFN-γ suggesting that the amount of IFN-γ produced by T cells may be less predictive of outcome than the ability of the cells to respond to this cytokine (26). In this regard, it has been shown that the level of IFN-γ produced by a mouse in response to a candidate vaccine does not always correlate with the effectiveness of the vaccine during *Mtb* challenge (26). Similarly, evaluation of the efficacy of human BCG vaccination using several assays demonstrated that mycobacterial growth inhibition did not correlate with IFN-γ response (27).

When IFN-γ production is present, there are situations where “more is not better, more IFN-γ production may mean more pathology or less protection e.g. bovine TB in calves”(20). Thus, although IFN-γ is essential for the development of an immune response that prolongs the life span of an infected animal, it is not sufficient to eliminate an *Mtb* infection. Therefore, much work is still needed to understand what IFN-γ production actually reflects.

1.1.4 Can we use the immune response as a surrogate measure of bacterial load?

Accurate diagnosis of TBI is scientifically challenging because of the low burden of tubercle bacilli, which are not directly detectable or quantifiable. However, the strong cellular immune response triggered by TBI serves as a signal for the presence of these bacilli.

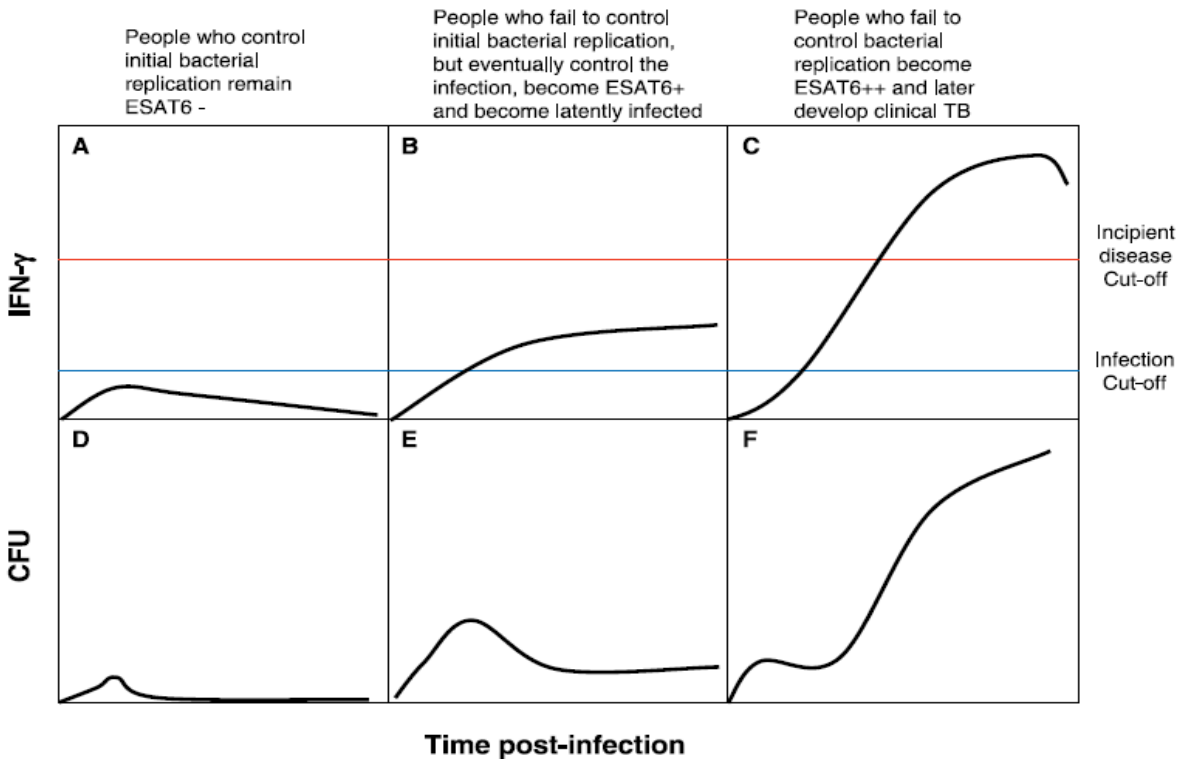
There is limited direct evidence on the correlation between immune response and bacterial load and pathology can only be inferred. The magnitude of the IFN-γ T-cell response to infecting *Mtb* is proposed to be proportional to the antigenic load of the infecting organism in human and animal models (28, 29). Hence the level of IFN-γ production is thought to be related to the bacterial load in the host (30). Although plausible and supported by some indirect evidence (31), there is no consensus yet, partly due to lack of a quantitative absolute measure of bacterial or antigen load in TB.

Studies have shown a correlation between infectious doses to which contacts have been exposed and the magnitude of their response to PPD and ESAT in vitro (32, 33). A rising ELISPOT count in a TB case contact with *Mtb* infection prior to onset of full-blown active disease showed how the quantitative ELISPOT read-out related to level of exposure, reflecting the infectious load (34). Other studies support this hypothesis as well (34-36).

Changes in the level of T-cell responses in persons undergoing treatment of both active and LTBI indicate some relationship to antigen load (37). Most studies have shown that TB patients produce lower concentration of IFN-γ in response to ESAT-6 than HHCs, community controls or recent converters (38-40). Significant qualitative and quantitative reversions of T cell responses to ESAT-6 and CFP-10 antigens following treatment of LTBI and active TB in different settings (37, 41-45) have been found. In two studies in the Gambia, a significant proportion of individuals who successfully completed TB treatment changed from a positive to a negative ELISPOT (46, 47). Similarly, in Cape Town, 81% HIV-negative patients who had successfully completed TB treatment were ELISPOT negative (48). While it is believed that most reversion cases indicate sterile cure of infection or active disease, other reasons may apply (49). Similarly, other studies have found that frequencies of ESAT-6 -specific IFN-γ secreting CD4 T cells were higher in infected healthy contacts and subjects with minimal disease and than in patients with advanced active TB (43).

Although such evidence examining the relationship between immune response and bacterial load is present, its interpretation remains unclear and contradictory. One interpretation of these findings is that, at the time of initial infection with *Mtb*, HHCs mount a strong, high frequency T cell response to *Mtb* and so limit bacterial replication (infection may be controlled). Limited bacterial replication induces a low IFN- γ response. Individuals who go on to develop active disease, in contrast, make a weak T-cell response, and the bacteria are allowed to reach a higher equilibrium bacterial load, resulting in disease. Disease is associated with progressive bacterial replication and a high IFN- γ response. **Figure 1.3** shows a schematic of the postulated correlation between bacterial load, T-cell responses, and clinical outcomes (50).

Figure 1.3: A Schematica of the Postulated Correlation between bacterial load, T cell responses and clinical outcomes.



Reproduced from [50].

Initial infection might be controlled at its onset with minimal bacterial replication (measured as colony-forming units (CFU)) and induction of ESAT-6 responses (A and D). In such cases, the T -cell response may be below the diagnostic cut-off. However, in most cases, initial bacterial replication reaches a point at which it induces an ESAT-6 specific IFN-γ response to increase above the established cut-off value (infection threshold), enabling the diagnosis of an individual as latently infected (B and E).In most cases, individuals control the infection, resulting in latent infection, but some develop active TB disease associated with progressive bacterial replication. This is accompanied by increasing and strong ESAT-6 responses and, as hypothesized here, an incipient (higher) disease cut-off value may predict development of progressive disease (C and F).

Other interpretations suggest that in TB patients with advanced disease, a high bacterial load stimulates weaker, less efficient proliferation of Ag-specific T cells in vivo (43). This model is analogous to that proposed for certain chronic viral infections, in which virus-specific CD8 T cells are believed to mediate protective immunity (43).

However, there is evidence suggesting that disease is associated with a defect in IFN-γ production that is improved by TB treatment (38, 39). A recent study done in Singapore among 275 sputum culture-positive, HIV negative TB patients showed significant declines in the positivity rates and quantitative results of IGRAs with treatment (42). It has been suggested that the progressive decline of IFN-γ response measured by IGRAs reflects the reduction of mycobacterial burden following a successful TB treatment. However, measurements using peripheral blood may be complicated by the sequestration of reactive cells to disease sites (20). Cytokines such as IFN-γ and the IL4/ IL4δ2 ratio have been shown to vary with treatment of

LTBI and active disease; these changes, however, are not always consistent and may be confounded by the natural variation in stimulated cytokine responses over time (51). Recent studies suggest that a profile using several cytokines, rather than one biomarker, may be more useful.

Despite this evidence there is no agreed consensus as studies have been inconsistent regarding whether T-cell responses in TB patients change during and after treatment. Thus while some studies have demonstrated that IFN-γ response decreased or became negative (41-44), others have reported persistently positive or even stronger responses during and after TB treatment (39, 52, 53).

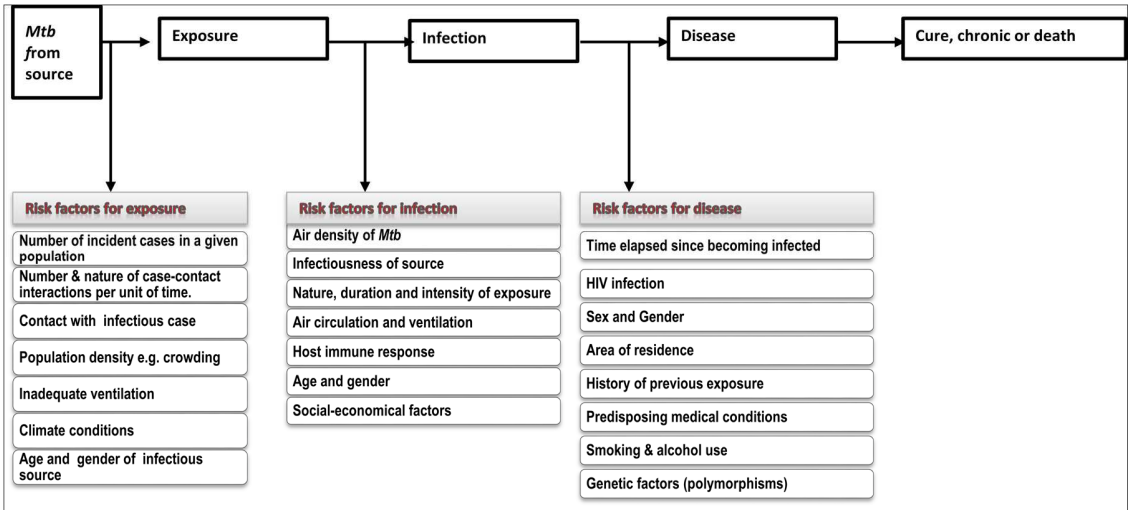
1.1.5 Risk factors for TB: From exposure to disease

Acquisition of active TB depends on the succession of various factors influencing:

1) The risk of exposure
2) The risk of infection and
3) The risk of developing disease.

While most factors that affect the risk of infection (such as crowding, urban residence, and low socioeconomic status (SES) are *extrinsic* to the susceptible host and are related to the environment, many that affect the risk of disease after infection are host related and also a consequence of human interaction with the environment (54, 55). Environmental factors may have an impact on the incidence of TB in a given population as a result of their effect on both the risk of infection and the risk of disease once a person is infected (55). A model of TB epidemiology showing risk factors for exposure, infection and disease is shown in **Figure 1.4** below:

Figure 1.4: Model of TB epidemiology showing risk factors for exposure, infection and disease.



Modified from (3)

1) Risk factors for exposure to *Mtb*

Risk of exposure to *Mtb* depends primarily on encountering an airspace that contains the tubercle bacilli. There are three major factors that determine the risk of becoming exposed to tubercle bacilli (3) which are as follows:

a) ***The number of incident infectious cases within a community***

Without the presence of infectious cases, no relevant exposure can occur. The more new cases of infectious disease there are, the greater the likelihood that a susceptible individual will inhale the organism in a given space. Nevertheless, there are other factors that need to be considered for any fixed number of incident cases. They are principally: how "infectious" a case is and how "susceptible" an individual (or population) is.

b) ***The duration of infectiousness of the individual case***

The risk of becoming exposed is greatly enhanced if infectiousness is prolonged, as compared with a short duration of infectiousness. On average, around 30-40% of contacts are infected around the time of diagnosis of a sputum smear-positive index case (56). Early intervention with chemotherapy reduces the time of infectiousness while inadequate treatment can prolong infectiousness.

c) ***The number and nature of case-contact interactions per unit of time****.*

The number and nature of possible case-contact interactions will vary greatly according to individual behavior and opportunities for interacting with other people in a community. Important factors include:

- ***Population density***

The nature of the dwelling in which people live and the number of persons who share that dwelling have an important impact on the risk of exposure, given that a case of TB lives in that dwelling. The number of possible contacts of a TB patient in a rural area may be smaller per unit of time than that of a patient living in an urban setting (given a similar dwelling and family size) because urban areas have a much higher population density than rural areas. Therefore, the likelihood that a susceptible person will be exposed to an infectious tuberculosis patient increases with population density, even if the incidence is the same.

- ***Family size and social arrangements within families.***

The most intense exposure is likely to occur among individuals who share the same household or who spend long periods of time in the same room with an infectious source case. The larger the size of the family, the greater the number of individuals at risk of exposure given that an infectious case of TB is part of the family and other factors considered like infectiousness of the case. Of further importance are social arrangements within the family, i.e., the sleeping proximity to the index case (sharing the same room or bed with the index case). Household studies conducted more than 30 or 40 years ago both in industrialized and non-industrialized

countries showed that the risk of becoming infected increased with intimacy of contact with a tuberculosis case (55). This may be enhanced by overcrowding and inadequate ventilation. Places of social networks in countries with a high burden of disease have been shown to be "hotspots"(57) for TB transmission. Although historically, household contacts (HHCs) were thought to have been infected mainly from within the households, it is now well recognized that transmission of TB occurs within households as well as the community (58).

- ***Differences in climatic conditions***

In a warm climate, outdoor social activities are much more common than in a colder climate. Tubercle bacilli expelled outdoors are dispersed rapidly, and exposed to sunlight they die very quickly due to the sun's ultraviolet rays. In contrast, tubercle bacilli expelled indoors by a patient in a confined space with poor ventilation may retain viability, and thus the potential to cause infection, for a prolonged period of time. A cold climate causes people to congregate inside, thus increasing the likelihood of exposure if there is a case of TB in the group. In contrast, indoor activities might be reduced in tropical climates, and indoor ventilation might be better, because windows can be kept open for much longer periods than in colder climates.

- ***Age of sources of infection***

Parents may expose their children to a much larger extent compared to other individuals. It is a common notion that people tend to socialize with those of their own age.

- ***Gender***

The degree of social interactions differs by gender to a great extent in different societies. The opportunity of becoming exposed to an infectious case will differ for men and women because of the different roles played by the two (see further details in section 1.1.5).

2) Risk factors for *Mtb* infection

Risk factors for becoming infected given that exposure has occurred are largely exogenous in nature (3). The probability of becoming infected with *Mtb* depends on the number of infectious droplet nuclei per volume of air (infectious particle density) and the duration of exposure of a susceptible individual to that particle density i.e.

$$\frac{\text{Particles}}{\text{Volume}} \times \text{Exposure time}$$

Key
Particles: *Production of infectious droplet nuclei*
Volume: *Volume of air and ventilation*
Exposure time: *Time of inhaling air with droplet Nuclei*

a) Air density of *Mtb* (infectious particle density)

To be transmissible through the medium of air, TB must remain airborne after being expelled from an infectious case. Successful transmission requires airborne infectious droplet nuclei, small enough to reach an alveolus in the periphery of the lung. Such droplets can remain suspended in air for several hours.

b) *Nature of the infectious case*

Patients with sputum smear-positive tuberculosis are by far the most potent sources of infection in the community. However, various studies have demonstrated, that patients classified as smear-negative can also transmit infection. A study in San Francisco, California, using molecular fingerprinting techniques demonstrated that 17 per cent of transmissions were attributable to index cases with sputum smears negative at diagnosis, and that the relative transmission rate from smear-negative compared to smear-positive TB was 0.22, or roughly one fifth of all transmissions (59). Household studies conducted more than 30 or 40 years ago both in industrialized and non-industrialized countries showed that the risk of becoming infected increased with intimacy of contact with a tuberculosis case (55).

c) *Air circulation and ventilation*

Given a defined number of tubercle bacilli expelled into the air, the volume of air into which the bacilli are expelled determines the probability that a susceptible individual breathing that air will become infected. Adequate ventilation plays an important role in diluting the concentration of bacilli.

d) *Host immune response*

The macrophage is the first cell from the immune system to encounter and ingest the TB bacillus. Macrophage function may vary due to genetic or acquired factors (3). Data from nosocomial outbreaks of TB in HIV-infected subjects suggest that susceptibility to infection is dependent on the health status of the person (55) and recent studies suggest that susceptibility to mycobacterial infection might be genetically modulated (60).

e) *Age and sex*

Age and sex variations in the prevalence of infection and disease have been reported worldwide, in both developed and developing countries (55, 61). The prevalence of tuberculin sensitivity is usually similar in males and females until adolescence, after which prevalence is higher among males (55). This difference after adolescence may reflect greater exposure among adult males because of differentiated social roles and economic activities, but it also may reflect a genuine sex difference in susceptibility to tuberculosis infection related to a different predisposition to responsiveness to delayed-type hypersensitivity.

f) *Socio-economic factors*

Crowding and urban dwelling have long been established as risk factors for exposure to TB infection (62). Housing conditions often reflect socio-economic status and can affect infection risk and outcomes through such mediators as poor ventilation and air quality in the home (62).

There is evidence that socio economic status (SES) is associated with risk of infection. Several studies have shown an association of socio economic status (SES) and infection with *Mtb* (55). A recent study done in two communities in Zambia (63) aimed to assess the association between household socioeconomic position and tuberculosis infection. In this study, higher socio-economic position, rather than lower, was associated with significantly higher risk of TB infection. None of the traditional risk factors for *Mtb* infection mediated this association, suggesting that in these two communities TB transmission occurred through exposure to as yet undefined risk factors that were associated with higher socioeconomic position. These results suggested emerging new patterns of TB transmission and a role of socioeconomic position on the risk of TB infection opposite to was expected.

3) Risk factors for tuberculosis

The most important risk factor for tuberculosis is infection with tubercle bacilli. Social, environmental and biological determinants of health have long been recognized as risk factors for TB (62). While the risk of becoming infected is largely exogenous in nature, the risk of developing TB given that infection has occurred is largely endogenous, determined by the integrity of the cellular immune system.

a) *Time elapsed since becoming infected*

The temporal association between infection and progression to disease has been well recognized. The risk is elevated in the first years following infection, rapidly falls off and then remains low, but measurable for a prolonged period of time (3). Only 5–10% of recently exposed individuals develop clinically active TB in the first two years after exposure. Preventive therapy trials using placebos have shown that the incidence of TB is highest in the first few years following infection and then rapidly falls off (64). Furthermore, studies have shown that individuals with recent TB infection rapidly progress to disease (64-66) and that recent infection is ten times more likely to produce a case than a long standing infection (3) in the absence of HIV infection.

b) *Infection with HIV*

HIV is the most powerful known risk factor for reactivation of latent TB infection to active TB. HIV fuels the TB epidemic in several ways. HIV promotes progression to active TB both in people with recently acquired and with latent *Mtb* infections. HIV increases the rate of recurrent TB, which may be due to either endogenous reactivation (true relapse) or exogenous re-infection (67). In populations at high risk of infection, re-infection might be a major contributor to the overall rate of TB in adults, whereas, in populations that have a low risk of infection, most cases of post primary disease in adults probably result from reactivation (55).

Studies from Sub-Saharan Africa have recorded HIV sero-prevalence rates of up to 50% in patients with TB (68). An HIV-positive person infected with *Mtb* has up to a 50% lifetime risk of developing disease as compared to an HIV-negative person who has only a 10% risk (69).

A TB prevalence survey conducted among 8044 adults sampled from 2 sub-districts in Lusaka province, Zambia, demonstrated that 36% of all prevalent TB cases in these communities were attributable to HIV. HIV appeared to have overshadowed previously described risk factors for prevalent TB such as male sex, urban environment and previous TB such that it was identified as the major driving force behind prevalent TB (68). Low CD4 and high viral loads have been found to be further risk factors for disease, while treatment with highly active antiretroviral therapy (ART) reduces risk (62).

c) *Age*

Large differences in TB incidence are often observed by age, with higher incidence of disease with increasing age due to the cumulatively increasing prevalence of tuberculous infection. Disease risk after primary infection with *Mtb* is greatest in children younger than 4 years, and declines slowly to its lowest at age 5–10 years (70). During adolescence (age 15–19 years), there is a rapid increase in risk with a second peak between the ages of 20–30 years (70).

d) *Gender*

In the pre-HIV era, TB notification rates were typically male-biased for all ages over 15 (3), but in several African countries with high rates of HIV infection, the majority of notified TB cases are now women. Women now account for up to 70% of HIV-infected adults in sub-Saharan Africa, which has shifted the male-to-female case-notification ratio such that more female than male cases of TB are now detected in countries with high HIV prevalence (71). In the pre-HIV era this gender disparity was mainly explained by access barriers, such as socio-cultural disempowerment, stigma, different patterns of health-care use, or lack of financial resources and poorly elucidated biological factors (3, 72). Most of these access barriers are still true today. A review suggested that not only was TB among women under-reported because of cultural and socioeconomic factors, but also these factors increased the chances of women to be exposed to TB bacilli and to progress from TB infection to TB disease (through for example malnutrition and HIV co-infection) (73).

e) *Area of residence*

TB tends to be more common in urban settings for reasons probably related to population density, crowding, environmental pollution and housing characteristics influencing ventilation issues. Living conditions are also known to be quite different between urban and rural areas, and are generally lower in rural areas.

Urbanization has largely increased in many resource-poor countries over the past 30 years and has dramatically enhanced HIV transmission at multiple levels; by bringing people closer together in time and space and in environments with fewer social control mechanisms, urbanization has enabled HIV infection to spread in densely populated areas (55).

f) *Socio-economic factors*

Historically, TB has been referred to as a "disease of the poor and socially disadvantaged" (see also section 1.1.4). Nevertheless, there is contrasting evidence linking SES and TB (74, 75). Overall, most studies (75) have traditionally linked lower SES to TB, including a recent one done in Zambia (74). In a case-control study nested within a population-based TB and HIV prevalence survey conducted in 2005–2006 in two Zambian communities, prevalent TB was significantly associated with lower household SEP (aOR = 6.2, 95%CI: 2.0–19.2 and aOR = 3.4, 95%CI: 1.8-7.6) respectively for low and medium household SEP compared to high (74).

g) *Medical conditions*

In persons infected with *Mtb,* any condition modifying the balance established in the body between the tubercle bacilli and the host's immune defenses can affect risk of developing the disease. Such factors include HIV infection, immunosuppressive treatment (corti-costeroid medication), malignancies, renal failure, measles, diabetes, malnutrition, and alcoholism, and all are considered *intrinsic* to the susceptible host (55). These factors are likely to reduce the efficiency of the body's means of defense.

h) *Smoking*

Smoking is a well recognized risk factor for both infection and disease (76). Suggested mechanisms include decreased immune response, CD4 lymphopenia, defects in macrophage immune responses, and mechanical disruption of cilia function in the airways.

i) *Alcohol*

One systematic review has examined this association between TB and alcohol. In 21 studies identified, all forms of TB were about three times higher, and pulmonary TB was four times more frequent, in heavy drinkers than controls (77).

j) *Body mass index and under-nutrition*

There is some evidence suggesting a relationship between nutrition or low BMI and TB. One review on TB and low BMI has indicated a strong dose-response relationship, with TB incidence increasing exponentially as BMI decreased in the six studies summarized (34). On a population level, the impact of this risk factor has been predicted to be enormous, due to the widespread global prevalence of under-nutrition (78).

k) *Genetic factors*

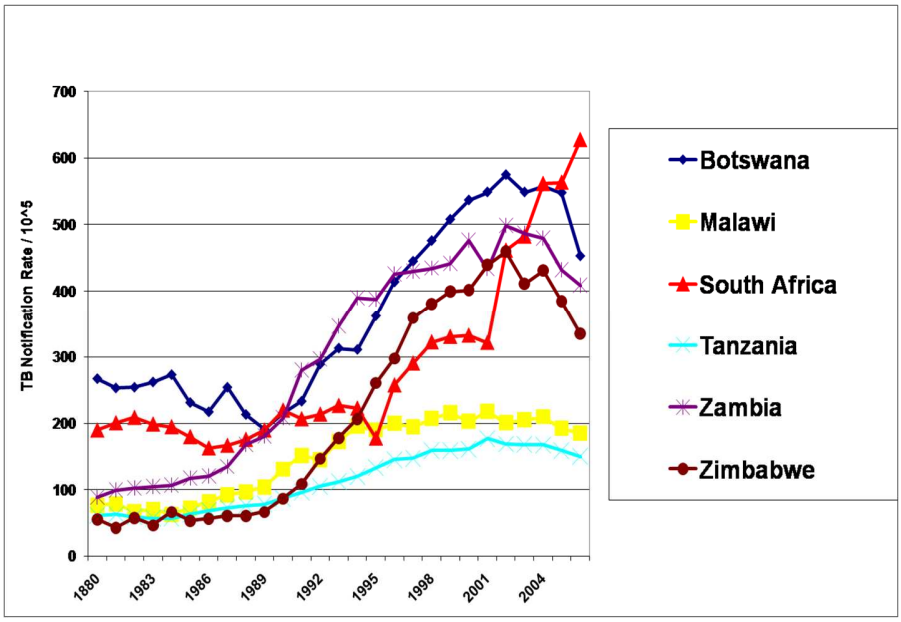
Evidence indicates that genetic factors may determine differences in host susceptibility to infection with mycobacteria and that they might contribute to the pattern of clinical disease (55, 79). A recent meta-analysis (79) consisting of 36 studies showed that the association between SLC11A1 polymorphisms and TB susceptibility supported the hypothesis that NRAMP1 might play an important role in the host defense to the development of TB.

1.2 Epidemiology and Control of Tuberculosis

1.2.1 TB and HIV epidemics globally

TB is among the most pressing public health problems globally. In 2009 there were an estimated 9.4 million incident cases (equivalent to 137 cases per 100 000 population) of TB globally, with the majority of these occurring in South-East Asia (35%), Africa (30%) and Western Pacific regions (20%)(80). Of the 9.4 million incident cases in 2009, an estimated 1.0–1.2 million (11–13%) were HIV-positive. Although the total number of incident cases of TB is increasing in absolute terms, the number of cases per capita is falling (less than 1% per year) since a peak of 142 cases per 100 000 population in 2004 . Sub-Saharan Africa has borne the brunt of the HIV and TB co-epidemics, accounting for 70% of the global burden of HIV **(Figure 1.5)(81)**.

Figure 1.5: TB notification rates in Southern Africa



In Africa, HIV has been the single most important factor in determining the increased incidence of TB in the past 10 years. The risk of developing TB is between 20 and 37 times greater in people living with HIV (PLWH), than among those who do not have HIV infection (68).TB is a leading cause of death in PLWH, accounting for almost a quarter of the 1.7 million AIDS deaths in 2009 (80). People with HIV infection also face the problems of multidrug-resistant (MDR) and extensively drug-resistant TB (XDR)(82).

The exact prevalence of latent TB is not known, but TST surveys done by WHO suggest that one-third of the world's population is latently infected with *Mtb,* corresponding to 2 billion people worldwide (83). It is from this reservoir that new cases responsible for transmission emerge. A review (64) of published literature and WHO/UN/ CDC databases, showed that the HIV epidemic was exacerbating TB transmission. HIV individuals infected with TB were responsible for an estimated 7% of all TB transmission in the African region (largely sub Saharan Africa). Of all individuals infected worldwide, the largest burden of TBI is in Southeast Asia (prevalence, 46%),

the Western Pacific region (32%), Africa (31%), and the Eastern Mediterranean region (27%)(64, 84). This finding contrasts with the lower prevalence of TBI noted in the Americas (15%) and Europe (14%).

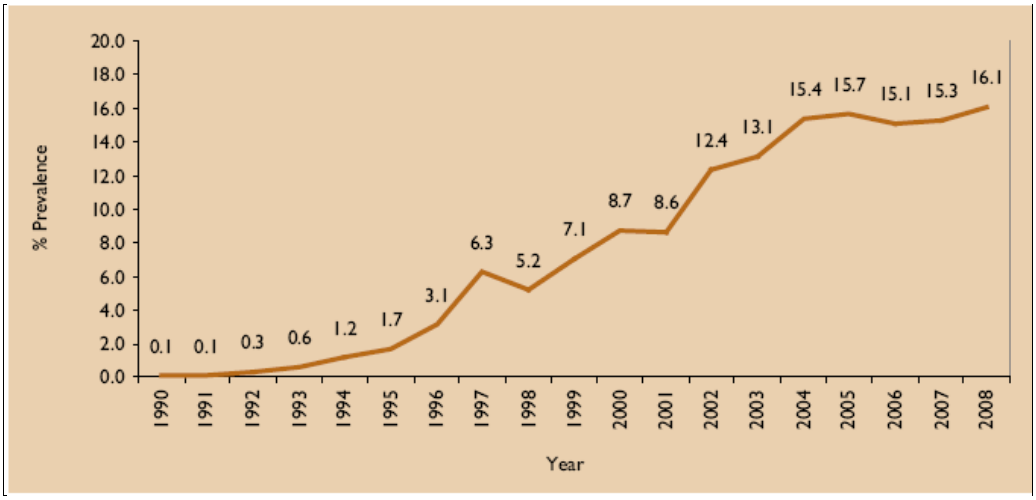
1.2.2 TB and HIV epidemics in Zambia and Western Cape, SA (the study sites)

Globally, WHO reports that new cases of TB have been falling for several years and fell at a rate of 2.2% between 2010 and 2011 (85). However, the burden of TB and HIV in sub Sahara Africa remains enormous. Zambia has one of the highest incidence rates of TB in the world today (86). In 2009 the estimated incidence rate was 482 per 100 000 population according to WHO (86). However, TB incidence has never been directly measured at national level, since this would require long-term studies among large cohorts of people at high cost and with challenging logistics. Estimates of TB incidence have been mostly based on assessments of the fraction of incident cases captured in notification data in the late 1990s (86). 70% of all new TB patients are co-infected with HIV. In 2007, 14.3 per cent of Zambia's estimated 12.9 million population was infected with HIV.

South Africa (SA) ranks among the worst afflicted countries in the world for both HIV infection and TB. This is as a result of the convergence of a major pre-HIV-era TB epidemic, rising numbers of TB cases associated with the maturing HIV epidemic, and growing resistance to antituberculosis drugs (87). Historically, the social, economic, and environmental conditions created by apartheid such as overcrowded squatter settlements, migrant labour, and deliberately underdeveloped health services for black people provided a favorable environment for efficient transmission of TB and HIV.

In 2009, SA was among the five countries worldwide which had the largest number of incident cases (0.40-0.59 million) of TB (80). TB case notification rates in South Africa have increased almost 4-fold from 163/100,000 in 1986 to 628/100,000 in 2006 (88). Overall, HIV prevalence has increased from 15.1% in 2006 to 16.1% in 2008 (89). HIV prevalence levels vary geographically between provinces and within provinces. In 2008, the Western Cape provincial HIV prevalence amongst 15-49 antenatal women was 16.1% **(Figure 1.6)**.

Figure 1.6: HIV prevalence epidemic curve among antenatal women, Western Cape, 1990-2008



The TB-HIV co-infection rate in SA is also high, with an estimated 73% of new TB patients co-infected with HIV (**Table 1.2**). In addition, the numbers of MDR-TB and XDR-TB patients have increased due to the concurrent HIV epidemic and inadequate management of TB (90). In 2009 the National Health Laboratory Services (NHLS) diagnosed 9 070 MDR-TB and 594 XDR-TB cases (90). A summary of the TB and HIV situation in both countries is shown below, **Table 1.2.**

Table 1.2: Selected TB and HIV indicators for 2009 (86, 89, 91, 92)

	Zambia	South Africa
Indicator		
Population	12.9 million	50.4 million
Incidence rate in all forms of TB/100 000	482 (433-533)	971 (791-1169)
Prevalence rate in all forms of TB/100 000	309 (108-562)	808 (362–1288)
TB patients that are HIV positive	70% (2007)	73% (2007)
MDR-TB among new TB cases	1.0% (0.4-2.3 %)[2]	1.8 (1.5–2.3) (2008)
Number of people living with HIV	1.1 million (1-2)	5.6 million (5.4-5.9)
HIV prevalence in antenatal women	16.4% (93)	29.4 (28.7-30.1)
Adults aged 15 to 49 HIV prevalence rate	14.3% (2007)	17.8% (17.2-18.3%)
HIV infected people receiving ART	55.1% (2008)	56%

All data are for 2009 unless otherwise stated.[2] Zambia National Drug resistance survey results, 2008 (unpublished)

Recent studies in Africa have estimated the annual risk of tuberculosis infection (ARTI) to be in the range of 1.5-4%, representing the proportion of the adult population which will be infected

with *Mtb* (94-96). Two recent large TST surveys found that the average ARTI of 24 high burden TB/HIV communities in Zambia and SA was between 0.8 and 2.8% and between 2.5% and 4.2% respectively (95). This survey provided an important source of data indicating the extent of TB transmission in Zambia and Western Cape, SA. The ARTI is an epidemiological index derived from TST surveys among children to measure the extent of TB transmission in a community (95).

The average annual risk of infection with *Mtb* is a calculated average from an observed prevalence of infection, approximating the incidence of infection (97).

1.2.3 Control of TB infection and disease

One of the targets of the WHO Stop TB Partnership is to reduce the annual incidence of new TB cases to less than one per million populations by 2050 (98). For many years, the cornerstone of TB control was focused mainly on the DOTS strategy which includes passive detection and treatment of infectious cases. Even though this still remains the cornerstone of most TB control programs, it is increasingly being accepted that effective TB control in endemic settings requires additional approaches. There are three recognized approaches to TB control: (i) early detection and treatment of active TB (ii) Prevent progression from infection to active TB and (iii) Reduce HIV transmission (99). Other recent approaches such as pre-exposure and post exposure vaccination against TB are being explored. Protection induced by Bacillus Calmette-Guerin (BCG) vaccination against adolescent and adult TB, is insufficient.

Early detection and treatment of active TB reduces transmission, reducing the number of carriers, who will later develop active TB. However, WHO has estimated that less than 50 per cent of all cases globally are currently diagnosed and treated (80) . These patients continue to act as reservoirs for new cases of TB. Limited access to quality health services, over stretched staff, stigma, poverty and inability to diagnose smear negative TB especially in HIV infected individuals are contributing factors in resource poor settings.

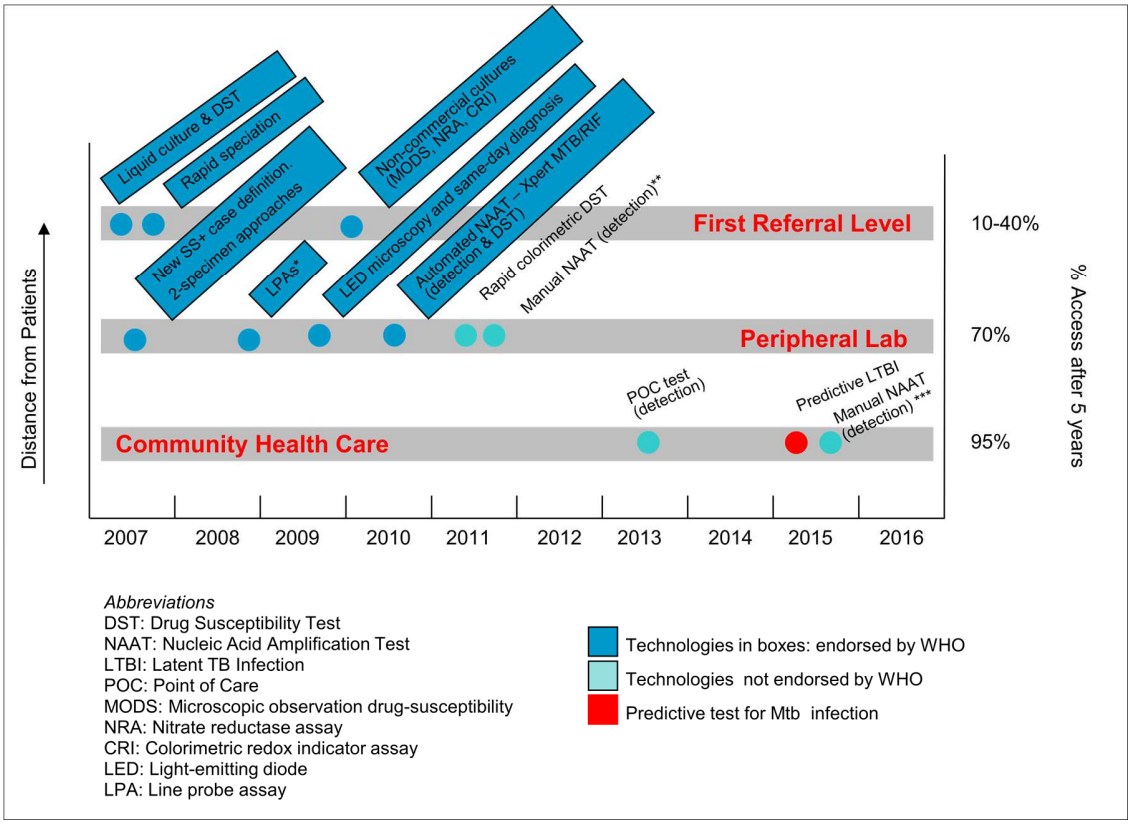
Reducing the risk of progression from *Mtb* infection to disease can be achieved through accurate diagnosis and treatment of individuals infected with *Mtb.* Several placebo-controlled trials have shown that preventive therapy (PT) substantially reduces the subsequent risk of active TB by 60-90% (100-102). Targeted testing and treatment of TBI especially among HIV positive individuals is an important strategy to reduce the incidence of TB (103) and contributes to closing the tap of new infections. The contribution of HIV positive individuals to the pool of TB transmission in high TB prevalence countries though small on a case-to-case basis has huge epidemiological implications and has increasingly drawn the attention to the need for PT. However, provision of PT to eligible individuals has been of higher priority in low than high TB prevalence countries.

Any strategy that successfully reduces HIV transmission will benefit TB control. The implementation of collaborative TB/HIV activities is a well practiced approach in many countries affected by the dual TB/HIV epidemic. Early initiation of ART in HIV positive individuals reduces plasma levels of HIV, which correlate closely with infectivity.

1.2.4 Predictive biomarkers for the development of active TB

Over the past decade, real progress has been made in the development of new TB diagnostics resulting in WHO endorsing several tests for use in TB endemic countries (104) whilst others are still pending. At the community health care level, no single test had been successfully endorsed by WHO by 2011 and among these is a predictive biomarker for TBI **(Figure 1.7)**.

Figure 1.7: TB diagnostics pipeline and their targets.



Modified from the Global plan to stop TB 2011-2015 (105) and (104).

However despite this progress the search for a predictive biomarker for the development of TB is still on. The need for a better tool than TST that will act as a predictive marker of progression to active disease is still among one of the important TB research priorities (106, 107). Several longitudinal studies in the recent past have explored whether Interferon Gamma Release Assays (IGRAs), such as the QuantiFERON-TB Gold assay (QFT) could provide the much needed answer (108) (see further details under literature review).

A new target to develop a predictive biomarker for active TB has been put forth by the Global Plan to Stop TB, 2011-2015 (105). The previous target by the WHO STOP TB New Diagnostics Working Group (Strategic Plan 2006-2015), to develop a rapid diagnostic procedure capable of predicting the future progression of latent TB infection to active disease, in both HIV-infected and uninfected subjects by 2012 now seems unachievable (85) . Therefore, despite the push and increase in TB diagnostics in the recent years endorsed by WHO; clearly not much has been

achieved for diagnostics for TB infection and disease progression. There is need to put much more continued emphasis on diagnostics for the prevention of active TB in high TB endemic countries.

FIND (Foundation for Innovative New Diagnostics) estimates that the largest potential available market for a new TB diagnostic would be for a test that both detects latent infection and predicts progression to active disease. Such a test, if widely implemented and accompanied by successful treatment, could revolutionize TB control (109). Such a test should be cost effective, robust enough to be used at peripheral levels of health-care systems, perform equally well in HIV infected individuals and should allow sensitive, specific and timely detection (110).

Even though the evidence so far seems to show that IGRAs have modest predictive ability (108) and may be by themselves only unlikely to be sufficient enough to act as predictive markers for TBI, longitudinal data especially in HIV infected populations is still lacking. Some of the unanswered questions (e.g. within subject conversions and reversions) may be important in understanding better platforms of designing and improving similar technologies. Recently WHO strongly recommended (111) that IGRAs should not be used as predictive tools because neither IGRAs nor the TST can accurately predict the risk of infected individuals developing active TB. However, this recommendation was based on three studies (one was submitted as an interim report because the study was ongoing-this is the main study under discussion in this thesis) of different quality done among individuals with varying risks.

This thesis is part of the many efforts by investigators to try and focus on issues that are still poorly understood about IGRAs due to paucity of longitudinal data. Furthermore, these assays have not only gained popularity in high income countries but slowly they are filtering in the private sector of high burden countries despite the recommendation by WHO Some of the key unresolved issues are the efficacy of preventive therapy on IGRA results; relationship between quantitative IGRA levels and the subsequent development of TB; immunological phenotypes of discordant-concordant TST/IGRA pairs and the subsequent rates of TB and the utility of repeated or serial IGRA results for predicting subsequent incident active TB (111). In this thesis my focus is on the relationship between quantitative IGRA levels and the subsequent development of TB.

1.3 Diagnosis of *Mtb* Infection

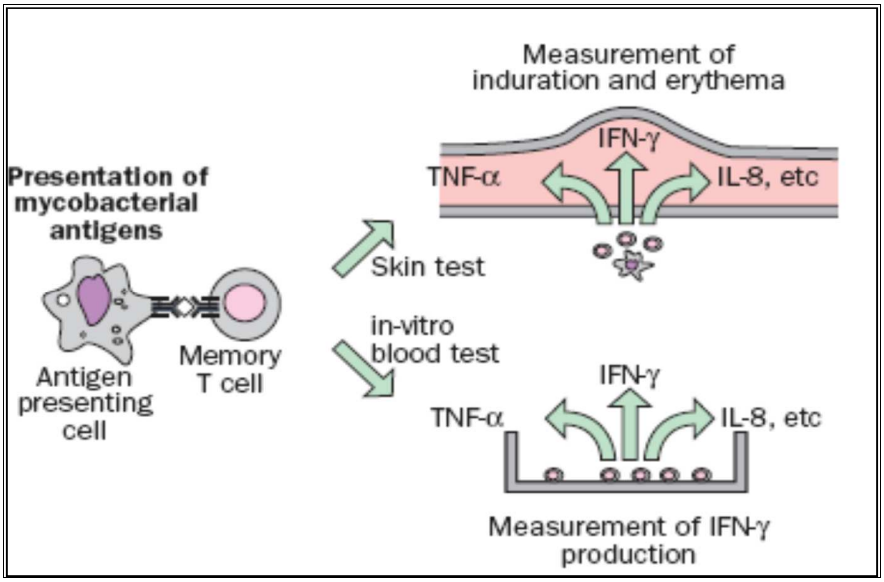
Introduction

The tuberculin skin test (TST) was until the past decade, the only test available for the diagnosis of TBI. Two commercially available Interferon Gamma Release Assays (IGRAs), the QuantiFERON-TB Gold assay (QFT-GIT; Cellestis Ltd.) and the enzyme-linked immunospot (ELISPOT)-based T-SPOT.TB (Oxford Immunotec Ltd, UK) assays have emerged as alternatives to the TST (112). The newer generation of QuantiFERON-TB Gold assay is called QuantiFERON-TB Gold In-Tube (QFT-GIT, Cellestis, Australia) and this is a whole-blood based enzyme-linked immunosorbent assay (ELISAs) measuring the amount of IFN-γ produced in response to three *M. tuberculosis* antigens (QFT: ESAT-6, CFP-10 and TB7.7). In contrast, the T-SPOT.TB (Oxford Immunotec, UK) measures the number of peripheral mononuclear cells that produce IFN-γ after stimulation with ESAT-6

and CFP-10 (112). However, both TST and IGRAs cannot distinguish infection from active disease. There is still no gold standard for diagnosing tuberculous infection. The only gold standard for a test of TBI is the future development of active disease.

TST and IGRAs are measures of cellular immunity and putative markers of latent infection. Both TST and IGRAs are currently available commercially and depend on the elaboration of inflammatory cytokines by T-cells previously sensitized to mycobacterial antigens (113) **(Figure 1.8)**.

Figure 1.8: Mechanism of action of TST and IGRAs.



Reproduced from (113)

Both tests simply determine that infection has at some point led to an acquired immune response that is detectable following re-challenge with antigen (8). Detection of an adaptive immune response towards mycobacterial antigens is only an indirect measure that represents "a footprint of a contact of the immune system with these organisms" (5).

Therefore, a positive IGRA only indicates a long-lasting T cell immune response against *Mtb*, but like the TST does not per se demonstrate the persistence of viable *Mtb* organisms. It is possible that viable *Mtb* organisms persist only in a fraction of individuals who have a persisting T cell immune response to *Mtb* antigens (114).

1.3.1 Tuberculin skin test

The principle of the TST is based on the intradermal injection of Purified Protein derivative (PPD) producing a cell-mediated immune response in the form of delayed type hypersensitivity response. The mycobacterial antigens injected below the epidermal layer, cause infiltration of antigen-specific lymphocytes and the elaboration of inflammatory cytokines. This inflammatory reaction results in the characteristic indurated area at the site of injection (figure 1.8).

TST reactions in humans are measured by the diameter of induration, measured 48-72 h after antigen injection. Clinically, the skin reaction may already start a few hours after the injection of tuberculin PPD with a white or rose-colored induration of the skin as a type-I or type-III immune reaction followed by the DTH reaction, which peaks after 48-72 hours and may last for up to 1 month, depending on the quality and quantity of the initial reaction.

The TST is one of the few tests that have been continuously in use for about 100 years in clinical medicine. Despite the shortcomings of the TST, large studies have shown that treatment of TBI, as defined by the TST, substantially reduces the risk of developing active disease (100-102). Several longitudinal clinical randomized trials have demonstrated the positive association between TST response and subsequent risk of active TB (115-118). These trials have demonstrated the two-year protective effect of PT, which reduces the risk of active disease by 60%. HIV positive individuals that have a positive TST have a 30% or more lifetime risk of developing active TB (119). In a review article by Watkins and others (116) across 10 studies the larger TST reactor category was found to have a greater risk of TB than the smallest reactor category indicating a positive association between tuberculin reactivity and the risk of TB.

Although TST has been widely used, it has well-known limitations (116, 120). Limitations in the skin test operating characteristics have a long history and have been extensively discussed (121-123). One such major limitation is that the PPD used in the skin test is a crude mixture of several antigens, many of which are shared among *Mtb, M.bovis* BCG and several non-tuberculous mycobacteria (NTM). As a result TST gives false positive and false negative results and has lower specificity in populations with high BCG and NTM exposure. In addition its sensitivity may be low in individuals with depressed immunity (AIDS, malnutrition, advanced TB, other immunosuppressive conditions). In one study in Zambia (124) only 30% of HIV-positive patients at a STI clinic had positive TST compared with 62% of HIV-negative individuals. The administration and reading of TST pose many problems such as reader variability and digit preference.

1.3.2 Interferon Gamma Release Assays (IGRAs)

IGRAs are based on the principle that T-cells of individuals sensitized by *Mtb* produce IFN-γ when they reencounter mycobacterial antigens (figure 1.8). These tests measure in vitro IFN-γ production by circulating T-cells after 16–20 hours stimulation in response to specific *Mtb* antigens. Specific IFN-γ production in response to mycobacterial antigens, therefore, is presumed to be indicative of infection with *Mtb* and can be measured by the rapid ex vivo enzyme-linked immunospot (ELISPOT) assay or by the whole blood ELISA (125). The detection and subsequent quantification of IFN-γ is the basis of these tests.

The genes encoding these antigens are found in the region of difference (RD), either RD1 (CFP-10 and ESAT-6) or RD11 (TB7.7) of the *Mtb* genome, which are deleted from the genome of *M. bovis* BCG and not present in most environmental mycobacteria, including the *M. avium* complex(5). However, homologues of the antigens are encoded within the RD-1 of *M. leprae*, wild type *M. bovis*, and other NTM (including *M. kansasii, M. marinum, M. szulgai and M. Flavescens* (113, 126).

It has been suggested that T-cells responding to the RD antigens after 24 hours stimulation are predominantly CD4 T-cells of an effector memory phenotype, consistent with having recently encountered antigen in vivo (5). In contrast, long-lived central memory T-cells are less likely to release IFN- γ after 24 hours of incubation and more likely to produce IL-2 (5).

Systematic reviews have shown that, compared with the TST, IGRAs have a higher specificity in low TB incidence settings, correlate better with surrogate measures of *Mtb* exposure, and have no cross reactivity with the BCG vaccine (127, 128). Although the replacement of the TST by IGRAs may not be imminent in resource-limited settings, IGRAs are increasingly being recommended in resource-rich countries for the diagnosis of TBI in adults. A comparison of the performance and operational characteristics of TST and IGRAS is shown in **Table 1.3**.

Table 1.3: Comparison of the performance and operational characteristics of TST and IGRAs (5, 127, 129-131)

Performance &operational characteristics	Tuberculin skin test	QuantiFERON -TB Gold In Tube assay (ELISA)	T SPOT-TB assay (ELISpot)
Administration/format	In-vivo (intradermal)	In-vitro, ELISA based	In-vitro, ELISPOT based
Antigens	PPD	ESAT-6, CFP-10 and TB 7.7	ESAT-6 and CFP-10
Test substrate	Skin	Whole blood	Peripheral blood mononuclear cells
Cells involved	Neutrophils, memory CD4 T cells and CD8	CD4 Tcells in whole blood tubes	CD4 Tcells in the wells
Cytokines involved	IFN-γ, TNF-α, IL-4, IL-10,	IFN-γ, TNF-β, TNF-α	IFN-γ, TNF-β, TNF-α
Outcome measure	Size of induration	Plasma concentration of IFN-γ	Number of IFN-γ producing T cells
Units of measurement	Millimeters of induration	International units (IU) of IFN-γ	IFN-γ spot forming cells (SFC)
Positive results	5 mm, 10 mm, or 15 mm	≥0.35IU/ml and >25% of nil	≥8SPU
Time to result	48-72 hours	16-24 hours (longer if run in batches)	16-24 hours (longer if run in batches)
[1]Sensitivity (95% CI)	77% (71-82%)	77% (CI: 75% to 80%)	92% (CI: 90% to 93%)
Sensitivity in HIV positives	60 (50 - 70%)	61% (47-75%)	72% (62%-81%)
[2]Specificity (95% CI)	97% (95-99%) non BCG vaccinated; 59% BCG vaccinated	99% (98-100%) Non BCG vaccinated; 96% (94-98%) BCG vaccinated	93% (86-100%) BCG vaccinated
Cross-reactivity with BCG and NTM	Yes	Less likely but can cross react with some NTM	Less likely but can cross react with some NTM
Inter-reader variability	yes	No	Variation in counting spots (if manual)
Laboratory infrastructure	No	Requires laboratory expertise & equipment.	Requires laboratory expertise & equipment.
Reliability (reproducibility)	Moderate	High	Limited evidence but may be high
Correlation with *Mtb* exposure	Yes	Yes (correlated better than TST)	Yes (correlated better than TST
Association between test positivity & risk of active TB	Moderate to strong positive association	Modest but evidence still limited	Modest but evidence still limited
[3]Benefits of treating test positives	Yes	No evidence	No evidence
[4]Boosting phenomenon	Yes	No	No
Potential for conversions and reversions	Yes	Yes	Yes
Cost per test($)(106)	12.73	41	85

.[1]May be positive with M. *kansasii* and M. *marinum* species.[1]**Sensitivity**: TB patients with active disease, most studies had no HIV positive participants.[2]**Specificity**: healthy individuals with low risk or without known exposure to TB. [3]Based on randomized control trials.[4]Boosting phenomenon: initial test influencing the subsequent test.

1.3.3 What is the current use of IGRAs in clinical practice?

IGRAs are now licensed for clinical use in many countries such as USA, UK and Canada (132, 133).

There has been growing interest in the use of IGRAs especially in low TB incidence high-resource countries(134). There are now 33 guidelines and statements from 25 countries (135) with considerable diversity in the approaches. However, these guidelines are predominantly from high-income countries with established TBI screening programs. In low TB incidence and high-resource settings, the higher specificity of IGRAs and their logistical advantages seem to enhance their adoption and usage.

In high TB incidence and low resource countries, the TST is still preferred because there is no strong evidence that IGRAs are superior to the TST in such settings, especially given the significantly higher costs associated with IGRAs. There is currently no high incidence and low-resource country that has published guidelines on IGRAs. However, IGRAs are available in some countries, such as India and South Africa, although they are being used mostly in the private sector and in research settings (135).

Current guidelines are characterized by four main approaches as shown in **Table 1.4** (134):

1) A two-step approach of TST first, followed by an IGRA for confirmation.
2) IGRA only, replacing the TST
3) Both TST and IGRA together
4) Either the TST or an IGRA, but not both.

These considerations are based mainly on cost effectiveness calculations. One recent study found that the use of IGRAs as a screening tool, either alone or in conjunction with the TST, for detecting TBI was a more cost effective alternative than using the TST alone (136).

Table 1.4: Recommendations for contact Investigations in adults (134)

Recommendation	Guideline or position statement*
TST alone	WHO, Brazil, ECDC (high-incidence countries)
TST followed by IGRA, if TST positive (either IGRA only in BCG vaccinated persons or independent of BCG vaccine)	Canada (low –risk contacts), Germany, Italy, Switzerland, Spain, Saudi Arabia, the Netherlands, Norway, Bulgaria, Portugal, Ireland, ECDC (low incidence countries), and for the UK and South Korea only in adults <35 years old.
Both TST and IGRA	Canada (High–risk contacts), Czech Republic, Croatia, Austria, Australia (IGRA may be considered in addition).
Either TST or IGRA	USA, Denmark, Finland (IGRA preferred if BCG-vaccinated all three countries, South Korea (only adults <35 Years old), Austria.
IGRA alone	Slovakia, Japan France
CDC, US Centers for disease control and prevention; ECDC, European centre for disease prevention and control: *Some countries /organizations are listed more than once because their recommendations vary across risk groups	

Most of these guidelines are based on expert opinion and not evidence based. Future guidelines will need to consider impact of IGRAs on patient outcomes and cost-effectiveness in various settings (135).

1.3.4 Limitations of using IGRAs as diagnostic tests for *Mtb* infection.

1) As with the TST, both false negative and false positive results can occur with IGRAs such as QFT-GIT.
   a) A negative IGRA result does not preclude the possibility of *Mtb* infection or disease: false-negative results can be due to the stage of infection (e.g. specimen obtained prior to the development of cellular immune response), medical conditions which affect immune functions, incorrect handling of the blood collection tubes following venepuncture, incorrect performance of the assay or other immunological variables.
   b) A positive IGRA result is not a definitive basis for determining infection with *Mtb*. Incorrect performance of the assay may cause false positive responses. Assessing the probability of TBI requires a combination of epidemiological, historical, medical and diagnostic findings that should be taken into account when interpreting results. Therefore, additional medical and diagnostic evaluations may be needed to make a diagnosis.

c) While ESAT-6 and CFP-10 are absent from all BCG strains and from most known nontuberculous mycobacteria, it is possible that a result may be due to infection by *M. kansasii, M. szulgai* or *M. marinum*.

2) Blood samples must be processed within 8-16 hours after collection while white blood cells are still viable.

3) Errors in collecting, transporting and processing blood specimens may influence the actual results obtained. For instance, technical issues that affect results include:
   a) Time from blood drawing to incubation longer than 16 hours.
   b) Storage of filled blood collection tubes outside the recommended temperature range (22° C± 5°C) prior to incubation.
   c) Insufficient mixing of blood collection tubes.
   d) Incomplete washing of the ELISA plate.
   e) Variation to the stated pipetting and washing techniques.

   Variations caused by these factors may be difficult to distinguish from variations caused by immunologic responses to *Mtb* exposure.

4) IGRA results may also be affected by other individual factors that are a result of immunologic responses to *Mtb* exposure e.g. within-personal biological variation, new infection (conversion), transient infection (137) or reversions. Existing studies suggest that IGRAs are highly dynamic tests whose T-cell responses especially weakly positive responses tend to fluctuate over time and that just like the TST; they are prone to conversions, reversions and non-specific variations (138-140).

5) IGRAs have higher resource demands when compared to the TST, as they require laboratory access, trained personal and implementation of quality-assured procedures. As they are technically more demanding, IGRAs are a more costly diagnostic tool. However, the reading and analysis of test results can be done by batch, thus reducing the cost but also increasing the time until results are known.

6) IGRAs require phlebotomy

1.4 Aim and Objectives

1.4.1 Hypothesis

Household contacts with high (≥10 IU/ml) levels of IFN-γ in response to *Mtb* specific antigens (ESAT-6, CFP-10 and TB 7.7) in the QFT-GIT assay are at higher risk of developing TB compared to those with low levels (> 0.35-<10 IU/ml).

There is data indicating that a high response to ESAT-6 and CFP-10 reflects *Mtb* infection . However, what constitutes a 'high' response may be context-dependant and might differ from TB endemic to TB non-endemic regions and from HIV uninfected to HIV-infected individuals (50). The estimate of 10 IU/ml has been used elsewhere (141) and is the maximum value the QFT-GIT ELISA software can read.

Other literature have defined strong responders or those with high levels as those with IFN-γ levels tenfold higher than the cut-off point of 0.35 IU/ml (142).

1.4.2 Aim

To determine the association between the magnitude of T-cell Interferon-γ response to *Mtb* specific antigens (ESAT-6, CFP-10 and TB 7.7) in the QFT-GIT assay and the risk of progression to TB post *Mtb* exposure.

1.4.3 Primary Objective

To determine whether household contacts with high (≥10 IU/ml) levels of IFN-γ in response to *Mtb* specific antigens (ESAT-6, CFP-10 or TB 7.7) in the QFT-GIT assay are at higher risk of developing TB compared to those with low level (> 0.35-<10 IU/ml).

1.4.4 Secondary objectives

1) To determine the performance and operational characteristics of QFT-GIT in a field setting.
2) To determine risk factors associated with positive QFT-GIT and TST results at baseline.
3) To determine the level of agreement or concordance between QFT-GIT and TST results.
4) To determine the incidence rates of TB in household contacts with positive and negative QFT-GIT and TST results, also stratified by HIV status.
5) To determine the association between QFT-GIT and TST positivity and the subsequent risk of development of TB.
6) To determine risk factors associated with incident TB.

7) To determine the association between the magnitude of QFT-GIT and TST responses and the subsequent risk of development of TB.

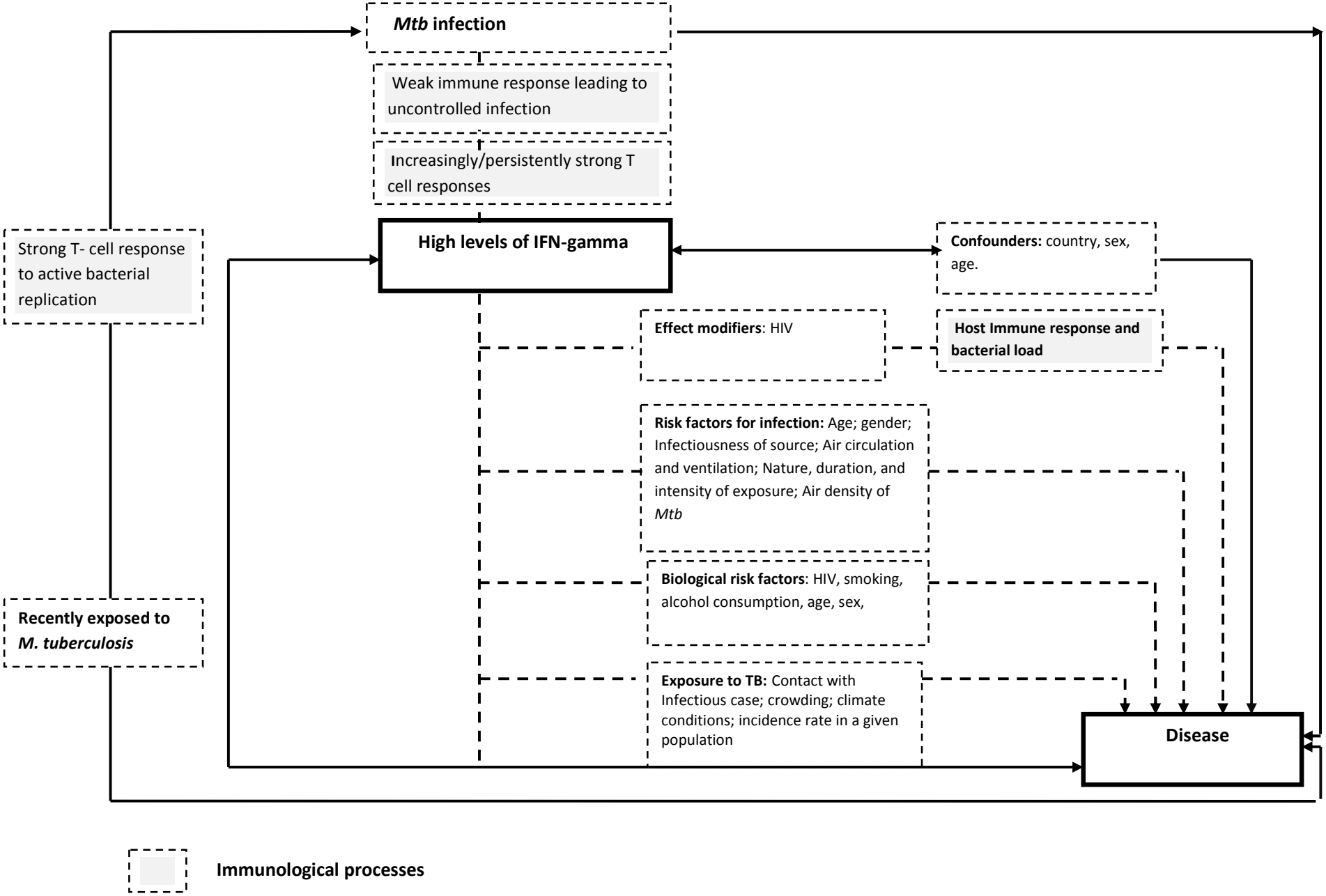
1.4.5 Research questions

1) Are household contacts with high (≥10 IU/ml) levels of IFN-γ in response to *Mtb* specific antigens (ESAT-6, CFP-10 or TB 7.7) in the QFT-GIT assay at higher risk of developing TB compared to those with low level (> 0.35-<10 IU/ml)?
2) What is the performance and operational characteristics of QFT-GIT in a field setting?
3) What risk factors are associated with positive QFT-GIT and TST results at baseline?
4) What is the level of agreement or concordance between QFT-GIT and TST results?
5) What are the incidence rates of TB in household contacts with positive and negative QFT-GIT and TST results, stratified by HIV status?
6) What is the association between QFT-GIT and TST positivity and the subsequent risk of development of TB?
7) What risk factors are associated with incident TB?
8) What is the association between the magnitude of QFT-GIT and TST responses and the subsequent risk of development of TB?

Conceptual Framework

There are various factors to consider when contextualizing the impact of high levels of interferon-γ on TB incidence. It is by setting in place this context that this study will facilitate an understanding of how high levels of interferon-γ in the QFT-GIT assay can affect risk of progression to TB. There is currently limited understanding and evidence of this relationship. The suggested conceptual framework is shown in **Figure 1.9**

Figure 1.9: A conceptual framework showing host immune response following exposure, IFN-γ levels and disease within a 2-3 year period.

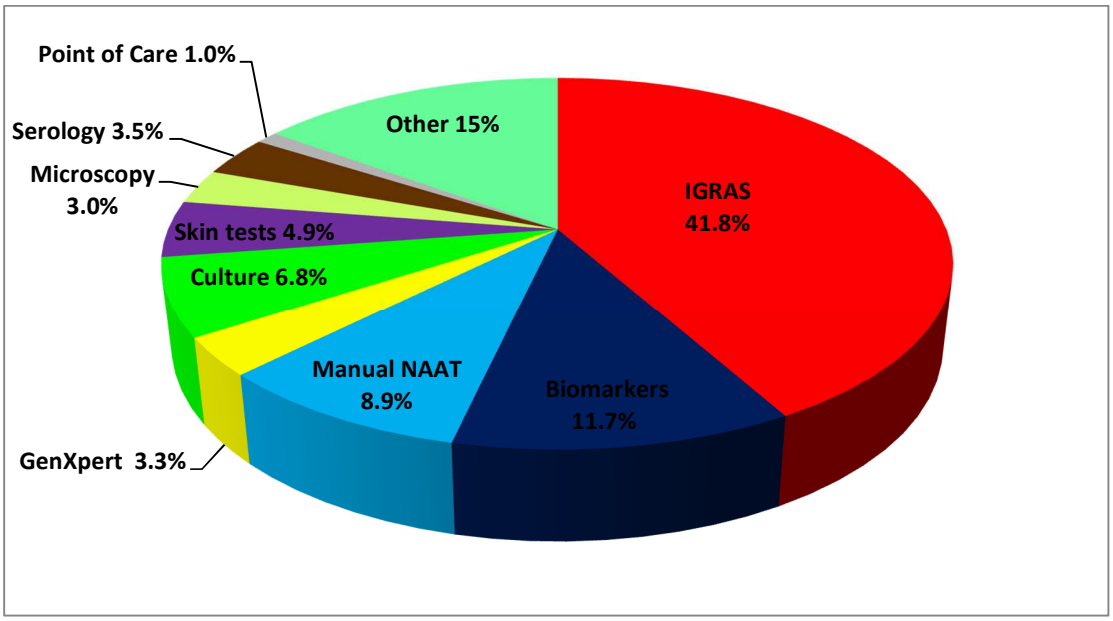


2.0 Literature Review

An overwhelming publication of IGRA literature

Research on IGRAs has been ongoing at unprecedented rate with an extremely large number of papers cumulating over the recent years. There are more than 250 published papers on IGRAs since 2001 (143). This field has been an extremely rapidly evolving field with large series of expert reviews and expert opinions and numerous guidelines issued worldwide as well as systematic reviews or meta-analyses. A PubMed survey of studies looking at TB diagnostics: 1st January 2008 to 7th August 2011 showed that almost 42% of studies on TB diagnostics focused on IGRA (143) **(Figure 2.1)**

Figure 2.1: A PubMed survey of studies looking at TB diagnostics: 1st January 2008 to 7th August 2011.



Modified from (143)

In this chapter I will attempt to review IGRA evidence in relation to the most relevant and critical analytical studies. The objectives of this chapter are:

1) To collate and review the most relevant analytical studies on the association between the magnitude of T-cell Interferon-γ responses to *Mtb* specific antigens and risk of progression to disease.
2) To critically review the above evidence with reference to the following:

a) Whether *Mtb* infected individuals with strongly elevated IFN-γ levels are most likely to progress to active disease?
b) Whether IGRAs are better than the TST at predicting individ*u*als who have a high risk of progression to active TB?
c) The concordance between IGRAs and TST results.
d) Effect of HIV on IGRAs.

2.1 What is the performance and operational characteristics of QFT-GIT in a resource-limited field setting with a high burden of TB and HIV?

Each time a new product is launched on the market, one of the important questions that consumers would want to know is accessibility, practicability, affordability and whether it is better than existing products and in what ways. This was no different for IGRAs. The development of IGRAs brought a lot of hope for TB diagnostics after a decade of using one test for the diagnosis of TB infection, the TST. To be useful, diagnostic tests need to be accurate, simple and affordable for the population they are intended. It is still not widely acknowledged whether IGRAs represent "true hope for TB diagnostics" as an alternative to the TST for poor resource settings with a high burden of TB and HIV.

The evaluation of any diagnostic test involves assessing performance and operational characteristics. Test performance involves determining the sensitivity and specificity, and the positive and negative predictive values of a test (144) and reproducibility. In evaluating the operational characteristics of a test, important considerations include stability of the test under ideal and less ideal conditions, time taken to perform the test, user acceptability, ease of use from the field to less specialized laboratories as well as some financial costs.

In poor resource settings with a high burden of TB and HIV, a lot of factors are at play that may determine how IGRAs work**.** According to WHO, novel technologies successfully introduced into developed countries require adaptation to match the needs of developing countries (145), which includes feasibility and cost effectiveness considerations. In developing countries potential drawbacks include the cost, the need for highly skilled staff and a good laboratory infrastructure (with capacity to run ELISA or ELISPOT), the high HIV prevalence as well as logistical issues (e.g. power shortages) peculiar to these settings. In addition, stringent control of multiple variables, from blood collection, processing and storage in the field to performance of the test in the laboratory may be difficult to control in resource-limited settings. QFT-GIT indeterminate results can occur due to improper assay techniques (e.g. time delays that lead to loss of viable T cells in the specimens)(146). Many studies have now shown that HIV has an effect on the performance of IGRAs (131, 147, 148).

Many countries with low TB incidence now have guidelines on the use of IGRAs for the diagnosis of tuberculous infection. In these settings, IGRAs are believed to have many advantages over TST in particular an increase in specificity (127, 128). However, their use is limited in high TB incidence countries as there is a dearth of data on their performance in these settings.

Systematic reviews have suggested that IGRA performance differs in high versus low TB and HIV incidence settings, with relatively lower sensitivity in high-burden settings (149).

Other factors that affect the immune response and hence the outcome of IGRAs (150) are at play in high TB incidence poor resource settings. In particular, the presence of universal BCG vaccination, poverty related malnutrition, tropical infections, helminths and widespread exposure to NTM might pose unique challenges. Protein-calorie malnutrition is associated with impaired cellular and humoral immunity, the basis of immunodeficiency thought to be metabolic disturbances, which adversely affect maturation and function of cells of the immune system (151).

Although the specific antigens encoded within the RD-1 of *Mtb* are absent in many NTM, their presence in other species like in *M. leprae* and *M. kansasii* can pose challenges if prevalent. Theoretically, the presence in the host of some NTM could cause a false-positive IGRA result. The presence of environmental mycobacteria in Zambian communities was evident in two recent large tuberculin skin test surveys in the ZAMSTAR study (95). A study in Malawi (152) found a high rate of IFN-γ responses to PPD of atypical mycobacteria, especially to those of MAIS complex (*M. avium*, *M .intracellulare, M. scrofulaceum* and *M. marinum*) which was strongly associated with positive TST response to human PPD. Only *M. kansasii* can cause disease similar to tuberculosis and it does so only infrequently (113).

A small number of cost and cost-effective analysis studies comparing different TBI screening strategies have been conducted. However, most studies were conducted from low TB incidence settings where TB control effort focuses on high-risk subgroups (153-155) and where healthcare costs may be different to high incidence countries. The potential savings from switching to IGRAs such as the reduced costs of unread tests, missed cases, preventable cases and reduced false positive tests need to be studied further in specific endemic populations. Currently, the cost-effectiveness of IGRAs in routine programmatic settings; in high incidence settings is unknown.

Recently WHO strongly recommended (156) that IGRAs should not be used in the diagnosis of active TB, diagnosis of TBI in children and HIV infected individuals; screening health care workers, contact screening, outbreak investigations and predicting development of TB in low- and middle-income countries, typically those with a high TB and/or HIV burden. WHO noted that the majority of IGRA studies had been performed in high-income countries and mere extrapolation to low- and middle-income settings with high background TB infection rates was not appropriate (156). However, the evidence upon which these recommendations were made was insufficient and was of low quality. It was also noted that given the comparable performance of IGRAs and TST but increased cost and technical complexity of IGRAs no real advantage was seen (156). These recommendations did not apply to high-income countries or any national guidelines that may have been written for such settings.

2.2 Are *Mtb* infected individuals with strongly elevated IFN-γ levels most likely to progress to active TB?

Despite a growing body of literature on IGRAs, several questions remain unanswered (24, 157). There is still a widely recognized need for large longitudinal studies with clinical outcomes to determine the prognostic value of positive IGRA results in the development of TB (106,141, 158). This is particularly important for highly endemic TB settings with large numbers of latently infected HIV positive individuals who may require preventive therapy.

There are few studies available that describes the association between the changes in IFN- γ responses in IGRAs and disease prediction (9). It has been postulated that individuals with strong and/or rising IFN-γ responses to QFT-GIT may be at greater risk for progressing to active disease (28, 33, 50, 141). Literature from studies done among healthy HHCs suggest that recently exposed individuals have different ESAT-6/CFP-10 response levels (22, 28). Some of the earliest studies done among HHCs in Ethiopia have demonstrated this (22, 28) and recently similar evidence has emerged (35, 141, 142, 159). It is thought that among such an exposed group of individuals are those with strong ESAT-6/CFP-10 responses or high levels of IFN-γ in response to *Mtb* specific antigens. It is believed that these recently exposed individuals offer a vigorous T-cell response as a result of active bacterial replication (39).

A study done in the Gambia (39), found increased proportions of responders and intensities of responses to ESAT-6 and TST in HHCs than community controls. In addition, higher frequencies of lymphocytes producing IFN-γ in response to ESAT-6 and TST were reported in recent converters (PPD) than in healthy controls in Germany and the United States (38). In another study done in Pakistan among 109 HHCs, CFP-induced IFN-γ showed consistent increases during the first 6 months until 12 months post exposure and then dropped to the baseline level at 24 months (13).

It has been suggested that such high responses are not only typical of recently exposed individuals but are suggestive of an early stage of infection by *Mtb*, which could in time result in overt disease or containment of the infection (160). Consequently, such strong increases in T-cell response after recent exposure might predict progression to active disease (28, 107). It has been hypothesized that IGRAs detect responses of partially activated, effector T cells that have recently encountered antigens *in vivo,* and can therefore rapidly release IFN-γ when stimulated *in vitro* (52). It is plausible that unlike the TST, IGRAs may be measuring recent rather than old infection and therefore associated with higher rates of progression to active TB.

Several studies of HHCs of TB patients suggest that in HIV negative individuals, high or increasing concentrations of TB-specific IFN-γ production might predict overt TB, although the numbers of cases in these studies are small (28, 33, 141). A recent study done among HHCs in Colombia (35) explored whether IFN-γ production in response to CFP-10 and non-specific (CFP) *Mtb* antigens at baseline were predictive of active TB . In this study, individuals with high IFN-γ levels in response to CFP-10 were at higher risk of developing TB than those with low levels early after exposure to

a pulmonary TB case. The results showed an almost 3-fold difference in incidence rate between the highest IFN-γ producers at baseline compared with negative responders. Disease incidence was found to be highest in strong responders to CFP-10, whilst those with medium and low levels of IFN-γ production had intermediate incidence levels. There was also a significant tendency of increasing hazard rates of disease development with increasing IFN-γ production levels in response to CFP-10 observed during follow-up. A limitation of this study is that an in-house assay was used that incorporated a 7 day whole blood culture in contrast to the 24-hour incubation used by commercial IGRAs. By virtue of the extended lymphocyte culture times used, these measures may primarily detect central memory responses while short incubation IGRAs detect mostly effector memory T cell responses of recently activated lymphocytes.

In a study conducted in Germany (141), all six subjects who developed TB showed a high IFN-γ above 10 IU/ml as well as in the follow up study (142) in which a larger cohort was examined. In this follow up study, 16 of the 19 people who developed active TB were strong responders in the QFT-GIT assay, with IFN-γ levels more than tenfold higher than the cut-off of 0.35 IU/ml. 10 of the 19 had levels above the 10IU/ml upper limit for the test's ELISA. Progression to disease was significantly associated with IFN-γ level; the chance of developing TB nearly doubled with every unit of IFN-γ /ml.

In a study done in Senegal (159), there was a quantitative relation between the level of ELISPOT response and the risk of development of TB within two years follow-up. HHCs with an ELISPOT response of 32 SFC/10^6 PBMC or greater had nearly a two-fold increased rate of developing TB in follow-up compared to those with a response <32 SFC. In a contact investigation study done in Japan (161), contacts with high levels of IFN-γ production in response to either ESAT-6 and/or CFP-10 had a higher risk of developing active TB than those with lower levels. In this study there was an increasing trend of disease risk with increasing quartile (odds ratio of 4^{th} quartile compared to 1^{st} quartile was 4.04). However, the study was not based on a pure prospective design, the diagnosis of TB was done using radiological findings only and the sample size was small. In addition the authors did not specific the quantitative values of IFN-γ for the quartiles indicated.

Other studies have found contrasting findings. In the study in Turkey in children contacts (158), although a positive ELISpot result was prognostic of progression to TB, the magnitude of this response did not further refine the risk for progression. Within ELISpot-positive contacts, the size of the baseline IFN-γ response to peptides did not significantly differ between contacts who developed TB and those who did not.

Some studies that have looked at the prognostic ability of IGRAs have used a single test showing an elevated IFN-γ response which does not provide information on when the response became elevated (whether this was recently or in the past) or whether it has stayed elevated for a long time (whether it has been persistently elevated or not)(162).

There have been suggestions to use quantitative as well as qualitative results of IGRAs. However, the significance of the quantitative test results is unclear at present, especially whether very high test results could indicate an increased risk of progression. The quantitative results from IGRAs have not yet been shown to have prognostic value. Hence it is important to determine whether quantitative IFN-γ responses are predictive of those who have a high risk of progression to active TB. Evidence provided by studies described in this section suggest that this approach is worth exploring (107).

2.3 Are IGRAs better than the TST at predicting individuals who have a high risk of progression to active TB?

Much has been speculated about the possible advantages of IGRAs over the TST in diagnosing TB infection. However, few studies to date have addressed the actual purpose of these assays, namely, the prognostic ability of IGRAs to accurately identify individuals with latent infection who are at the highest risk for progression to active TB (24, 157, 163). This is particularly important for highly endemic TB settings with large numbers of latently infected HIV positive individuals who may require preventive therapy. If an IGRA has better sensitivity of predicting active TB than the TST, its use would lead to fewer people being ‘‘identified’’ as positive and, thereby, fewer people to evaluate for active TB and fewer being offered preventive therapy (141) . Such an outcome would focus scarce TB-control resources on those individuals most likely to progress to active TB and reduce transmission (107, 157).

To date, 13 longitudinal studies have been published assessing the predictive value of IGRAs (35, 159, 164-166). However, some of these are designed as contact investigation studies (8-12) in low TB burden countries. Out of the 13 studies, only five are large cohorts with over 2000 participants. Only two studies have been done from countries with a high burden of TB and HIV; one among adolescents in SA and another among pregnant HIV infected women in Kenya (166, 167). Given the paucity of data from such high burden countries, the present study was conducted in two countries with high burden of TB and HIV.

The evidence from studies evaluating the predictive value of IGRAs has been conflicting .The value of IGRAs in predicting the development of active TB has been reported to be higher than the TST in some studies (141, 168) and similar (158, 164, 169, 170) in others. The positive predictive value for the development of TB is most likely to be higher with an IGRA than with the TST because of the higher test specificity and sensitivity (although this is similar in high TB burden settings). It is unclear if these different outcomes can be attributed to the different IGRAs used, different cut-offs used for TST, the different populations, or to differences in TB prevalence.

Some studies have shown that IGRAs perform similarly to the TST in predicting risk of subsequent TB. The largest prospective study investigating the predictive value of TST versus an IGRA was conducted among 5244 adolescents in Cape Town, SA (169). This study showed that adolescents with either a positive QFT-GIT or TST result at baseline had a three-fold increased

risk of active TB than those who were negative. Another large longitudinal study (164) done in Gambia among 2348 HHCs found that the presence of either a positive ELISPOT or TST result at baseline was associated with a two-fold increased risk of developing active TB compared to those who were ELISPOT or TST negative. These findings are similar to those of a study among 2679 HHCs in Senegal (159). However, the studies done in Gambia and Senegal used in house EC ELISPOT assays in contrast to the commercial types reported in other studies. Among child contacts of persons with active TB in Turkey, positive ELISpot or TST predicted a three-fold increased risk of active TB compared to those that had negative results, although the evidence was stronger for ELISPOT than for TST.

Other studies have shown that IGRAs perform better than the TST in predicting risk of subsequent TB. A study following 601 exposed HHCs in a German metropolis found a progression rate to active TB in QFT-IT positive individuals of 14.6% compared with 2.3% in those who were TST positive (141). However, all TB cases occurred in persons who refused PT. In another study done in China among 308 silicosis patients, a positive T-SPOT test independently predicted the subsequent development of active TB while TST did not, irrespective of the cutoff values (168). However, silicosis patients have a much higher risk (2.8 to 39 times higher, depending on the severity of the silicosis) of developing TB compared to healthy controls (171).

In a systematic review done by WHO on the use of IGRAs in low- and middle-income countries (111), both IGRAs and the TST appeared to have only modest predictive value and did not help to identify those who were at highest risk of progression to active disease. The vast majority of individuals (>95%) with a positive IGRA results did not progress to active disease during follow-up, although a modest but statistically insignificant increase in incidence rates of TB in IGRA-positives compared to IGRA-negatives was observed. However, the quality of evidence used to determine the predictive value of IGRAs was poor.

There are only three large studies that have evaluated IGRAs for prediction of active TB in HIV positive adults (167, 172, 173). All of these studies were done among HIV positive individuals within a clinic or hospital setting and lacked a comparison group of HIV negative individuals. Therefore, these results cannot be extrapolated to immunocompetent populations. However, the studies do show that IGRA positivity at baseline is associated with a much greater risk of active TB, in comparison to studies involving immunocompetent HHCs.

The largest longitudinal study among 822 HIV positive individuals was conducted in Austria (172). Of 37 individuals who were QFT-IT positive, three (8.1%) developed active TB during a follow-up period of 19 months. None of the QFT-GIT-negative individuals progressed to active TB, indicating a very high negative predictive value. Limitations of this study are that 60% of individuals were on ART at enrolment hence lowering the risk of active TB. In addition, even though all individuals were screened for TB symptoms at baseline, more intensive screening with chest X-ray, smear, culture, PCR & TST were done in those who were QFT-GIT positives.

This additional work up introduced verification (work-up bias) making QFT-GIT positive individuals more likely to have disease than negatives.

Similar results were observed in a study done in Kenya among 333 HIV positive pregnant women (167). In multivariate analysis, adjusting for baseline CD4 count, IGRA positivity was associated with an almost 5-fold increased risk of active TB. Among women with baseline CD4 cell counts of less than 250 cells/mL, IGRA positivity was associated with a 5-fold greater risk of active TB or death. In the study done in Gambia described above (164) similar results were obtained despite the small number of HIV positive HHCs (2%) i.e. ELISPOT positivity was associated with a 6-fold greater risk of active TB.

Although IGRAs seem to predict subsequent active TB better than or similar to the TST, the majority of the high risk people with positive tests will not develop active TB (142). Thus, studies so far have shown that IGRAs have a low-modest positive predictive value (PPV)(142). The PPV for active TB represents the probability that an individual who tests positive is truly at risk of developing active TB disease later in life. In a systematic review, PPV was determined among individuals who had refused PT in two-year follow up studies (141, 170, 172, 174). The PPV range for QFT-GIT, T-SPOT and TST was 2.8% to 14.6% ; 3.3% to 10% and 2.3%to 3.1%, respectively (175). However, only four studies were included, the study designs varied widely and all of them were done in low TB burden settings. Nevertheless, the results fall within similar range when compared to studies done in high TB prevalence settings. For instance, the PPV for IGRAs for the studies done in Kenya among HIV positive pregnant women and among adolescents in SA were 5.5% and 1.7% respectively (166, 167).

A NPV for active TB reflects a test's ability to correctly predict that an IGRA negative individual will not develop active TB in later life, provided they have no further exposure to infection. In HIV negative individuals, the NPV of IGRAs for active TB is very high either alone or combined with a negative result of the TST. The NPV of IGRAs seems similar to that of TST, falling within a range of 98%-99.8% (142). Hence, among individuals with negative IGRA results, subsequent active TB in the next 2-3 years following infection seems to be extremely low (142, 164).

Despite the evidence presented above, there is still no consensus yet. This is because comparison between studies is limited by diagnostic flaws. Some of these flaws include studies with small number of participants, verification bias, lack of blinding leading to interpretation bias, short duration of follow-up, incorporation bias, use of an inappropriate reference standard and failure to report design features. The PPV is influenced by the prevalence of disease in the population that is being tested. If the test is done in a high prevalence setting, it is more likely that individuals who test positive truly have disease than if the test is performed in a population with low prevalence.

NPV and PPV for IGRAs remain to be established in the pediatric population and in immunocompromised individuals especially in HIV positive individuals (131).In contrast, randomized controlled trials in HIV infected persons demonstrate that PT confers a 20% to 60%

reduction in the risk of active TB among persons with positive TST (115, 131). Unfortunately, similar high-quality data on predictive value of IGRA testing are currently lacking. The studies evaluating the predictive value of IGRAs are summarized in **table 2.1.**

Table 2.1: The association between positive IGRAs results and progression to active TB in selected studies

Author, year of publication, country	Study population	Follow up time (years)	Assay used	Positive test n/N (%) or baseline prevalence	[1]aHR for positive IGRA results (IRR or PPV (95% CI)	aHR for positive TST (≥ 10 mm)
High TB incidence						
Mahomed et al, 2011, SA	5244 adolescents	2.4(median)	QFT TST10	51% (2669/5244) 42% (2214/5244)	IRR: 2.9 (1.6-5.2)	IRR: 2.7 (1.4-5.0)
Jonnalagadda et al, 2010, Kenya	333 HIV-+ve pregnant women	2	T-SPOT.TB	36% (120/333)	4.5 (1.1-18.0)	
Medium TB incidence						
Lienhardt et al, 2010, Senegal	2679 HHCs adults & children	2	ELISPOT (20 SFC) TST10	65% (615/952) 65% (1591/2458)	2.0 (0.8-5.1)	1.6 (0.7-3.2)
del Corral, 2009, Columbia	2060 HHCs adults & children	2-3	ELISA (CFP-10) TST10	66.3% (1310/1977) 66% (331/502)	1.8 (0.79-4.20)	
Hill et al, 2008, Gambia	2348 HHCs adults & children	2	ELISPOT TST10	37% (649/1736) 38%	1.8 (0.8-4.2) HIV +ve:6.2 (1.7-22.5)	1.8 (0.8-4.1)
Doherty et al, 2002, Ethiopia	24 HHCS adults	2	ELISA (PPD)	9/24 (38)		
Low TB incidence						
Diel et al, 2010, Germany	954 contact adults & children	4	QFT-GIT TST10	21% (198/954) 25%(242/954)	PPV:12.9%	PPV:4.8%
Yoshiyama et al, 2010, Japan	3102 contacts adults & children	19 months	QFT-G TST many cutoffs	13.5% (419/3102)	6.7 (3.6-12.6) (RR)	
Harstad et al, 2010, Norway[1]	823 asylum seekers	23-32 months	QFT-G	30% (246/823)	IRR: 18.8 (2.4-149)	
Leung, et al, 2010, Hong Kong	308 silicosis patients	2.5	T-SPOT.TB TST10	66% (204/308) 66% (203/308)	4.55 (1.1-20.4)	1.8 (0.5-6.0)
Aichelburg et al, 2009, Austria	830 HIV positives	1.6	QFT-GIT	5.6% (44/783)	PPV :8.3% (1.8-22)	
Kik et al, 2009, Netherlands	339 contact immigrant adults	2	QFT-GIT T-SPOT.TB TST10	54% (178/327) 61% (181/299) 85% (288/339)	PPV: 2.8% (1.0-4.6) PPV: 3.3% (1.3-5.3)	PPV: 3.1% (1.3-5.0)
Diel et al, 2008, Germany	601 close contacts	2	QFT-GIT TST10	11% (66/601) 18% (110/601)	PPV: 14.6% (6.9-28.4)	PPV:5.6%
Bakir et al, 2008, Turkey	908 contact children	1.3	T-SPOT.TB TST10	42% (381/908) 61% (550/908)	IRR: 3.4 (1.1-10.7)	IRR:2.6 (0.7-14.6)

Notes: In all studies TB diagnosis was largely based on smear and culture results.IRR: incidence rate ratio. [1] HR: Hazard ratios of positive IGRAs/TST in comparison to negative IGRAs/TST at baseline. If aHR is not reported then IRR (incidence rate ratio) or PPV (positive predictive value) is used.

2.4 Concordance between IGRAs and TST results

Evaluating the strength of agreement (concordance) between TST and IGRAs avoids the use of either test, as the gold standard (129) and comparisons cannot demonstrate which test is superior. Discrepancies encountered may be the result of limitations in the TST or limitations in the IFN-γ assays.

There are several studies that have looked at concordance between TST and IGRAs (129, 176, 177). However, evidence so far is conflicting. Comparison between studies is difficult due to differences in the type of assays (in-house assays vs. commercial, different formants); incubation times, type of antigenic preparations, TST cut offs, PPD dose, BCG status, HIV status, other predisposing individual risk factors for *Mtb* infection as well as prevalence of TB and NTM in the population. Like TST, IGRAs are susceptible to factors affecting host immune responses, although the degree of susceptibility may differ between the two tests. Overall, such factors may influence concordance between assays and need to be taken in consideration when comparing studies.

Agreement studies comparing TST and IGRAs have commonly used the percent agreement and kappa statistics. However, there is wide disagreement about the usefulness of kappa statistics to assess rater agreement (178, 179). Although Kappa statistics are appropriate for testing whether agreement exceeds chance levels for binary and nominal ratings, controversy still exists. Others argue that kappa statistics should not be viewed as the unequivocal standard or default way to quantify agreement due to various reasons. Kappa is not really a chance-corrected measure of agreement and it does not make distinctions among various types and sources of disagreement. It is also influenced by trait prevalence (distribution) and base-rates. As a result, kappas should be compared with caution across studies, procedures, or populations. Furthermore, kappa may be low even though there are high levels of agreement and even though individual ratings are accurate. There is not, however, agreement about what to do about all these issues and meanwhile studies have continued reporting kappa statistics.

A systematic review (24) comparing TST with different interferon-γ assays among 17 studies found modest to high agreement (60% to 80%) even though the kappa statistics were highly inconsistent ranging from –0.03 to 0.87. In this review, QFT-TB assay (PPD) was used as a comparison in 59% of the studies; none of these studies used QFT-GIT and those using TSPOT were of small sample size apart from one (180). Another review among HCWs found poor levels of agreement between TST and QFT-G in 25 studies and a predominant pattern of TST+/QFT- results (181). In a recent community survey among 652 apparently healthy adult pastoralists in Ethiopia, there was poor agreement between TST and QFT-GIT (66%; k=0.2) (182). In a study in Cape Town among 43 HIV positive adults and children there was moderate agreement between the TST and QFT-GIT (k=0.46) in adults (147).

A study (129) conducted in India among 726 health care workers in a rural hospital reported a high degree of agreement (81%, k=0.61) as compared to the poor agreement rates reported in

most studies from high TB incidence settings (176, 183, 184). Similarly in a study done in hospitalized children in India, substantial concordance between TST and QFT-GIT (95.2%; k= 0.73) was reported (177) despite the high level of malnutrition in these children. Results from these two studies showed much higher levels of agreement compared to most studies probably due to differences in PPD dose (TU was used in both studies).

Agreement levels have been shown to be better among HIV positive individuals compared to HIV negative ones. However, most of such studies, especially from high TB incidence settings are limited by small sample sizes. A small study done in Zambia using TSPOT among 49 healthy adults (29% were HIV positive) found a higher concordance rates in HIV positives (64%, kappa 0.26) compared to HIV negative individuals (185). In a study done in Cape Town (186) among 160 adults (74 HIV infected), fair agreement between the TST and the IGRAs was seen in HIV-infected people (k= 0.52–0.6). In contrast, poor agreement between the TST and QFT-GIT tests was observed in the HIV- negative group (k= 0.07-0.30). The pattern was similar for T-SPOT.*TB* (k= 0.18–0.24).

A recent systematic review conducted in HIV positive individuals (131) among 15 studies from various countries evaluated agreement between TST and IGRAs. In this review, results were reported according to whether the study was done in a low/middle income (Sub-Sahara Africa, China, and South Africa) or high income country (Switzerland, Italy, Spain, Germany, USA).

Overall, estimates of percent concordance were higher in high income countries compared to low/middle income countries. In high income countries TSPOT and TST results were concordant in 89% (95% CI, 81–98%) of cases and QFT and TST results were concordant in 94% (95% CI, 91–96%). Eight of the nine studies that reported test agreement using kappa values reported poor or moderate agreement (kappa 0.4-0.6).

In low/middle income countries, TSPOT and TST results were concordant in 77% (95% CI, 67–88%) of cases but there was significant heterogeneity among individual studies. Two of three studies that reported test agreement using kappa values reported poor or moderate agreement (kappa 0.4–0.6). In addition, IGRA+/TST- results were more common than IGRA-/TST+, as shown elsewhere in HIV negative populations (181, 187). However, in this review the exact proportions of discordant results were not given. The authors in the review did not discuss the reasons behind differences in percent concordance between low/middle and high income countries which could have been a result of differences in test performances in the different settings.

In 12 studies (meta-analysis) comparing TST with IGRAs in healthy populations with varying risk for LTBI, (157), IGRA−/TST+ discordance (24%) was more common than IGRA+/TST-(5%), similar to findings of a community survey in Ethiopia (182). However, in this meta-analysis overall concordance rates and kappa statistics were not reported, making comparison difficult.

It is increasingly being accepted that concordance between IGRAs and TST in high TB incidence settings is lower than that seen in low TB incidence settings. Many factors that modulate the sensitivity of IGRAs may explain this difference in addition to those already mentioned. Additional factors include high exposure to *Mtb;* transmission dynamics and repeat exposures; high exposure RD-1 homologue producing NTM, disease phenotype and severity(188).

More recently it has been shown that just like for TST, QFT-GIT has a window period as well (the time between infection with *Mtb* and the test becoming positive). Agreement between the TST and QFT-GIT, and the correlation with the intensity of exposure to *Mtb*, are thought to be better after the window period (189). It has been suggested that the test should be carried out 2 months after exposure, either for the first time or as a confirmatory analysis in cases with a positive TST. Although this sounds plausible, this is restricted to contact tracing in low TB incidence settings. In high incidence settings, the repeated exposure and re-infection makes it difficult to know exactly when an individual was exposed.

Agreement between IGRAs and TST should not be expected to be excellent because they may be measuring different aspects of cell mediated immune response (new vs. old infection) as well as using different antigens. It is not yet understood to what extent a positive IGRA result suggests previous (remote) infection (either cleared or still persistent) versus recent infection (190). However, even though some literature suggests that QFT positivity is related to relatively recent acquisition of *Mtb i*nfection, there is still no consensus (61).

Concordance may be maximized by choosing the appropriate cut-off (191). Studies have shown that agreement between QFT-GIT and TST increases at higher TST cutoffs (150, 192). Agreement between the two tests improved markedly if a > 10mm cut-off was used for the TST, but resulted in a large number of QFT+/TST- contacts (193). Three studies showed that agreement could be improved by employing a more stringent TST cut-off i.e. 15 mm vs. 10 mm (181).

2.5 Discordance between IGRAs and TST results

Discordance between IGRA and TST and its interpretation (24) is still an area of considerable confusion. Discordance analysis is helpful since there is no gold standard for TB infection. Flaws in discordance studies have been observed, some studies assume IGRAs results are “gold standard” and that it is more likely to be true and that any discordance (TST+/IGRAs-) is because of TST false positives. This approach ignores the fact that this century old test, subjected to dozens of very large studies pertaining to its diagnostic and prognostic abilities still performs well in certain populations where BCG is given at birth (194-196). Although some discordance may be explained by superior specificity of IGRAs, it would be overly simplistic to assume that the results of IGRAs were always correct and those of TST were always incorrect.

Discordance between IGRA and TST has been frequently observed. In a meta-analysis comparing TST with IGRAs in healthy populations with varying risk for LTBI, discordant results were found in 21% (T-SPOT) or 29% (QFT) of participants (157). In a study in Spain among 135

HIV positive individuals and controls, TST+/QFT- results (78.1% of discordant results) predominated in the control group. Contrary, the HIV group showed a predominance of QFT+/TST- (75% of discordant results). HIV was negatively associated with a QFT-/ TST+ result.

The reason for discordance has been generally explained by BCG vaccination or NTM infection in cases where TST is positive and the QFT-GIT test is negative and by a past history of TB infection, poor TST sensitivity or false positive IGRA where TST is negative and that of QFT-GIT test to be positive (197).

Beyond this common explanation for discordance, few studies have gone a step further by looking at other reasons for discordance (198). Discordance, especially one where TST ≥ 15mm and QFT-G assay negative has been reported. Studies in South Africa (183) India (129) and Italy (199) have found that among those with large TST reactions (≥ 15mm and therefore, high likelihood of infection) approximately 31%, 43%, and 11% were negative by QFT-G respectively. In contrast, the community survey among 652 adult pastoralists in Ethiopia found that those with TST ≥ 10 mm were 13.6 times more likely to have positive results using QFT-GIT than individuals with skin indurations of 0 mm (182).

While false positives TST (due to BCG, NTM) could be an explanation is it still possible that IGRAs are measuring only a fraction of those with latent TB infection (recent versus remote infections). The possibility of false positive results due to NTM infections is greater using TST than IGRAs but in settings where NTM are prevalent, this may explain QFT-GIT false positives results (182). One study in Ethiopia attributed IGRAs false positives due to the present cut-off value recommended by the manufacturer which is thought to be too high (182). Some of this observed discordance may in part be resolved through further exploration of IGRAs cut-off points and by analyzing the results of both tests as continuous outcomes. Discordance of the reverse type (TST-/IGRAs +) has been documented as well (182, 199) but not well explained.

Explaining discordance should be context specific taking into account the epidemiological and individual factors contributing to infection. For example, confounding factors, such as birthplace, may be a risk factor for TST+/IGRA− discordance in low TB incidence settings but not in high incidence settings. In low incidence settings, it is currently well accepted that most of the TST+/QFT− discordant results can be explained by BCG vaccination or birth in a foreign country (usually a high incidence setting). In a pooled analysis of two studies conducted in Germany, being foreign-born and BCG-vaccinated explained 95.7% of the TST+/QFT− results (198). Furthermore, age explained 49.1% of all TST-/QFT+ discordance. The influence of age on IGRA outcomes may be explained either by IGRA ability to detect recent infection and also reflect age-related differences in immune maturation and IFN-γ production (147). In high incidence settings, where BCG is given at birth and hardly repeated in adolescence, its effect on TST results several years after the vaccination is minimal (95). Summaries and agreement levels of selected studies are shown in **Tables 2.2 and 2.3.**

Table 2.2: Concordance of QuantiFERON-TB Gold with TST in low incidence countries in adult individuals

Authors, Year, country , reference	Population enrolled	[1]Total with both TST & QFT results (% BCG)	PPD dose, 10mm	Concordant results		Discordant results		% overall concordance (95% CI)	Kappa statistic
				TST+QFT+ n (%)	TST- QFT- n (%)	TST-QFT+ n (%)	TST+ QFT- n (%)		
Healthy individuals									
*Lee et al,2006, Korea (157, 200)	224 adults	131 (100)	2TU	3 (2)	95 (73)	8 (6)	25 (19)	73.3	0.43
Santin et al, 2011, Spain(173)	135	135	2 IU, 5mm	22 (16.3)	81 (60)	7 (5.2)	25 (18.5)		
Contacts of TB patients (high risk groups)									
*Brock et al, 2004, Denmark (157, 201)	85	45 (0)	2TU	23 (51)	19 (42)	1 (2)	2 (4)	94 (89-99)	0.87
*Kang et al,2005, Korea (106, 202)	273	120 (73)	2TU	24 (20)	40 (33)	4 (3)	53 (44)	53.3	0.16
Diel et al, 2006, Germany(150, 176)	311	309 (50.8)	5TU	28 (9)	169 (55)	3 (1)	109 (35)	63.8	0.20
Arend, 2007, Netherlands,(192)	909	785 (0)	2TU	74 (9)	518 (66)	7 (1)	186 (24)	75.4	0.33.
Diel et al, 2008, Germany(141)	629	601 (67)	2TU, 5 mm	62 (10)	354 (59)	4 (1)	181 (30)	69	0.28
Diel et al, 2011, Germany(142)	1414	954	5 TU & 2 TU	138 (15)	652 (68)	60 (6)	104 (11)	82.8	0.52
Anibarro, 2011, Spain(203)	152	136 (0)	2TU, 5 mm	50 (37)	37 (27)	4 (3)	10 (7)		0.73
HIV positive individuals									
Luetkemeyer, et al, 2007, USA(204)	294	196	5TU, 5 mm	8 (4)	167(85)	11 (6)	10 (5)	89 (84-93)	0.37
Jones et al, 2007(205)	207	191	5TU, 5 mm	5 (3)	172 (90)	6 (3)	8 (4)	93 (88-96)	0.38
Balcells et al, 2008, Chile (206)	116	109 (88)	2TU, 5 mm	9 (8)	90 (83)	8 (7)	2 (2)	91 (84-96)	0.59
*Stephan et al, 2008, Germany (207)	286	275 (6.9)	2 IU, 5mm						0.33
Talati et al, 2009, USA(208)	336	273 (9.2)	5TU, 5 mm	2 (1)	259 (95)	7 (2)	5 (2)	96 (92-98)	0.23
Richeldi et al, 2009 (209)	369	116 (6)	5TU, 5 mm					95.4 (90-98)	0.52
Santin et al, 2011, Spain(173)	135	135	2 IU, 5mm	7 (5)	118 (89)	6 (4)	2(1)		

[1] **Excludes QFT indeterminates and TST not read;* QFT-G as used**

Table 2.3: Concordance of QuantiFERON-TB Gold with TST in moderate-high TB incidence countries in adult individuals

Authors, Year , country	Population enrolled	[1]Total with both TST & QFT results (% BCG)	PPD,dose, 10mm	Concordant results		Discordant results		% overall concordance (95% CI)	Kappa statistic
				TST+QFT+ n (%)	TST-QFT- n (%)	TST-QFT+ n (%)	TST+QFT- n (%)		
Healthy individuals									
Legesse et al, 2011(182)	652	271	2TU	151 (56)	28 (10)	76 (28)	16 (6)	66	0.2
Mahomed et al, SA, 2006 (183)	367	358 (81)	2TU	189 (53)	57 (16)	12 (3)	100 (28)	69	0.32
*Rangaka et al, 2007, SA (186)	160 (86 HIV –ve)	74 (71)	2TU	32 (43)	9 (12)	4 (6)	29 (39)	55	0.12
[2]Chapman et al, 2002, Zambia (185)	49 (29% HIV +ve)	49 (67)	5TU	22 (45)	8 (16)	8 (16)	11 (22)	HIV +ve:64 HIV –ve:60	0.26 0.03
Contacts of TB patients									
Machado et al, 2009, Brazil(210)	301 (28% children)	255 (76)	2TU	100 (39)	94 (37)	17 (7)	44 (17)	76	0.53
*Hesseling et al, 2008, SA (211)	82	53 adults (62)	2TU			0%	36%	60	0.34
Adetifa et al, 2007, Gambia (212)	194	175 (48)	2TU	69(39)	57 (33)	33 (19)	16 (9)		0.43
[2]Hill et al, 2006, Gambia(213)	775	693 (46)		165 (24)	413 (60)	55 (8)	60 (9)	75	0.43
[2]Hill et al, 2004, Gambia(214)	856	735 (45)	2TU	162 (22)	382 (52)	58 (8)	139 (19)	74	0.43
Health Care workers									
Costa et al, 2009, Portugal(187)	1686	1686 (100)	2 TU	525 (31)	332(20)	33 (2)	792 (47)	54	0.22
Lien et al,2009, Vietnam(215)	300 (37)	255	5 TU	114 (45)	71(28)	21 (8)	49(19)	72	0.44
Pai et al, 2005, India(129)	1081	726 (71)[3]	1 TU	226 (31)	359 (50)	62 (9)	72 (10)	81	0.64
HIV positive individuals									
Mandalakas et al, 2008(147)	43 (53% children)	43	2TU			0%	26.9%		0.49
*Rangaka et al, 2007, SA (186)	160 (74 HIV +ve)	74 (51)	2TU, 5 mm					76	0.52

[1] Excludes QFT indeterminates and TST not read* QFT-G was used. [2]ELISPOT used. [3]BCG scars.

Note: The following risk groups were excluded: prisoners, army personnel, drug users, individuals with other causes of immunosuppression.

2.6 Effect of HIV on IGRAs

The effect of HIV on the interpretation of IGRAs is now widely discussed (157, 216). There is little doubt that the performance of IGRAs is affected by immunosuppression just like TST. High rates of indeterminate results and lower sensitivity of IGRAs in immunocompromised people have raised concern about their applicability in routine clinical practice (209, 217). As an advantage, IGRAs contain an internal positive control allowing the reader to discriminate true negative from false negative results. The TST lacks an internal control to distinguish false-negative results due to anergy from true negative results, leading to a poor diagnostic accuracy, especially in HIV positive individuals (121).

Earlier studies in HIV positive individuals from a variety of settings had shown that ELISpot results were robust in HIV infection and independent of CD4 cell count (186, 218, 219). ELISPOT was found to be relatively unimpaired in the detection of either TBI or active TB in patients with moderately advanced HIV infection (186, 220, 221). A study done in Zambia using ELISPOT assay (185) showed high sensitivity in all of HIV-negative (100%) and HIV-positive (90%) TB patients although the study was small. In another study of HIV-infected individuals routinely attending a HIV-clinic in a high-prevalence setting, the ELISA was found to have a lower rate of positivity than either the ELISpot or the TST (147). Previously, it has been generally accepted that the TST has a lower sensitivity than IGRAs in HIV-positive individuals and that IGRAs (especially ELISPOT) are less prone, but not unaffected by T-cell anergy at lower CD4 cell counts (186, 218).

This picture seems to be changing as data coming from recent studies appears less convincing (131). Data from a meta-analysis that identified 37 studies (5736 HIV infected individuals) showed that although TSPOT.TB appeared to be less affected by immunosuppression than QFT-GIT and the TST, overall, differences among the three tests were small or inconclusive.

Sensitivity estimates (using culture confirmed TB as a surrogate reference standard for LTBI) were higher for T-SPOT (72%) than for QFT-GIT (61%) in low-/middle-income countries. The results were similar for high income countries but sensitivities were higher than those in low/middle countries (T-SPOT: 94%; QFT-GIT: 67%). However, in this meta-analysis, apart from two studies all studies were done in low/middle income countries. In addition, neither IGRA was consistently more sensitive than TST in head-to-head comparisons.

Furthermore, studies done in SA, Uganda and China showed a large reduction in the proportion of positive test results for both QFT-GIT and T-SPOT in HIV-infected individuals with advanced immunosuppression . Reasons for the stronger impact of immunosuppression on IGRA performance in low/middle-income vs. high-income settings were unclear but may be related to disease severity and anti-retroviral treatment status.

Other authors have found that in HIV positive individuals with very low CD4 cell counts, the ELISA's performance is adversely affected, as exemplified by a higher proportion of negative and indeterminate results. In a study done in Zambia (222) using QFT-GIT, low CD4+ counts in HIV positive TB patients were associated with increases in both indeterminate and false-negative results. A number of studies have also found that indeterminate results increase in severe immune-suppression, a fact that may affect the diagnostic utility of the ELISA in HIV-positive

individuals (147). In a study conducted in Zambia among TB patients, a marked decrease in sensitivity was observed in HIV positives compared to HIV negative (63% v 84%, p=0.027) (148). In addition, low CD4+ count (<200 cells) was associated with increases in both indeterminate and false-negative results, similar to findings in other studies (129, 217, 222-224).

Both IGRAs are known to suffer from indeterminate results. A high response to the nil or a low response to the mitogen will give an indeterminate result. However, in HIV positive individuals indeterminate results most commonly arise due to a failed positive control, which usually reflects underlying cellular immune suppression. Technical errors during the testing process can also result in indeterminate results as well. There is now sufficient evidence showing an increase in indeterminate results in HIV positive individuals with advanced immune-suppression (222, 225) as well as in those with other immunosuppressive disorders (226).

A recent systematic review and meta-analysis (131) assessed the proportion of indeterminate IGRA results among healthy HIV-infected individuals screened for TBI. The proportion of indeterminate results was less than 5% in nine of 13 studies evaluating T-SPOT (range,0–13%) and six of 10 studies evaluating QFT-GIT (range, 2–11%). Indeterminate ELISA results seem to be associated with young (<5 years) and old age (>80 years) and immune-suppression, either due to HIV infection or immuno-suppressant medication (130). Co-morbidities such as malaria or hepatitis in children have been associated with indeterminate results (227). Other studies have found a significant relationship between increasing age and IFN-γ response to the mitogen (228).

2.7 QFT-GIT cut offs

The recommended cut-off values for positive IGRA results are probably not optimally related to the risk for disease progression. The determination of new cut-off values for QFT-GIT may improve the assay sensitivity, especially in high endemic populations (229). The challenge is not to identify positive responses *per se* but to establish a cut-off point within the positive category that predicts subsequent development of TB (107).

Current cut-off values for commercially available IGRA assays are set at relatively low levels in order to maximize the detection of individuals with *Mtb* infection who might benefit from chemoprophylaxis, especially amongst contacts of TB cases (159).

The cut off value of QFT-GIT is based on receiver operating characteristics curve analysis and just like the TST can only be meaningfully discussed in a specific context. The suggested manufacturer's cut off points are increasingly being questioned as to whether they should be applied universally regardless of the context. It has been postulated that IGRA cut off points should perhaps be used in the same way as TST taking into consideration the epidemiological status and underlying diseases like HIV (200, 230). Authors that have evaluated new cut off points (229) pointed out that the determination of new cut-off values for QFT-GIT may improve the assay sensitivity, especially in high endemic populations.

It has been suggested that the cut-off value for QFT-GIT should be reset at a lower level when the test is used in high-prevalence situations (231). Lee and others (200) investigated new cut-off values for the QFT-G and T-SPOT assays in 87 active TB and 131 healthy control subjects in South Korea. They found that for QFT-GIT, the cut-off value of >0.13 IU/ml of IFN-γ increased the sensitivity of the test from 70.1% to 86.2%, with a minor decrease in specificity (from 91.6% to 87%). Similarly, another study (229) showed that using a cut-off value of ≥0.818 IU/ml of IFN-γ improved the sensitivity of QFT-GIT with no decrease in specificity. This finding suggests that using a lower cut-off value in the QFT-GIT assay may increase the sensitivity in diagnosing *Mtb* infection, especially in immune-compromised patients and children (229). Arend and others (192) also evaluated new cut-off values for the QFT-GIT and T-SPOT assays in TB contact tracing.

Experience with the QFT-IT indicates that individuals can have variability in IFN-γ responses (232). Some patients with positive results, particularly at or near the cutoff point, can revert to negative if retested (and vice-versa) (232). Similar to interpretation of the TST, higher values are more likely to be truly positive than values near or below this cutoff point and lower negative values are more likely to be truly negative than values that are closer to the cutoff point (232).

3.0 Methods

Introduction

The aim of this chapter is to give a detailed description of the study methods. This chapter first describes the background study (ZAMSTAR trial) within which the cohort study under discussion in this thesis, the QFT cohort study was nested. The rest of the chapter describes the QFT cohort study with regard to sample size, study duration, study population, measurement tools, baseline and follow-up activities, data collection and statistical analyses. The specific objectives of this chapter are to:

1. To give a brief description of the background studies within which the QFT cohort was nested i.e. the ZAMSTAR and SOCS studies
2. To describe study procedures and activities in the enrolment and follow-up of HHCs in the QFT cohort study.
3. To describe the statistical analyses used to answer primary and secondary study objectives.

3.1 Background Studies

3.1.1 The ZAMSTAR Study

The ZAMSTAR (The Zambia South Africa TB and AIDS Reduction) study was a community randomised trial involving 24 communities with a total population of over 1,000,000, in Zambia and the Western Cape Province of South Africa. The ZAMSTAR study was the background study within which the cohort study under discussion in this thesis, the QFT cohort study was nested.

ZAMSTAR primary objectives

The ZAMSTAR study evaluated two public health interventions that aimed to reduce the prevalence of TB at community level. These interventions were community-based enhanced case finding for tuberculosis and household counselling and provision of combined TB/HIV prevention services at the household level. The ZAMSTAR study was conducted from 2005-2010. The primary study questions in the ZAMSTAR study were:

1 Does enhanced tuberculosis case finding (ECF) by a strategy of community mobilisation and enhanced access to sputum microscopy, reduce prevalence of tuberculosis in the community?
2 Does a strategy of combined TB/HIV activities at the household level (HH), that includes active case-finding, Isoniazid preventive therapy, psychosocial and adherence support for both treatment and prevention of tuberculosis and HIV, reduce the prevalence of tuberculosis in the community?

Institutions involved in the ZAMSTAR study

This study was conducted by ZAMBART (Zambia AIDS Related TB) Project, a collaboration between the University of Zambia and the London School of Hygiene and Tropical Medicine) and the Desmond Tutu TB centre from the University of Stellenbosch Cape Town. In addition it was also implemented in close collaboration with the departments of Health in Zambia and SA, NGOs, civil society and community advisory boards.

Study setting

The ZAMSTAR study was conducted in Zambia and South Africa, two countries highly affected by the TB and HIV epidemics. A total of 24 communities, 16 communities in Zambia and 8 communities in the Western Cape Province of South Africa were selected for the study. Study communities were selected based on TB notification rates greater than 400/100,000 per annum, high HIV seroprevalence and proximity to a TB diagnostic centre. A ‘‘community’’ was defined as the population (minimum size of 25,000) accessing one TB diagnostic centre and was the unit of randomization for the ZAMSTAR trial (233). The communities selected were in five provinces of Zambia and in Western Cape Province of SA and included both urban and rural communities (**Figures 3.1 and 3.2**).

Figure 3.1: Study communities in Zambia

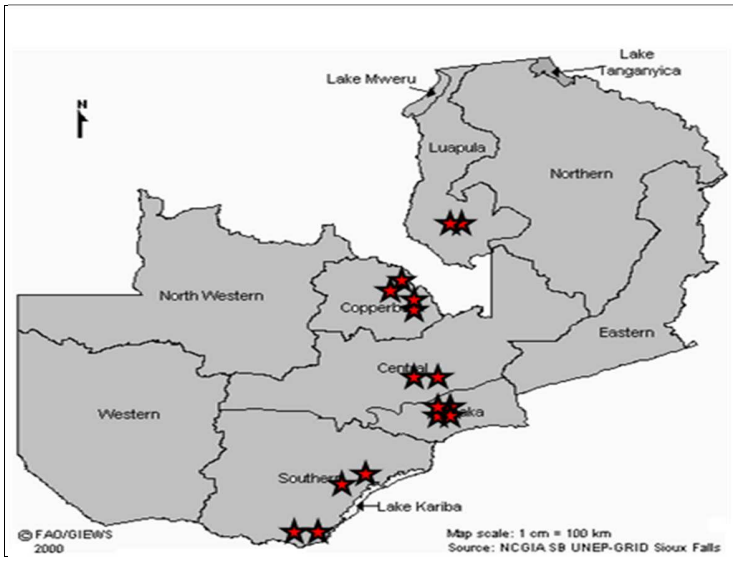
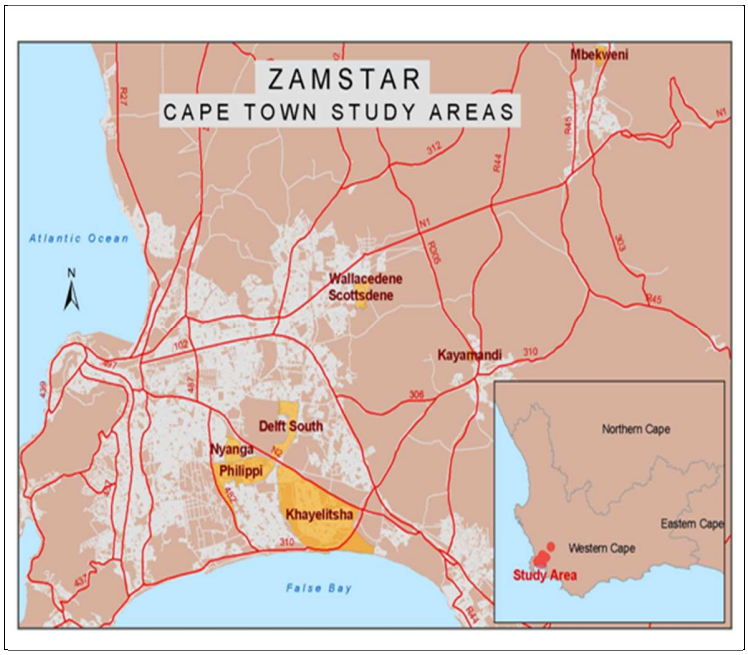


Figure 3.2: Study communities in Western Cape, SA



ZAMSTAR study sampling and randomization.

The communities formed the primary sampling units. The design and randomization of the ZAMSTAR study is described in detail elsewhere (233, 234). However, briefly for the randomization, stratification and restriction was used to randomize 24 communities into four intervention arms with 6 communities in each arm in a 2x2 factorial design. To ensure that

intervention effects were not distorted due to enrolment imbalances between intervention groups, communities were ranked according to their TBI prevalence within each country. TBI prevalence estimates within each community were determined through a TST survey done among primary school children (95). Stratification was by country and TBI prevalence and restriction by HIV prevalence, TBI prevalence, urban/rural, social context, and geographical location.

The 24 communities were randomly allocated to one of 4 trial arms, with 6 communities in each arm (**Figure 3.3**).

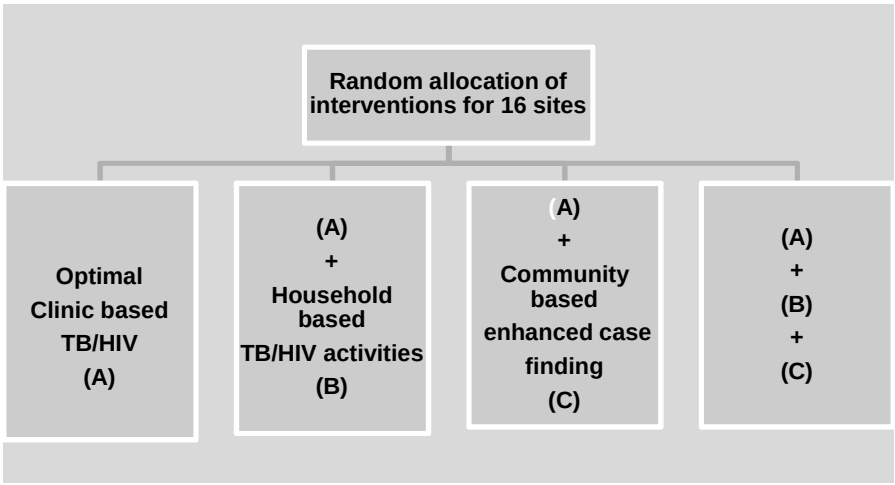
Arm A: Strengthened TB/HIV activities at the clinic only (Clinic)

Arm B: Clinic plus enhanced case finding (ECF)

Arm C: Clinic plus household intervention (HH)

Arm D: Clinic plus ECF and HH interventions

Figure 3.3: Interventions randomly allocated into four arms.



3.1.2 ZAMSTAR study secondary objectives

To determine the secondary objectives of the ZAMSTAR trial, a cohort of consecutive TB patients and their household members were followed up for the duration of the interventions to allow analysis of household-level outcomes, using both epidemiological and social science methods (234).

These cohorts of TB patients and their household members were called secondary outcome cohorts (SOCS). The aim of SOCS was to recruit a cohort of 350 adults from 150-200 households in each community in order to be able to measure the secondary outcomes of ZAMSTAR.

Specific secondary outcomes of the ZAMSTAR trial at household level, as measured on this cohort of households were:

• Cumulative HIV incidence measured by serial HIV testing among those over 15 years old.
• Uptake of HIV testing and counselling and uptake and adherence to tuberculosis preventive therapy among household members of TB patients.
• TB transmission within the household measured by TST.
• Cumulative incidence of TB disease in household HHCs.
• Stigma levels measured using a quantitative stigma score.

SOCS study population

Adults ≥ 15 years and children < 5 years old were invited to participate in the SOCS study.

SOCS sample size

From each of the 24 communities, 150 adult TB index cases were expected to be enrolled into the study. A household with one TB index case was expected to have at least 2 adult HHCs, giving a total of 7,200 adults (150 indexes x 24 communities x 2 HHCs). Therefore, the SOCS was designed to enroll a total of at least 7,200 adults across all communities at enrolment.

SOCS study duration

Enrolment of study participants was done between July 2006-2008 (SOCS1). Follow-up visits were scheduled at 18 months after enrolment (SOCS2) and 36 months after enrolment (SOCS3).

3.2 The Quantiferon (QFT) Cohort Study

The QFT cohort study is the study under discussion in this thesis, described in detail in the subsequent sections of the methods. The QFT cohort study focuses on adult HHCs enrolled in the SOCS study and accepting to have a QFT-GIT test performed.

After being enrolled into the SOCS study HHCs were free to choose whether or not to take part in the QFT cohort study (details below). HHCs had to be enrolled in the SOCS study first before being offered being invited to participate in the QFT cohort study.

Since the QFT cohort was nested within SOCS, the enrollment and follow up of HHCs described in the subsequent section of this thesis are similar to those of SOCS (apart from adults being offered QFT-GIT and TST tests).

3.2.1 Study setting

The study setting of the QFT cohort study was as for the ZAMSTAR trial described above.

3.2.2 Study duration

The QFT cohort study began approximately 10 months after the SOCS study had started enrolment of study participants. Study duration was from April 2007 to December 2010. In Zambia, the study began at different times in some communities (difference in the range of 3-4 weeks because training was done in phases by one multidisciplinary team to ensure quality assurance*)*. The first QFT-GIT test in Zambia was done on April 2007. In SA, however, the study largely began at the same time in all communities on January 2007.

3.2.3 Sample size

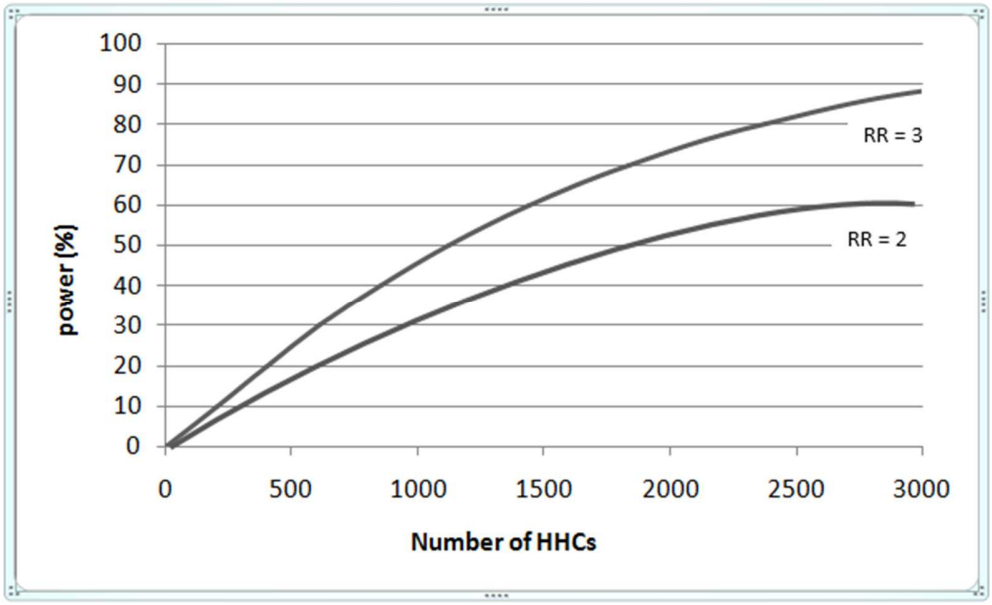
The sample size for the QFT cohort study was approximately one third of the HHCs recruited in SOCS at visit 1 (1,600 and 800 HHCs from Zambia and SA respectively) giving a total of approximately 2,400 HHCs. The sample size was also estimated following various considerations including logistical feasibility; some resources were shared.

Based on data from other longitudinal studies (35, 159, 235) sample size was estimated expecting a 3% TB incidence and risk ratio of 3 among HHCs with high IFN-γ responders during the 2-3 years following exposure. Approximately 20% of HHCs were expected to have high IFN-γ responses (≥10 IU/ml based on a similar study done in Colombia (35)and data on community controls from a rural district in Zambia (unpublished).

Under these conditions, an approximate cohort of 2,374 was adopted, consisting of 1,899 individuals with low interferon-γ levels and 475 with high interferon-γ levels. A cohort of 2,374 HHCs was expected to detect a minimal significant difference (2-sided $p < 0.05$) of 2% in TB incidence in the two groups of IFN-γ producers (high-responders vs. low responders) at 80% power,95% confidence level and significance level $\alpha = 0.05$.

Figure 3.4 shows power as a function of sample size. Power curves were obtained for the cohort study where the rate ratio (RR) in the exposed group is 3 and the two sided level of statistical significance is 5% as the hypothesis. Two different hypothetical scenarios are shown. The lower curve shows the power to detect RR of 2 and the upper curve a RR of 3. If the RR is 3 as hypothesized in the study, and the aim is to achieve a minimum of 80% power this suggests that the sample size of approximately 2500 would be required (**Figure 3.4).**

Figure 3.4: Power as a function of sample size for the QFT cohort study



3.3 Primary and Secondary Exposures and Outcomes

3.3.1 Primary exposure

High interferon-γ levels (≥ 10 IU/ml) at enrolment in response to *Mtb* specific antigens (either ESAT-6 and/or CFP-10).

3.3.2 Secondary exposure

The secondary exposure variables included the following:

1. Age
2. Sex
3. Level of education
4. Socioeconomic status
5. Alcohol
6. Smoking
7. HIV status
8. Smear status of the index
9. Sleeping proximity to the index TB patient
10. Country of residency

3.3.3 Primary outcome

TB incidence rate in HHCs with high IFN-γ responses

3.3.4 Secondary outcomes

1. Performance and operational characteristics of QFT-GIT in a field setting.
2. Risk factors associated with positive QFT-GIT and TST results at baseline.
3. Level of agreement or concordance between QFT-GIT and TST results.
4. TB incidence rates in HHCs with:
   a. Positive and negative QFT-GIT and TST results, stratified by HIV status,
   b. Concordant and discordant QFT-GIT and TST results.
5. Positive and negative predictive values of QFT-GIT and TST tests.
6. The association between QFT-GIT and TST positivity and the subsequent development of TB.
7. Risk factors associated with incident TB.

3.4 Ethical Considerations

The study protocols for the ZAMSTAR and QFT cohort study were approved by the Ethics committees of the University of Zambia and Stellenbosch University as well as the London School of Hygiene and Tropical Medicine (**Appendix 1 and Appendix 2**). Participation was voluntary. All HHCs involved in the study gave written informed consent. Participation in this study was not independent from participation in the SOCS study as HHCs were requested to fill in one consent form.

Ministries of Health, Community leaders, district and local health staff and neighbourhood health committees agreed to the study taking place.

3.5 Instruments and Tools

3.5.1 Study population

The study population was comprised of adult HHCs of newly notified TB cases at least 15 years of age who were enrolled in SOCS and in whom QFT-GIT was performed.

Definition of Household contacts

HHCs were defined as individuals, who generally slept in the home, ate with the index case and who identified a common household head.

Eligibility criteria

1. HHCs who were ≥ 15 years of age.
2. Had QFT-GIT performed at enrolment
3. Was not on TB treatment at enrolment
4. Provided informed written consent

Note: HHCs on TB preventive therapy and on antiretroviral therapy were also eligible for inclusion in the study.

The following HHCs were excluded from the study at enrolment

1. Did not have QFT-GIT results at enrolment.
2. Were less than 15 years of age
3. On TB treatment at enrolment

3.5.2 Baseline Activities

The clinic visit by index TB patients

Newly notified adult TB patients (index cases) were used as entry points into households where HHCs were invited to participate. Index cases (pulmonary smear positive, smear negative or extrapulmonary) recruited within a month after being notified in the TB register were consecutively enrolled into the study from a government clinic by study nurses.

The household visit

Permission was sought from index cases to visit their households where HHCs were invited to participate. Household visits were done either on the same day the index was recruited or on another day by appointment. Once in the household, the study was fully explained to HHCs; these were enumerated and individual (name, sex, age, consent) and household (address and GPS coordinates) data recorded on an enumeration form (**Appendix 3**). The address and GPS coordinates were important in relocating the same household at a later stage, during follow-up visits. Information about the study was given to household members individually and in some instances, as a household, if they so preferred (**Appendix 4**). All HHCs were asked for individual signed consent before participating in the study (**Appendix 5**).

The consent form and information sheet used in this study were originally designed for use in the SOCS study. These were modified for the QFT cohort study to include information on getting blood for QFT-GIT and having a TST done in adults.

Baseline measures

In summary, the following measures were done at enrolment:

a. A standardized questionnaire (**Appendix 6**) was administered to all consenting HHCs by trained research assistants (see details under data collection).
b. Blood was drawn for HIV antibodies and QFT-GIT testing (see details below).
c. TST were performed on the same day, after blood for QFT-GIT testing was drawn (see details below).

Enrolment screening for active TB was not conducted because the ZAMSTAR study design allowed little flexibility to accommodate this.

HIV Testing

HIV status was both self-reported (through the questionnaire) and laboratory-reported. The HIV results from the laboratory were used to determine the prevalence of HIV infection in this study.

Consenting HHCs had blood drawn for HIV antibodies. For each contact, 6 mls of venous blood was drawn into a vacutainer tube labeled with an individual barcode. The blood was sent to central laboratories in Lusaka and Stellenbosch University where it was tested in batches. HIV testing (**Appendix 7**) was done using the Abbot Axsym system HIV Ag/Ab Combo Assay (4^{th} generation assay) (Max-Planck-Ring 2, Germany) and confirmed with the Vironostika algorithm.

For HHCs who wanted to know their HIV results a small sample of blood was tested either on the spot or at the clinic using routine rapid HIV tests. Determine (Determine™ HIV-1/2) was used as the first line test and Uni-Gold (Uni-Gold™ HIV) as the confirmatory test for individuals with a positive result. HHCs referred to the clinic for HIV testing were encouraged to attend counseling and HIV testing before the test was done.

HHCs that tested HIV positive on Determine had a confirmatory test using Unigold. If the 2 tests were discordant the study staff took the participant to the clinic to allow re-testing and confirmation using a tie-breaker test.

Management of HIV positive HHCs was done according to the standard of care in the clinics. In South Africa, HIV positive individuals were advised to go for TB preventive therapy in accordance with National TB Control Program guidelines (91) while in Zambia this was not yet government policy. However, in Zambia, preventive therapy was offered to eligible HHCs through collaboration with another study operating in the ZAMSTAR sites.

QuantiFERON-TB Gold In Tube (QFT-GIT) assay testing

QFT-GIT testing was performed according to the manufacturer's instructions (Cellestis Ltd, Victoria, Australia) (236) (**Appendix 8**). 1 ml of blood was drawn into each of the three blood collection tubes coated with either heparin (negative control) or Phytohemagglutinin (PHA, a T-cell-activating mitogen (positive control) or peptides of ESAT-6, CFP-10 and TB 7.7. Samples were mixed vigorously by hand and delivered to the laboratory within 16 hours of collection. Tubes were incubated for 16- 24 hours at 37°C and plasma was harvested and frozen at –20°C. A Quantiferon recording form (**Appendix 9**) was used to record data (details in data collection section). ELISA was performed using standard kits supplied from the manufacturer. The test results were interpreted using the software supplied by the manufacturer. The raw data were entered into the QFT-GIT analysis software which reported results as negative, positive and indeterminate (**Table 3.1**).

Table 3.1: Interpretation criteria for QFT-GIT

TB antigen minus Nil (IU/mL)	Nil (IU/Ml)	Mitogen minus Nil(IU/Ml)	QFT-GIT Result	Report/ interpretation
<0.35 or ≥0.35 and<25% of Nil value	≤8.0	≥0.5	**Negative**	*Mtb* infection NOT likely
≥0.35 and≥25% of Nil value	≤8.0	Any	**Positive**	*Mtb* infection likely
<0.35 or ≥0.35 and< 25% of Nil value	≤8.0	<0.5	**Indeterminate**	Results cannot be interpreted as a result of low mitogen response
Any	>8.0	Any	**Indeterminate**	Results cannot be interpreted as a result of high background response

QuantiFERON®-TB Gold IT Analysis Software (version 2.17 and latter version) was used to analyze raw data and calculate results (237). The software performed a Quality Control assessment of the assay, generated a standard curve and provided a test result for each subject. The software reported all concentrations greater than 10 IU/mL as ">10" as such values fell beyond the validated linear range of the ELISA.

Results were calculated by subtracting the IFN-γ level of the negative control tube from the IFN-γ level of the respective TB-antigen and mitogen tubes. A test result was positive if the net IFN-γ response to the TB antigens was ≥0.35 IU/mL, regardless of the mitogen response; a test result was negative if the net IFN-γ value was <0.35 IU/mL and there was sufficient mitogen response (> 0.50 IU/mL); and a test result was indeterminate if there was excessive IFN-γ production from the negative control tube (>8.0 IU/mL) or an insufficient net mitogen response (< 0.50 IU/mL) plus insufficient net TB antigen response (< 0.35 IU/mL)(236).

For four Zambian and all the South African communities, QFT-GIT processing was done centrally at the research laboratories in Lusaka and Stellenbosch University Medical School respectively. However, for twelve Zambian remote communities, QFT-GIT processing was decentralized. In these communities, blood samples were collected, incubated, separated and stored locally. Frozen samples from these sites were transported monthly to the central laboratory in Lusaka where the ELISA was performed manually in batches.

Feedback of QFT-GIT results to participants

Feedback of QFT-GIT results was given to HHCs within a period of less than 4-8 weeks since results were centrally processed and had to be sent back to individual sites. Study staff was trained to give standard explanations of the results as shown in **Appendix 10**.

Quality Assurance of QFT-GIT process and results

A multi-disciplinary Quality Assurance (QA) team was constituted in Zambia due to the decentralized nature of sample collection, incubation, separation and storage. The aim of the QA team was to ensure that sample processing was done accurately as well as identify and correct gaps and weaknesses. A QA monitoring tool was designed which captured the following data on various aspects of the process and the test itself (Appendix 11). There were also other routine laboratory checks each time samples arrived at the central laboratories and these included: checking for leakages during transportation, quality of the sample, correct use of barcodes, type of specimen bottles and packaging used

Tuberculin skin testing

Skin tests were carried out using 2 TU (Tuberculin Units) of PPD RT23 with Tween, supplied by the Statens Serum Institute (Copenhagen, Denmark). A dose of 0.1 ml was injected intradermally on the left forearm. Skin reactions were read using calipers 72 hours later as the largest palpated transverse diameter. The TST was considered positive if the induration was >=10 mm (even for HIV positive HHCs) unless otherwise stated. A TST recording form (**Appendix 12**) was used to record data (details in data collection section).

Tuberculin skin testing Quality assurance

All tests were administered and read by nurses who were trained according to the standard IUATLD protocol (238). Survey staff was trained in the placement and reading of TST according to the standard IUATLD protocol. Training included exchange visits between Zambia and SA so that the trainers were using the same methods throughout.

3.5.3 Follow up Visits

After enrolment HHCs were followed up at two time points over the study period. The first follow-up visit was at 18 months and the second follow-up visit was at 36 months after enrolment into the study.

All HHCs were followed up regardless of their baseline QFT-GIT and TST results until they developed disease or were lost to follow up (due to death, moved or untraceable). Therefore all HHCs positive or negative by either test were followed up.

1st follow up visit

The following activities occurred at the first follow up visit at 18 months from enrolment

a) For each household, a print out of the enumeration form filled in at enrolment was given to the field teams by the data department at Head Office. The GPS coordinates and the address on this form were used to locate households.
b) Households were revisited and particulars of the households (number and composition) were checked against those collected at enrolment. Any HHC who no longer lived in the household on a permanent basis was identified and marked and coded on the enumeration form. HHCs that were absent but were expected to return within 30 days were still included

in the study. Those who were unlikely to return within 30 days were not included at this point but were considered for the 2nd follow up visit. Those who had died or who had left on a permanent basis to live outside the community were censored at this visit. Those who had moved to another house in the same community were retained in the cohort and were visited in the house that they currently lived in.

c) A structured questionnaire was administered to collect information on risk factors for tuberculous infection and disease as well as whether individuals were or had been on TB treatment since they were last seen (see data collection section).
d) HHCs were educated on the signs and symptoms of the disease to encourage self-referral to the clinic in the event of suspected disease. However, at each follow-up visit no active screening for TB was done (HHCs were not queried on signs and symptoms of TB).
e) At least three attempts were made to visit HHCs who were absent during the 1st follow up visit.

2nd follow-up visit

The second follow-up visit occurred at 36 months from enrolment

The procedure for the second follow-up visit was the same as for the 1st follow-up visit including the following:

a) All households recruited at enrolment were revisited at this follow up visit regardless of whether HHCs had previously been recorded as died, moved or absent during the1stfollow-up visit. This was done to minimize losses to follow-up.
b) HHCs recorded as moved out in the 1st follow-up visit were revisited to confirm that this was truly the case. If they had subsequently returned, but were absent for 6 months or more they were censored at this point as "moved out of the community". If they have returned and were absent for a period of less than 6 months they were invited to participate in the second follow up visit.
c) Any HHC who was absent in the 1st follow-up visit was actively followed up on this occasion. At least three attempts were made to trace such individuals. If such attempts were not successful these HHCs were censored as "absent".
d) If a HHC had died or was recorded as dead in the 1st follow-up visit, condolences were first extended after which the research assistant asked for the date of death. This was recorded on the enumeration form (Note: the protocol was revised to include the date of death for this follow up). No verbal autopsy was conducted to identify whether the causes of death were related to the outcome in this study or not. Although the date of death was recorded this was not used in this study since death was not an outcome in study.
e) Sputum specimens were collected from all HHCs and processed using manual MGIT (see details below).

3.5.4 Sputum processing, and identification of *Mycobacterium tuberculosis* complex

All HHCs were asked to produce a respiratory sample on the spot. Instructions were given by the study staff to assist individuals to produce the best possible sample for culturing *Mtb.* Sputum

samples were collected from HHCs at home and transported (**Appendix 13**) to the laboratory in cool boxes with ice packs within 72 hours of collection. Sputum sample containers were labeled with individual barcodes. Upon receipt, samples were electronically logged and assigned a laboratory number before processing (Appendix 13). A work sheet was then printed for each sample that was registered. Sample processing was done according to standard laboratory procedures for cultures of *Mtb* (239).

Digestion and decontamination

Once sputum specimens were logged in, they were centrifuged. In instances where the volume was less than 3mls, it was adjusted with normal saline to 3 mls to create a better decontamination and digestion. All sputum samples were processed within 72 hours after arrival at the laboratory. The sputum samples were digested and decontaminated using the Sodium hydroxide (NaOH)-N-Acetyl L-Cysteine (NALC) method. Commercially prepared NaOH-NALC solution (BBL™ MycoPrep™ KIT, BD) was used with a final NaOH concentration of 1.5%. After decontamination, the samples were neutralized by adding phosphate buffer up to the 45mls. The sample was centrifuged at 3000 rpm for 20 minutes, left to sit for 5 minutes to allow aerosols to settle and the supernatant discarded thereafter. The pellet sediment was re-suspended in 2mls phosphate buffer. The re-suspended pellet was used for making smears and for inoculation of MGIT (Mycobacterium growth indicator tube) tubes.

Inoculation

Inoculation was performed according to recommended laboratory standard operating procedures (239). Each sputum sample was inoculated into two manual mycobacterial growth indicator (MGIT BD) tubes. The two manual MGIT tubes containing OADC (enrichment broth) and PANTA (antibiotic mixture) were inoculated with 0.5mls from the 2 ml re-suspended pellet. After inoculation the tubes were incubation at 37°C in a standard stand alone incubator for a maximum of 6 weeks (42 days). The remainder of the pellet was aliquoted into a labeled 1.5ml screw cap tube and stored at -20^{o}C until further need.

Reading cultures

The MGIT instrument was inspected for tubes that were flagged as having growth. Cultures were read once a week until 42 days had elapsed using the manual reader. Culture results were scored as positive, negative or contaminated. Once a MGIT tube was flagged as having growth by the manual reader it was logged in the database as a "*positive MGIT culture*". All MGIT tubes that showed no growth after 6 weeks of incubation were scanned into the database as "*negative MGIT cultures*" and discarded. A culture with growth was considered *contaminated* if the ZN stain was negative for AFB. For the MGIT tubes that were flagged as having growth, a slide for *Ziehl-Neelsen (ZN)* staining was prepared and a MPB64 antigen test (TBc ID) was done. At the same time an archive and a DNA sample was prepared and stored at -20 until further need.

Culture isolates that were ZN stain positive and MPB64 antigen strongly positive were defined as *Mtb* isolates. Follow up was initiated for such individuals. In South Africa, a slightly different

SOP was used*;* follow up was initiated only if the concentrated smear slide was FM (fluorescence microscopy) positive for individuals with *Mtb isolates* as defined above. Cultures with growth that were ZN stain negative and MPB64 negative were scored as contaminated (**Appendix 14).**

Quality control (QC)

All staff was trained according to standard laboratory procedures for cultures of *Mtb (239)*. Reference *Mtb* complex strain-H37Rv stock cultures prepared from the central laboratories were used as positive controls for every batch (positive control being scanty" growth of M37RV *Mtb)*. These were subjected to the digestion and decontamination process as described above. Normal saline was used as a negative control. The control samples were processed in the same way as the sputum samples. A randomly selected number of ZN slides were sent to the central laboratories for QC to be reread by a second reader in a blinded manner. Discordant slides were read by a third reader and the result of this was regarded as final. All readers were experienced laboratory scientists.

Follow-up of culture positive individuals

Follow up was only conducted in individuals who were AFB positive (with cording) and MPB64 positive results (**Appendix 14**). These individuals were revisited so as to refer them to a clinician for confirmation of clinical TB and subsequent treatment. Test result forms were automatically printed from their respective laboratories and sent to the data department at Head office. A follow-up form was generated where an individual's details were filled in (name, age, individual barcode, household barcode, PDA code, and GPS coordinates) (**Appendix 15**) after which it was sent to the site team leader. The site field team then visited all individuals scheduled for follow-up to explain the results whilst keeping the process and all information confidential.

A trained study clinician used a questionnaire to screen individuals for TB symptoms and examined anyone suspected of TB. Two further sputum samples (spot and early morning sample) were collected from individuals with a culture positive for *Mtb*. One sputum sample was for smear at the local clinic and another one for culture at the central laboratory. TB suspects whose smear or culture results were positive for AFB as per algorithm were treated as cases of TB and referred for treatment at the local TB diagnostic centre. Counseling and HIV testing were offered to all those found to have TB.

All efforts were made to find individuals with a positive culture. If the house was found but the individual was absent, two return visits were made to attempt to find them. If the individual was not found on the third attempt a confidential letter was left asking him/her to contact the study team and the local health centre. If the individual could not be traced, then the positive result and name of person were handed over to the clinic staff for further follow up.

Definition of incident Cases of TB (Incident Cases)

Incident TB was defined as self reported TB that was confirmed in the TB treatment register (or patient treatment card). In a sensitivity analysis three other definitions of incident TB were explored as follows:

1. Self-reported TB confirmed in the TB register but excluding co-prevalent cases. Co- prevalent TB was defined as active TB diagnosed within 2 months after entry into the study

2. Bacteriologically confirmed TB (presence of one or more sputum smears positive for acid fast bacilli (as notified in the TB register).

3. Bacteriologically confirmed TB **plus** one or more positive cultures for *M.tuberculosis* **plus** positive on MPB64 antigen test.

3.6 Data Collection Tools

3.6.1 The Questionnaire

Questionnaires were administered to consenting HHCs at three time points: enrolment, 1st follow up visit and 2nd follow up visit. These questionnaires were standardized and administered by trained research assistants. Since the questionnaires were originally designed for the ZAMSTAR study, they also captured other information that did not pertain to this study. All questionnaires were translated and back translated from local languages mainly spoken in Zambia (Bemba, Nyanja, Tonga, Lozi or English) and South Africa (Xhosa, Afrikaans or English).

The enrolment questionnaire (**Appendix 4**) captured data on the following (for the purpose of this study):

1. Demographic characteristics of the cohort (age, sex, marital status, and religion).
2. Risk factors for tuberculous infection and disease (age, sex, level of education, HIV positivity, smoking, alcohol, sleeping proximity to index, household size).
3. Whether the contact was on TB treatment or not at the time of administering the questionnaire
4. TB treatment registration number (for those on were on treatment).
5. Whether the contact was taking ARVs (Antiretroviral therapy) or not at the time of administering the questionnaire
6. Date of commencement of ARVs, for individuals taking ARVs.

The 1st follow up visit questionnaires captured data similar to that administered at enrolment. At the 2nd final follow-up visit, the questionnaire was modified to include information from the TB treatment card (**Appendix 16**).

3.6.2 The TB register and patient TB treatment cards

In addition to the questionnaire, during the 2nd follow up visit, individuals were asked for their TB cards from which the research assistant recorded the following (in instances where they were recorded):

1. Date TB treatment started,
2. Type of TB (smear positive, smear negative, extra pulmonary),
3. Type of patient (new, retreatment),
4. The name of the TB treatment centre
5. Number of TB episodes during the study period.

The purpose of using the TB register and the patient TB treatment cards was two-fold a) to verify that an individual was notified at the clinic b) To collect additional data that may not have been collected using the questionnaire such as date of registration, type of TB and smear results. This was especially important for individuals who were only seen at the 1st follow up visit as the questionnaire was less detailed.

To identify individuals who developed TB during each time point the questionnaire data was linked to the TB register. Key variables (TB registration number, serial no, name, age, sex and date of registration/date treatment) recorded in the questionnaire were used to trace the individual in the TB register.

Manual match with the TB-register data was done. TB registers in 24 communities were captured electronically from the beginning of the ZAMSTAR study. Expert judgment was used to match the variables: community and sex needed to match exactly, differences in age of up to 2 years were allowed, differences in date of registration of 3 months were tolerated, misspellings of names, TB registration number and mixing up of the TB registration number and serial number were taken into account. The matched records were exported into Stata 11 for analysis.

3.6.3 HIV recording form

In addition to the data collected through the questionnaire at enrolment, blood for HIV antibodies was drawn and recorded on the enumeration form (**Appendix 3**).

3.6.4 TST and QFT-GIT recording forms

For QFT-GIT processing a recording form (**Appendix 9**) captured the following data: date and time of sample collection, date and time of incubation start and end, temperature at the beginning and end of incubation, date of plasma extraction, storage and delivery to the central laboratory.

A TST recording form captured the dates the TST was administered, read and the size of the induration (**Appendix 12**).

3.6.5 TB culture recording form

A transport form (Appendix 13) was filled in before sputa were taken to the laboratory for processing. This recorded the number of sputum samples sent to the laboratory from the field on a particular day.

Amendment of the data collection tools at the 2nd follow-up visit

The enumeration form was amended to include a column for sputum. Collection of sputum for culture was included at the 2nd follow-up visit. The consent and information forms were amended to include consent and information regarding the need for participants to provide a sputum sample.

3.7 Quality Assurance and Data Management

3.7.1 Data quality and management

This study was conducted by local experienced staff that was fluent in most of the commonly spoken local languages. This ensured a better acceptance of the study from the local population and ensured good knowledge of the study sites. In addition, each study site had community advisory boards that ensured that the study procedures and content were locally appropriate and acceptable by the community. Both nurses and research assistants were trained in Good Clinical Practices (GCP). Data collection was preceded by intensive training. The questionnaire was piloted using all major languages spoken in Zambia and SA and was revised following unclear issues arising from the piloting.

During the study, site team leaders checked the quality of data that was collected such as whether the questionnaires were filled in correctly, completely or whether bar codes matched samples collected. In addition, a multidisciplinary Quality Assurance team was constituted at the beginning of the ZAMSTAR study whose roles included assessing compliance with standard operating procedures and the quality of the laboratories.

3.7.2 Data entry and data cleaning

Questionnaire data, laboratory worksheets, HIV results, QFT-GIT and TST results were dually entered into a "Microsoft SQL Server" database and checked for errors. Data entry, query generation and correction were performed on a daily basis. Data entry differences were corrected by referring to the source documents. Data was imported into STATA (version 11.0) using Microsoft Access 2000.

3.8 Confidentiality

All data collected in this study was kept strictly confidential. The study information did not identify or disclose any person's name, status or test result and samples were kept anonymous. Bar codes were assigned to individuals. No HIV testing was carried out for the purpose of this study but HIV test results were obtained through data collected from the ZAMSTAR study. Data security was ensured through password-protected databases accessible only to a selected group of people involved in the study.

3.9 Statistical Analyses

Data analysis was performed using STATA (version 11.0). Analysis was performed at individual level taking into account the effect of clustering at household and country levels due to the sampling design of the ZAMSTAR study. A p-value ≤ 0.05 was defined as statistically significant.

General description of qualitative and quantitative data analysis

Frequencies and percentages were used to describe categorical variables and the median and interquartile range for quantitative variables. Categorical data were compared by the Pearson's chi-square test (or Fisher's exact test, when expected sample sizes comprised fewer than five individuals). Wilcoxon rank-sum test was used to compare non-parametric distribution of two

groups. Kruskal-Wallis test was used to compare non-parametric distributions of more than two groups. Non-parametric tests were used to avoid the assumption that data were sampled from a normal distribution.

Prevalence of TB infection

Prevalence of infection was defined as the number of QFT-GIT or TST positive results divided by the total number of HHCs with interpretable (positive and negative) results. Individuals with missing TST or indeterminate QFT-GIT results were excluded from the analysis.

Risk factors associated with positive QFT-GIT and TST results at enrolment

The distribution of positive reactions to each test in relation to established individual and household level risk factors for TBI was described. The strength of relationship between risk factors and QFT-GIT / TST positivity was assessed using random effects logistic regression. The random effects approach specified the household of residence as the clustering variable. All models were adjusted for age, sex, and community of residence. Adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for each risk factor were calculated. A single "chunk" test was performed to assess the significance of all the interaction terms.

Agreement between TST and QFT-GIT

Test concordance and discordance were estimated using the kappa statistic and McNemar's test respectively. TST and QFT-GIT results were analyzed as continuous variables and as dichotomized binary values. Concordance (Agreement) between TST and QFT-GIT was measured using the Cohen's Kappa (k) coefficient (240), where $\kappa > 0.75$ represent excellent agreement, κ values from 0.4-0.75 represent fair to good agreement and $\kappa < 0.4$ represent poor agreement, beyond chance (241). Agreement was explored stratified by different TST cut-off points and by HIV status. The concordance rate was determined from individuals positive for both plus those negative for both divided by the total, and the discordance rate was the complement of the concordance rate.

Strength of association between two variables (Correlation)

Correlation coefficients were used to measure the strength of association between two variables. Correlation between continuous INF-γ values (IU/ml) and TST induration (mm) was assessed using Spearman's correlation coefficient. Spearman rank correlation is a non-parametric test that is used to measure the degree of association between two numerical variables and does not assume any assumptions about the distribution. Pearson correlation was used to measure the degree of the relationship between linear related variables, both of which were normally distributed and continuous.
Intraclass correlation (ICC) analysis was also used in the background reproducibility study.

A positive correlation coefficient indicated a positive relationship between the two variables while a negative correlation coefficients expressed a negative relationship. A correlation coefficient of 0 indicated that no relationship between the variables existed at all.

Likelihood Ratio Test (LRT)

The LRT was used to examine the following

1. The association of the exposure with the outcome
2. The association of the exposure with the outcome after adjusting for other variables
3. Interaction
4. Linear trend
5. Departure from linear trend

Definition of low and high IFN-γ levels

High and low levels of IFN-γ were defined as ≥ 10 and ≥ 0.35 and <10 IU/ml respectively. IFN-γ responses greater than 10 IU/ml were truncated and reported as 10 IU/ml as they fall beyond the linear range of most ELISA readers. This is consistent with how the QuantiFERON®-TB Gold IT software provided by the manufacturer reports results. Missing data and QFT-GIT indeterminate results were excluded from analysis.

IFN-γ levels were analyzed using dichotomous measures and as per Manufacturer's recommendations. In a sensitivity analysis, different thresholds were explored to determine "high levels" of IFN-γ. Continuous measures were converted to quartile category values to explore the risk of developing disease with each quartile and the trends with increasing value of responses. Further exploration was through use of positive quartile category values. Definition of "strong responders" used by other authors were also explored (235). Geometric means and medians of IFN-γ levels in high and low responders were tested with Wilcoxon-Mann-Whitney.

Survival Analysis

Basic goals of survival analysis were:

1 To describe, estimate and interpret survival characteristics using Kaplan-Meier survival function estimation
2 To compare survival in different groups using non-parametric tests
3 To assess the relationship of explanatory variables to survival time using Cox regression models.

Descriptive survival analysis

The Kaplan-Meier estimates and the generalized log-rank test were used to construct and compare the TB-free survival probabilities curves of HHCs with positive and negative QFT-GIT and TST results. Survival estimates were analyzed by enrolment test and also stratified by HIV status.

The median survival time was also calculated; this was described as the time with probability of survival equal to 0.5 (or the length of time during which the first 50% of the sample developed the event, TB). Nelson-Aalen cumulative hazard estimates were also explored as an alternative to showing survival using Kaplan-Meier curves.

Examining the Proportional Hazard Assumption (PHA) in the Cox model

A check for departure from the proportional hazard assumption (which assumes that the ratio of the hazards in the two groups remains constant over time) was done. This comprised of checking whether the HRs estimated by the Cox model change with time or vary over different intervals of the time scale. Three general methods were used to examine model adequacy: Graphically by using the Kaplan-Meier curves (to check whether survival curves intersect); mathematically by using log-minus-log survival plot (Schoenfeld test) and by using time-dependent variable (test for interactions with time).

Incidence rates

TB incidence rate was defined as the number of new cases of TB during a specified period of time divided by the total person-time-at-risk throughout the observation period.

The period of risk started at the date of HHCs were enrolled into the study. The period of risk ended at either the second follow-up visit, the midpoint of the last visit seen (if earlier than visit 2) and the following scheduled visit date or date of TB diagnosis (defined as the start date for treatment) whichever came first.

TB incidence rate was expressed per 1000 per years as follows:

TB incidence rate*= (Number of new TB cases/Total person years of observation) x 1000*

The incidence rate ratio was defined as the incidence rate in the exposed group divided by the incidence rate in the unexposed group. It was expressed as follows:

Incidence rate ratio*: Incidence rate in exposed group/Incidence rate in unexposed group*

TB incidence rates were determined and incidence rate ratios compared for various groups:

- QFT-GIT positive vs. negative.
- TST positive vs. TST negative.
- Concordant QFT-GIT and TST results.
- Discordant QFT-GIT and TST results
- High vs. low IFN-γ levels and for the groups used in sensitivity analysis

TB incidence rates and IRR were stratified by country and HIV for some groups

Cox regression was used to estimate incidence rates of TB per 1000 person-years of follow-up, together with 95% CIs. Incident and non-incident cases were compared with respect to socio-demographic and clinical characteristics using the chi-square test.

Positive and negative predictive values

Positive predictive value was defined as the proportion of HHCs with test positive results and had incident TB. Negative predictive value was defined as the proportion of HHCs with test negative results and did not have incident TB. These were calculated as follows:

$$\text{Positive predictive value} = \frac{\text{all testing positive and develop TB}}{\text{All testing positive}}$$

$$\text{Negative predictive value} = \frac{\text{all testing negative and do not develop TB}}{\text{All testing negative}}$$

Evaluation Of Confounding, Mediation and Effect Modification

Three concepts about the relationships of variables were examined and these were:

- Confounding
- Mediation
- Effect modification or interaction.

An assessment of confounding and mediation and etiological pathways was conducted in line with the study objectives. Below is a summary of each of these concepts

1. Potential Confounding Factors

Confounding factors are variables that produce a mixing of the effect of the exposure under study on the disease (outcome) with that of a third factor. Confounders met the following criteria: associated with the exposure of interest; independently associated with outcome; not on the causal pathway

2. Mediating Factors

Mediation has been defined as the totality of processes that explains an observed relationship between exposure and disease. A mediator is also associated with both the exposure and outcome, but differently from a confounding factor it is part of the causal pathway linking the exposure and outcome. A mediator does not bias an association; rather it can help to explain it.

3. Effect Modifiers

Interaction occurs when the presence of one factor modifies the effect of another (the effect of the exposure of interest differs according to which category of the other factor is being examined).

Univariable and Multivariable Analysis

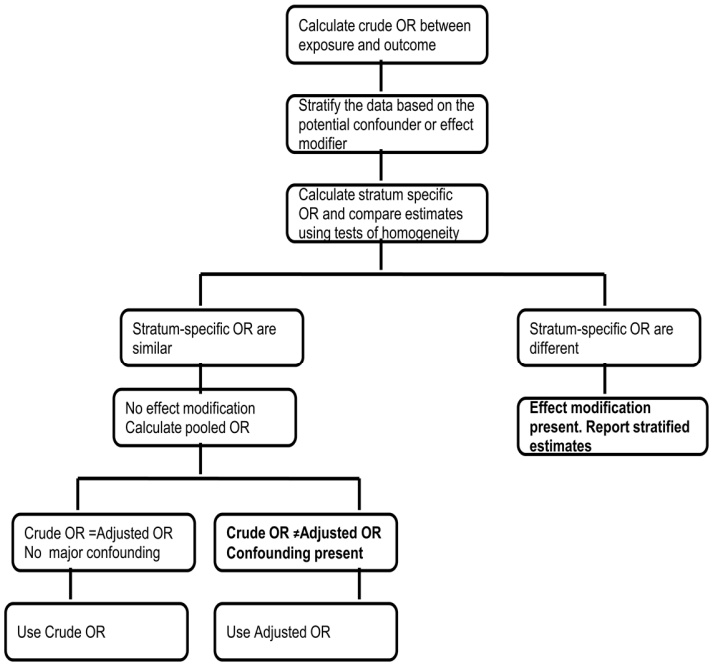
Univariable and multivariable regression models were used to assess the relationship between primary exposures variables and outcomes. For survival analysis Cox regression models were used. HRs were the main measure of association. P-values were calculated from likelihood ratio tests comparing models with and without the factor of interest. The Cox model assumes that all covariates act equally (proportionally) on the enrolment hazard over time (i.e. the effects of the covariates do not change with time).

Initially, a univariable analysis was performed among explanatory variables followed by multivariable analysis. In multivariable analysis, a hierarchical logistic regression model was used to explore the relationship between the study outcome and a range of explanatory variables for the primary research question. Since there is no evidence to determine the interrelations between HHCs with high IFN-γ levels and other known risk factors for incident TB, the objective of the study was to identify this, using a hierarchical approach. In addition it still unknown whether the effect of the high IFN-γ levels on TB incidence is direct or mediated through other factors. Since there were many explanatory variables, the decision to include risk variables was not based on statistical significance only (as in the case of step-wise logistic regression) but was determined through a conceptual framework describing the hierarchical relationships between them.

To construct a hierarchical logistic regression model firstly, I defined the conceptual, hierarchical framework for the relationships between the different variables and the disease outcome as reflected in the conceptual framework presented in the introduction. Secondly, I identified variables that were known *a priori* to be important confounders based on existing evidence.

Thirdly, I identified other possible confounders and effect modifiers through exploratory analysis. Exposure-confounder interactions were examined paying particular attention to those thought *a priori* to be worth investigating. I used a combination of external knowledge with statistical associations in the following strategy summarized in **Figure 3.5**.

Figure 3.5: Evaluation of confounding and interaction



Modified from Webb. Essential Epidemiology, Cambridge Univ Press

Note: Odds ratio or Hazard ratio

Lastly, exposure variables were categorized into levels and regression models were fitted as shown in the results section. A final model was derived after assessment for interaction between the relevant exposures. Exposure-exposure and exposure-confounder interactions were examined one at a time, paying particular attention to those thought *a priori* to be worth investigating. In instances were interaction was weak, groups were combined in the interaction parameter to try and increase the power of tests for interaction.

Cluster-level analysis

After the final model was built, further analysis was performed at individual level taking into account the effect of clustering at household level due to the design of the ZAMSTAR study. Analysis was also performed at country level.

Cluster level analysis was considered because of the following reasons: individuals within the same cluster are likely to be more similar to each other than they are to individuals in other clusters; infectious disease tends to cluster in time and space; clusters may share risk factors for infection, and for progression from infection to disease.

There are 3 methods that allow individual-level analysis but account for within-cluster and between-cluster correlation: "Robust" standard errors (SE); Generalized estimating equations (GEE) and Random-effects (multi-level) model. In this analysis Random-effects (RE) was method of choice but because the model could not work, the next preferred method was chosen, "Robust" standard errors.

"Robust" SE is expected to work well if the number of clusters is more than 30. Although this method is simple and one can always fit this model to the data it does not take account of within-cluster correlation. However, this is relatively less important when the size of each cluster is similar (as in the design of the ZAMSTAR study).

Risk factors for incident TB

Risk factors for time to TB episode during follow-up were analyzed using Cox proportional hazards regression. The primary risk factors of interest were age, sex, high IFN-γ levels, QFT-GIT positivity, TST positivity, HIV positivity, socio-economic status, smear status of index, smoking, proximity to the index case and country.

Risk factors associated with high IFN-γ levels:

For the analysis in this section and subsequent ones in this chapter some variables (age, smoking, education, socioeconomic status and sleeping proximity to the index) were re-coded due to small numbers

Summary: Methods

This study, the QFT cohort study was nested within a large community randomized trial called ZAMSTAR (Zambia South Africa TB and AIDS Reduction) implemented in 16 communities in Zambia and 8 communities in the Western Cape Province of South Africa (SA). More specifically it was nested within cohorts of TB patients and their household members called secondary outcome cohorts (SOCS) of the ZAMSTAR study whose aim was to determine the secondary objectives of the ZAMSTAR study at household-level.

The sample size for this study was approximately 2400 adult HHCs enrolled in the SOCS. The study was conducted from April 2007 to December 2010. The study population was adult HHCs of newly notified TB cases at least 15 years of age who were enrolled in SOCS and in whom QFT-GIT was performed. The study protocols were approved by all the relevant authorities in both countries.

In SA, 4 teams conducted the study in all the 8 communities, while in Zambia, 16 teams (one team per community) located in the different geographical areas conducted the study. However, training and operating procedures were standardized as much as possible.

Newly notified adult TB patients (index cases) were used as entry points into households where HHCs were invited to participate. QFT-GIT, TST and HIV testing were performed at baseline according to standard procedures. HHCs were not screened for active disease at baseline. They were followed-up twice at 18 and 36 months post-enrolment to ascertain who was still in the cohort and those that were lost to follow-up. A structured questionnaire was administered to collect information on risk factors for tuberculous infection and disease. At the final follow-up visit HHCs submitted a sputum sample to determine active disease using culture; liquid medium (MGIT960 *H* BD). Quality assurance procedures of all study processes, the tests and data were developed and followed.

Incident TB was defined as self reported TB that was confirmed in the TB clinic register or patient treatment card. In a sensitivity analysis three other definitions of incident TB were explored as follows: exclusion of HHCs who developed TB within 2 months of enrolment; bacteriologically confirmed TB (smear) and smear together with culture.

Cox regression models were used to assess the relationship between primary and secondary exposures and incidence of TB.

DISCLAMER: Kwame Shanaubes' specific roles in the studies described in this thesis:

1. The feasibility studies (Chapter 4)

The performance of QFT-GIT in a field setting in Zambia (study 1)

Edward Raby was the principal investigator of this study. He conceived, designed, conducted the study and analyzed some results as per his publication (**Appendix 20).** My specific role was that I used data collected in this study and analyzed it in line with my objectives since not all the data was analyzed and reported by Edward Raby. More specifically I analyzed data on reproducibility of QFT-GIT i.e. test-retest reproducibility of QFT-GIT and the effect of a 24-hour delay in incubation as in my published paper (Appendix 20).

The effect of power outages on IFN-γ responses (study 2) and the effect of storage temperature on IFN-γ responses (study 3)

For these two studies (study 2 and 3), I designed the studies together with colleagues, Petra De Haas and Maureen Moyo. I also supervised the studies and analyzed the results with help from Ab Schaap.

The financial cost of using QFT-GIT for the diagnosis of TB infection (Study 4)

The study was designed and conducted by Lawrence Mwenge. My specific role was that of supervision. Although the main analysis and summary write-up in this thesis was done by Lawrence Mwenge, I was involved in these processes as well.

2. The background study: ZAMSTAR study

I was involved in the conduct of the background study, the ZAMSTAR study. Although not part of this thesis I was responsible for the day to day running of the two tuberculin skin test surveys and one regional TB prevalence survey.

3. The QFT-GIT cohort study (main study of the thesis)

For the main study described in the thesis i.e. the QFT cohort study, my roles were that of co-investigator and study manager. I contributed towards the design of the QFT cohort study together with Peter-Godfrey Faussett and Helen Ayles and Nulda Beyers. I was responsible for the day to day running of the study, the supervision of teams and quality assurance during the entire duration of the study. I analyzed the results of the QFT cohort study and received statistical guidance from colleagues from ZAMBART project and LSHTM (see acknowledgements).

4.0 Performance and operational characteristics of QFT-GIT in Zambia

Introduction

The aim of this chapter is to determine the performance and operational characteristics of QFT-GIT in a field setting. The evaluation of any diagnostic test involves assessing its performance and operational characteristics, preferably in a setting where the test will be used. For this reason, before we rolled out QFT-GIT on a large scale we decided to do some preliminary studies to determine factors that may affect the performance and operational characteristics of this test in our setting. Few studies have been published on the performance of QFT-GIT in resource limited settings with a high burden of TB and HIV (242).

To assess test performance, we evaluated the sensitivity and test-retest reproducibility of QFT-GIT as well the effect of CD4+ T-lymphocyte counts on IFN-γ responses. We also looked at some process and storage factors affecting the operation of this test.

More specifically, we aimed to determine the effect of three factors on IFN-γ responses namely; delayed incubation of blood samples; power outages during incubation and increased storage temperature of blood collection tubes. Lastly, we determined the financial cost of diagnosing TB infection using QFT-GIT.

Four small studies were used to examine these objectives. The first study (study 1) addressed four objectives namely; the sensitivity and test-retest reproducibility of QFT-GIT as well as the effect of CD4+ T-lymphocyte counts and delayed incubation of blood samples on IFN-γ responses. The second study (study 2) addressed the effect of power outages during incubation on IFN-γ responses. The third study (study 3) addressed the effect of increased storage temperature on IFN-γ responses. Lastly, the fourth study (study 4) addressed the financial cost of diagnosing TB infection using QFT-GIT.

4.1 The performance of QFT-GIT in a field setting in Zambia (Study 1).

Introduction

The basic performance characteristics of a test designed to distinguish infected from uninfected individuals are sensitivity, that is, the probability that a truly infected individual will test positive, and specificity, that is, the probability that a truly uninfected individual will test negative (144). Since there is no gold standard for the diagnosis of TB infection, both sensitivity and specificity cannot be directly measured. For assessing sensitivity, patients with bacteriologically confirmed (culture or smear positive) active TB are used as a proxy measure. Sensitivity of IGRAs in active TB is considered a surrogate of sensitivity of IGRAs in TB infection, as has been done for the TST in the past (190). For assessing specificity, healthy, low risk individuals without known exposure to TB are used. An estimate of specificity would be difficult to determine in both Zambia and SA due to the high prevalence of TB infection (222).

Another important performance characteristic of a test is its reproducibility. The reproducibility of a test is an assessment of the extent to which the same tester achieves the same results on

repeated testing of the same samples, or the extent to which different testers achieve the same results on the same samples (144).

In this study we aimed to evaluate factors affecting the performance of QFT-GIT in a field setting. The specific objectives of this study were four-fold. Firstly, we aimed to determine the sensitivity of QFT-GIT in smear positive TB patients and secondly, determine the test-retest reproducibility of QFT-GIT. Thirdly, we aimed to determine the effect of CD4+ T-lymphocyte count on IFN-γ responses and lastly, determine the effect of a 24-hour delay in incubation of blood samples on IFN-γresponses. Some of the results in presented in this study have been published (61, 222).

Sample size

The sample was limited by time and budget constraints. It was estimated that there were around 30 new cases of smear positive TB per month in each of the four large health centers in Lusaka. Over a recruitment period of four weeks at the four health centers it was hoped to recruit an overall number of 200 people to the study. It was expected that around half of these would be HIV positive and up to a third will have a low CD4 count. Assuming overall sensitivity of QFT-GIT to be around 80%, with 200 subjects' sensitivity could be estimated with confidence intervals of around +/- 5%.

Statistical analysis

Overall, statistical analysis was done as described in the methods (statistical analysis) section of the thesis. However, a brief description is given here.

We determined the sensitivity of QFT-GIT to assess test performance and compared it to that of TST using STATA version 11.0. Sensitivity was defined as the proportion of individuals with a positive QFT-GIT result among those with smear-positive TB. Sensitivity was defined using two methods; firstly, by including indeterminate QFT-GIT results and then secondly, excluding these results from the denominator.

QFT-GIT results in these patients were also examined quantitatively, by looking at the IFN-γ responses to TB antigens in relation to grades of smear and duration of TB treatment. This is because there is some literature, although inconclusive suggesting that TB antigen-specific IFN-γ responses may correlate with antigen (bacterial load)(28).

To determine test-retest reproducibility of QFT-GIT, test concordance and discordance were estimated using the kappa statistic and McNemar's test respectively. Continuous measures were also compared using Inter Class Correlations (ICC).

4.1.1 Methods

a) Study population

The study population was adults (over 18 years) with smear-positive TB who are untreated or had less than 4 weeks of TB treatment. These patients were enrolled from four government clinics in Lusaka from July to October 2007.

b) Eligibility and recruitment

TB patients were invited to participate from outpatient chest clinics by study nurses. The study nurses explained the study to TB patients and invited them to participate. Informed written consent was obtained from all patients using a standard information sheet (Appendix 17 and 18). This was read out to patients if necessary and they were then given the opportunity to ask questions. Patients who were unable to write were asked to provide a thumbprint. Eligible patients were identified through the review of TB treatment cards. Patients were defined as smear positive if acid-fast bacilli were identified by microscopy in at least one sputum smear.

Demographic data, information on risk factors for TB infection, height, weight and mid-upper arm circumference measurements was recorded for each patient by means of a short questionnaire (Appendix 19).

c) Ethical approval

Ethical approval was obtained from the London School of Hygiene and Tropical Medicine and the University of Zambia ethics committees.

d) Venepuncture

Approximately 9 mls of venous blood was drawn into three sets of QFT-GIT collection tubes (3 ml for one set x 3) which were processed as follows: two sets were handled as duplicates and processed according to manufacturer's instructions (standard incubation 1 and 2) while for the third set of tubes, incubation was delayed for 24 hours (delayed incubation).

One set of the duplicate samples (standard incubation 1) was used to determine sensitivity of QFT-GIT and the effect of CD4+ T-lymphocyte count on QFT-GIT performance. To determine test-retest reproducibility of QFT-GIT, both duplicates samples were used while the third set whose incubation was delayed for 24 hours was used to determine the effect of a 24-hour delay in incubation on IFN-γ responses. In cases where the patient was offered and accepted to undergo routine diagnostic counseling and testing (DCT) extra blood was drawn for full blood count as well as liver and renal function tests in accordance with MoH guidelines at the time of the study.

For the duplicates samples, QFT-GIT was performed according to manufacturer's instructions (see methods section). In brief, whole blood was drawn and placed directly into three 1 ml tubes each containing ESAT-6, CFP-10 and TB7.7 or PHA (phytohaemagglutinin-a mitogen used as a positive control) or heparin (used as a nil sample to measure the background level of IFN-γ).

Samples were mixed vigorously and transported to the laboratory within 16 hours. Samples were incubated at 37°C for 24 hours, centrifuged and then plasma harvested to perform ELISA. One laboratory scientist processed the duplicate samples on the same ELISA plate.

For the samples whose incubation was delayed for 24 hours, QFT-GIT samples were processed as described above (manufacturer's instructions) except that the incubation period was delayed by 24 hours. This was done by leaving the blood collection tubes on the bench at room temperature (20-25° C) before incubation.

T-lymphocyte estimations were done in these patients as a prerequisite to assessing anti-retroviral therapy (ART) in line with standard Ministry of Health (MoH) guidelines at the time of the study. Blood CD4 and CD8 counts were obtained using FACS Count (Becton Dickinson) flow cytometric assay according to the manufacturer's instructions (BD).

e) Tuberculin Skin Test (TST)

TST was administered and read as described in the methods section of the thesis. Briefly, TST was administered using 2 TU (Tuberculin Units) of PPD RT23 (Copenhagen, Denmark). A dose of 0.1 ml was injected intradermally on the volar aspect of the left forearm. TST results were read using calipers between 48-96 hours after placement. All tests were administered and read by study nurses who were trained according to the standard IUATLD protocol (238).

f) Clinical care

Clinical standard of care was offered according to MoH national guidelines for patients with TB or those co-infected with TB and HIV. As part of the routine health service activity, TB patients were given general information on TB and HIV. The MoH recommends that HIV testing be done as a routine test for all TB patients in order to maximize the potential for ART referral for those found positive. Therefore, diagnostic counseling and testing (DCT) was encouraged and if accepted HIV status was recorded.

HIV status of the TB patients was obtained from VCT and TB registers as well as patient records. No HIV testing was done specifically for the study. Patients without documented HIV status were encouraged to take DCT and all those found positive were referred for further counseling and to discuss treatment options. Those who declined to take part in the study received TB treatment in the standard way. Those found to have a low CD4 count (<350 cells/microL) were referred for the consideration of ART.

4.1.2 Results

a) Subject characteristics

We enrolled 117 TB patients for the study. Of these, 5 patients were excluded due to missing data. The remaining 112 adult smear-positive TB patients were included in the analysis of this study. Out of the 112 TB patients, 72 (64.3%) were male and the median age was 31 years (IQR: 25-35) **(Table 4.1)**.

Table 4.1: Demographic and clinical characteristics of TB patients

	TB patients n (column %)
Total	**112**
Sex	
Male	72 (64.3)
Female	40 (35.7)
Age group (years)	
18-24	27 (24.1)
25-34	53 (47.3)
35-44	26 (23.2)
45-64	6 (5.4)
Body mass index (kg/m^2) (WHO definition)	
< 18.5 (underweight)	49 (46.2)
>18.5	57 (53.8)
Missing	6
BCG scar	
No	38 (35.8)
Yes	68 (64.2)
Missing	3
HIV status (n, 96)	
Negative	34 (35.4)
Positive	62 (64.6)
Missing	16
Currently taking ARVs	
No	97 (86.6)
Yes	15 (13.4)
CD4 count, cells/ µl (WHO AIDS defining)	
<200	35 (31.2)
≥200	77 (68.7)
Smear grade	
Smear 1+	42 (37.5)
Smear 2+	18 (16.1)
Smear 3+	52 (46.4)
Previous TB	
No	53 (72.6)
Yes	20 (27.4)
Missing	39
Period of TB treatment	
Not on TB treatment at enrolment	35 (31.8)
1-14 days	55 (50.0)
15-31 days	20 (18.2)
Missing	2

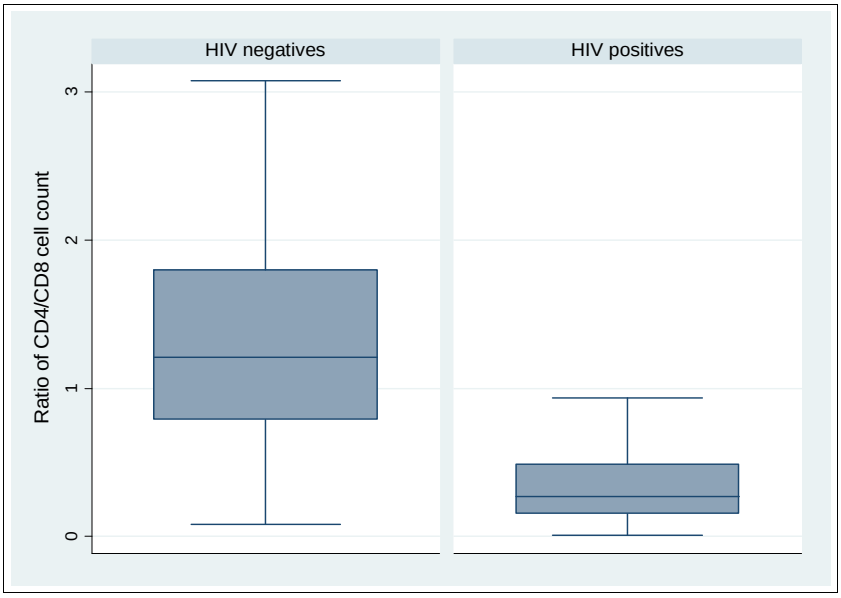
Using the WHO definition of malnutrition, 46.2% of TB patients were underweight (table 4.1). The median body mass index (BMI) was 18.6 kg/m^2 (IQR: 17-21).

As expected in this setting, there was a high rate of TB/HIV co-infection in these patients. HIV status was recorded for 96 patients, of these, 64.6% (62/96) were HIV positive (Table 4.1). At the time of the study, ART roll-out by the MoH was in its initial stages. Therefore, ART status was recorded for 24.2% (15/62) HIV positive TB patients. At enrolment, the median duration of being on ART was 7.4 months (95% CI: 2.33-21.2).

93.7% (105/112) patients had results available for T-lymphocyte estimations. Overall, the median CD4 count was 310 cells/µl (IQR: 153-571) and the median CD4/CD8 ratio was 0.45 cells/µl (IQR: 0.18-1.15).

T-lymphocyte estimations were also compared in HIV positive and negative patients. In HIV positive patients, the median CD4 count was 205 cells/µl (IQR: 115-312) and the median CD4/CD8 ratio was 0.27 cells/µl (IQR: 0.15-0.49). In HIV negative patients, both the median CD4 count, 537 cells/µl (IQR; 450-689) and the median CD4/CD8 ratio, 1.21 cells/µl (IQR; 0.79-1.80) were higher compared to HIV positive patients **(Figure 4.1)**.

Figure 4.1: Box plot showing CD4/CD8 cell count ratio by HIV status



There was a significant difference between HIV positive and negative patients in the ratio of CD4/CD8 cell count (ranksum, p<0.001).

Smear status was recorded for all the 112 patients; 37.5% (42/112) had sputum smear recorded as 1+, 16.1% as 2+ and 46.4% as 3+ (table 4.1). 27.4% (20/73) of TB patients had a history of prior TB treatment (relapse cases).

For the current episode of TB, there were 110/112 (98.2%) patients with accurate TB treatment dates recorded. The remaining 2 TB patients were excluded due to errors in dates (dates of current TB treatment were earlier than when TB was diagnosed). At enrolment, 31.8% (35/110) of patients were not on TB treatment; 50.0% (55/110) were within 1-14 days of treatment and the remaining 18.2% (20/110) were within 15-31 days of treatment.

b) IFN-γ responses

Overall, the median IFN-γ response to TB antigens was 1.89 IU/ml (IQR: 0.25-5.28). The median IFN-γ responses were lower in HIV positive (1.46 IU/ml (IQR: 0.14-5.03)) compared to HIV negative (2.63 IU/ml (IQR: 0.77-6.54)) patients (rank-sum; p=0.12), reflecting underlying cellular immune suppression in these patients.

TB patients who had smear grade 3+ had higher IFN-γ responses (2.08 IU/ml (IQR: 0.45-4.57)) compared to those who had smear grade 2+ (1.68 IU/ml (IQR: 0.35-5.02)) or smear grade 1+ (1.50 IU/ml (IQR: 0.06-7.07)). However, these differences were not significant (Kruskal-Wallis test, p=0.82).

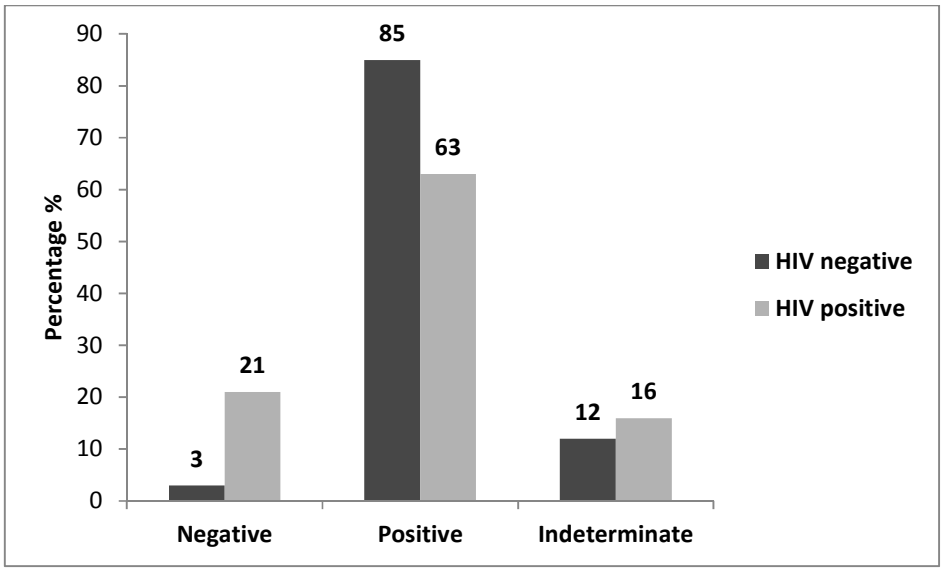
IFN-γ responses were lower in patients who were not on treatment ((1.30 IU/ml (IQR: 0.18-5.38)) compared to those who were on treatment for less than 2 weeks (2.8 IU/ml (IQR: 0.37-5.31)) or greater than 2 weeks (1.34 IU/ml (IQR: 0.2-5.69). However, these differences were also not significant (Kruskal-Wallis test, p=0.30).

c) Sensitivity of QFT-GIT and TST

Using QFT-GIT, 74.1% (83/112) of TB patients were positive, 12.5% (14/112) were negative and 13.4% (15/112) had indeterminate results. When patients with indeterminate results were included, the sensitivity of QFT-GIT was 74.1% (95%CI: 65.0-81.9). However, QFT-GIT sensitivity increased to 85.6% (95% CI: 77.0-91.9) when patients with indeterminate results were excluded. Using TST, 47/91 patients were TST positive giving a sensitivity of 51.6% (95% CI: 40.9-62.2) at the standard cut-off of ≥ 10 mm. At a TST cut-off of 15 mm, sensitivity of TST was 20.9% (95%CI: 13.1-30.7%) while it was 65.9% (95%CI: 55.2-75.5) at a TST cut-off of 5 mm.

In a subgroup analysis of patients with known HIV status, sensitivity was lower in HIV positives, 62.9% (95%CI: 49.7-74.8) compared to HIV negatives, 85.3% (95%CI: 68.9-95.0) (chi2: p=0.03). They were more indeterminate and negative results in HIV positive patients compared to HIV negative ones (chi2; p=0.03) (**Figure 4.2**). All the indeterminate results were due to poor response to mitogen (positive control).

Figure 4.2: QFT-GIT results in TB patients by HIV status



Similarly, when TST results were stratified by HIV status at the cut-off of ≥ 10mm, sensitivity was lower in HIV positives, 40.8% (95%CI: 27.0-55.79) compared to HIV negatives, 64.3% (95%CI:

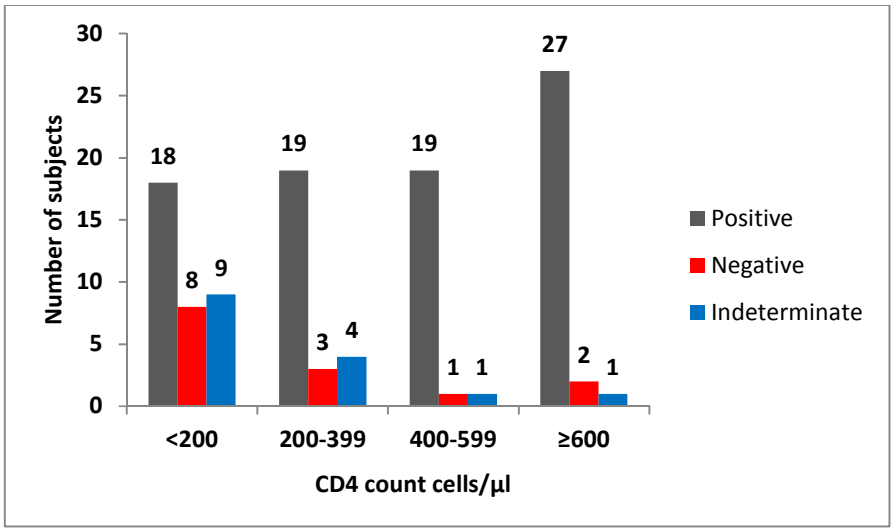
44.1-81.3)(rank-sum: p=0.05). However, sensitivity improved at a TST cut-off of ≥ 5mm to 55.1% (95%CI: 40.2-69.3) in HIV positives and 82.1% (95%CI: 63.1-93.9) in HIV negative patients (rank-sum: p=0.02). Nevertheless, at TST cut-offs of ≥ 10mm, ≥ 15mm and ≥ 5mm, TST sensitivity was lower than that of QFT-GIT overall and stratified by HIV status .

d) The effect of CD4+ T-lymphocyte cell count on QFT-GIT performance

Our second objective in this study was to determine the effect of CD4+ T-lymphocyte count on QFT-GIT performance. The median CD4 cell count was significantly lower for patients with indeterminate results, at 178 cells/µl (IQR: 39-238), than for patients with negative (189 cells/µl (IQR: 89-312)) and positive results (428 cells/µl (IQR: 215-615)) results (Kruskal-Wallis test, p=0.0003). The difference in median CD4 count remained significantly different between QFT-GIT positive and negative patients even when indeterminate results were excluded (ranksum, p=0.008).

With falling CD4 count there was a decrease in sensitivity of QFT-GIT with increases in both negative and indeterminate results (chi2, p =0.01) (**Figure 4.3**). Reduced sensitivity was particularly marked at CD4 counts less than 200 cells/ml, where only 18/83 (21.7%) patients had a positive QFT-GIT result (Figure 4.3). There was a trend towards an increase in both indeterminate and negative results with lower CD4 counts (test for trend, p<0.001).

Figure 4.3: The distribution of QFT-GIT results by CD4 cell count



As expected in this study, the majority of HIV positive patients, 46.8% (29/62) had CD4 counts below 200 cells/µl. In contrast, the majority of HIV negative patients, 41.2% (14/34) had CD4 counts ≥ 600 cells/µl. There was a significant trend towards an increase in both indeterminate and negative results with falling CD4 count for HIV positives (test for trend, p=0.03) but not for HIV negative patients (test for trend, p=0.2).

The median IFN-γ response to TB antigens was significantly lower in patients with a CD4-cell count <200 cells/µl than any of the three other groups (Kruskal-Wallis; p=0.01). There was a trend towards increased IFN-γ response to TB antigens with increasing CD4 cell count levels (test

for trend, p=0.001). This trend was also found when PHA stimulated IFN-γ response and CD4 cell counts were examined **(Figures 4.4 and 4.5)**.

Figure 4.4: IFN- γ responses to TB antigens (ESAT-6/CFP-10/TB7.7) by CD4 count category

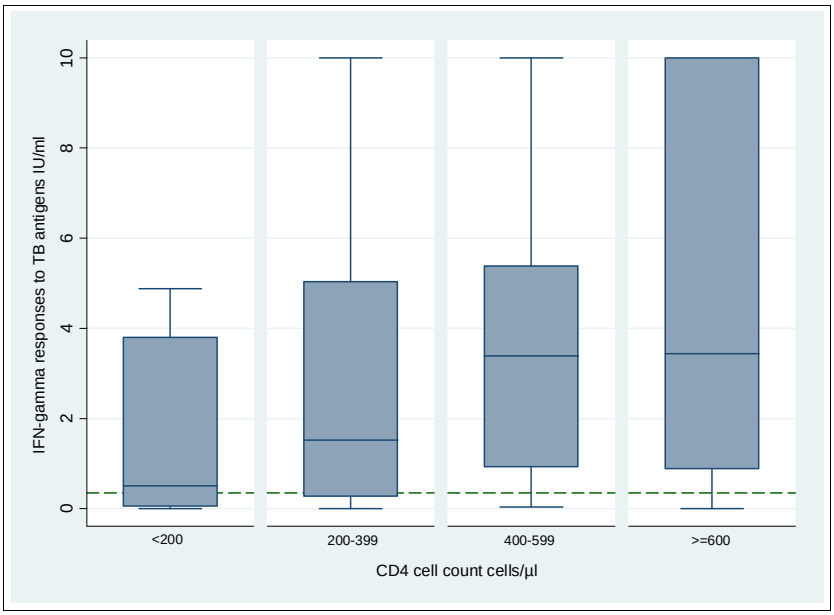


Figure 4.5: IFN- γ responses to PHA (mitogen) by CD4 count category

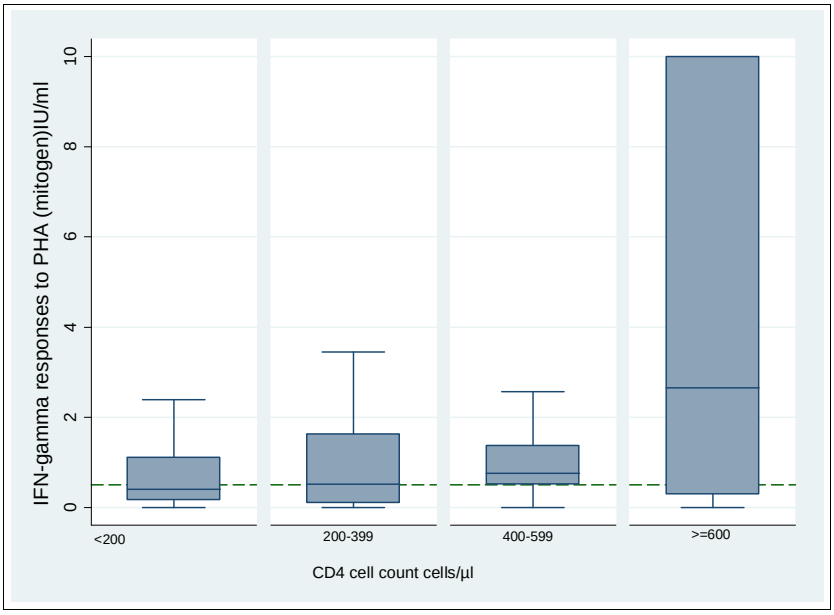


Figure 4.4 and 4.5: Distribution of IFN-γ responses to TB antigens (ESAT6/CFP10/TB7.7) and PHA (mitogen) by CD4 cell count (box plot showing median, IQR and range) respectively. The median IFN-γ values within each CD4-cell count interval are indicated with horizontal lines. Dotted lines represent cut-off values as recommended by the manufacturer: 0.35 IU/ml for TB antigens stimulated IFN-γ response and 0.5 IU/ml for PHA stimulated IFN-γ response.

4.2 Reproducibility of QFT-GIT and the effect of a 24-hour delay in incubation

Our third and fourth objectives in this study (study 1) were to determine the test-retest reproducibility of QFT-GIT and the effect of a 24-hour delay in incubation of blood samples on IFN-γ responses, respectively. Out of the 112 smear positive TB patients available for analysis, we enrolled 109 smear positive TB patients who had duplicate sets of results (standard incubation 1 and 2) and one set that was delayed for 24 hours (delayed incubation).

4.2.1 Test-retest reproducibility of QFT-GIT

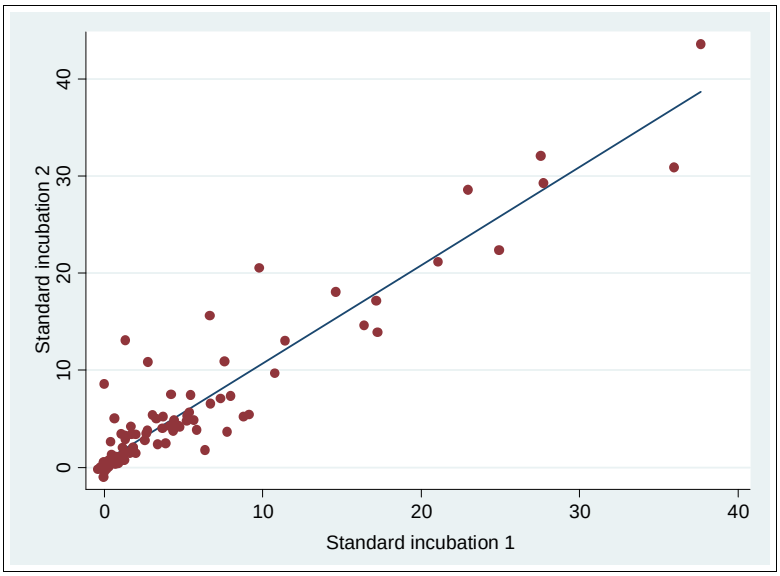
Out of the 109 patients, concordance was observed for 91.7% (100/109) and discordance for 8.3% (9/109) duplicate samples. Overall, the agreement between duplicate samples (standard incubation 1 and 2) was high at 91.74% (kappa=0.81 (95% CI: 0.78-0.85)) **(Table 4.2)**.

Table 4.2: QFT-GIT results of duplicate samples (standard incubation 1 and 2)

	Standard incubation 2			
Standard incubation 1	Positive	Negative	Indeterminate	**Total**
Positive	77	0	6	83
Negative	0	10	1	11
Indeterminate	0	2	13	15
Total	77	12	20	109

The test-retest agreement between these duplicate samples remained high when continuous measures were used (ICC: 0.90; 95% CI 0.82- 0.97) (**Figure 4.6**), similar to results obtained from using dichotomous measures.

Figure 4.6: IFN-γ responses to TB antigens for standard incubation 1 and 2



Although discordance was observed for 9 patients, all of which included indeterminate results (Table 4.3), this was not significant (McNemar test; *P* =0.18). There was no discordance between positive and negative results.

Of the 9 patients with discordant results, 6 were HIV positive and 3 had unknown HIV status (**Table 4.3**). The IFN-γ responses to TB antigens and PHA of the 9 patients with discordant results are shown in table 4.3. All the indeterminate results were due to poor response to PHA and not as a result of high background responses detected in the negative control tube (all nil values were ≤8.0 IU/ml for both TB antigens and PHA). There was strong evidence of an association between HIV positivity and discordant results (OR 1.98, 95%CI 1.06-3.67, p= 0.03).

Table 4.3: QFT-GIT results of nine discordant patients

	Standard Incubation 1	**Standard Incubation 2**					
Patient No.	**TB antigen-nil[1]**	**Mitogen–nil[2]**	**TB antigen-nil[1]**	**Mitogen–nil[2]**	**Overall results: Standard incubation 1/Standard incubation 2**	**HIV status**	**CD4 count**
1	0.5	0.24	0.00*	0.31	Positive/Indeterminate	Unknown	120
2	0.40	0.10	0.14	0.06	Positive/Indeterminate	Unknown	572
3	0.72	0.38	0.31	0.22	Positive/Indeterminate	Unknown	1056
4	0.00*	1.64	0.1	0.46	Negative/Indeterminate	Positive	75
5	0.14	0.3	0.04	0.62	Indeterminate/negative	Positive	26
6	8.6	1.50	0.00*	0.00	Positive/Indeterminate	Positive	246
7	0.35	0.46	0.15	0.45	Positive/Indeterminate	Positive	615
8	0.57	0.00*	0.01*	0.05	Positive/Indeterminate	Positive	342
9	0.07	0.46	0.02	0.53	Indeterminate/negative	Positive	238

***Negative values recorded as zeros; IFN-gamma response to TB antigens[1]or mitogen[2].**

Overall, the median IFN-γ responses to TB antigens of standard incubation 1 and 2 were 2.06 IU/ml (IQR 0.39-5.31, mean 5.16) and 1.36 IU/ml (IQR 0.15-5.24, mean 4.52) respectively. Although this difference was significant overall (Wilcoxon signed-rank test; p= 0.03), excluding the 9 patients with discordant results made this difference insignificant (Wilcoxon signed-rank test; p= 0.08). As for the 9 discordant patients, overall, there was a significant difference in the median IFN-γ responses to TB antigens of standard incubation 1 and 2 (Wilcoxon signed-rank test; p= 0.02) **(Figure 4.7)**.

Figure 4.7: IFN-γ responses to TB antigens for standard and delayed incubation

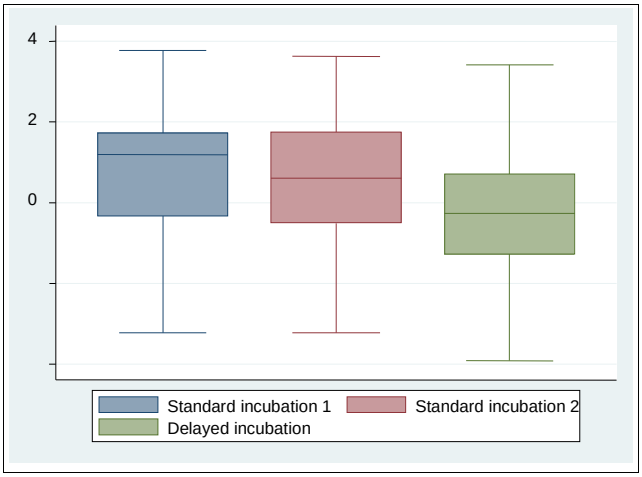


Figure 4.7: Box plots (log transformations) showing range, inter-quartile range and median Interferon gamma responses to TB antigens of standard and delayed incubations. Median IFN-gamma responses of delayed incubation are lower than those of either standard incubation 1 or standard incubation 2.

4.2.2 Effect of delayed incubation on IFN-γ response

The fourth objective in this study was to determine the effect of a 24-hour delay in incubation of blood samples on IFN-γ responses.

The overall agreement between standard incubation 1 and delayed incubation samples was fair to good (kappa= 0.54 (95% CI: 0.49-0.59)) at 77.1%. The sensitivity of QFT-GIT decreased from 74.1% (95%CI: 65.0-81.9) during standard incubation 1 to 58.7% (95% CI: 48.88- 68.06) during delayed incubation with indeterminate results included in the definition (Wilcoxon signed-rank test; p=0.002). Similar results were obtained when standard incubation 2 was compared with delayed incubation (Wilcoxon signed-rank test; p=0.03). However when indeterminate results were excluded in the definition, there was little difference between the sensitivity of standard incubation 1 and delayed incubation (standard incubation 1: (85.6% (95% CI: 77.0-91.9)). Vs. 85.33 (95% CI: 77.27-92.44)) (Wilcoxon signed-rank test; p=0.05). A greater number of indeterminate results were observed during delayed incubation, 31.2% (34/109) compared to standard incubation 1, 13.8% (15/109) (chi2, p<0.001).

There was a significant decrease in the median IFN-γ responses to TB antigens of patients from 2.06 IU/ml during standard incubation 1 to 0.52 IU/ml (IQR -0.04-1.61, mean 1.82) when it was delayed (Figure 4.7) (Wilcoxon signed-rank test; p< 0.001). Similar results were obtained between standard incubation 2 and delayed incubation (Wilcoxon signed-rank test; p<0.001; kappa= 0.57; 77.98%).

Overall there was a significant decrease in mitogen stimulated IFN-γ responses from 0.68 IU/ml (IQR: 0.18-2.52) during standard incubation 1 to 0.23 IU/ml (IQR: 0.07-0.77) when incubation was delayed (Wilcoxon signed-rank test; p<0.001).

Delaying incubation of QFT-GIT blood collection tubes by 24 hours changed the results of 22.9% (25/ 109) of patients while 77.1% (84/109) remained unchanged. Out of the 25 changed results, 20 changed from either negative (4) or positive (16) to indeterminate. The remaining 5 results changed as follows: positive to negative (4) and indeterminate to positive (1) (Fisher's exact, p <0.001).

Patients whose results changed were similar in age, sex (Fisher's exact, p = 0.63) and HIV status (Fisher's exact, p =1.0) to those whose results remained unchanged. There was no evidence that HIV status (OR= 1.04; 95%CI 0.65-1.65, p=0.87) or CD4 count levels (OR=1.00, 95% CI 0.99-1.00 p= 0.99) was associated with delayed incubation using standard incubation 1. A further comparison with standard incubation 2 showed similar results (OR 0.92, 95%CI 0.57-1.50, p= 0.75).

4.3 The effect of power outages on IFN-γ responses (Study 2)

Introduction

Power outages are common in resource limited settings, affecting mostly rural communities and high density urban and peri-urban areas. A new diagnostic test designed to be used in such settings requires a consideration of whether the test is dependent on uninterrupted electric power supply and whether results are grossly affected by power outages. Some tests require a stable electric power supply, and even short term interruption of power may cause indeterminate or incorrect results.

This study stemmed from frequent power outages that occurred in Zambia during the period 2008-2010. At this time the main study under discussion in this thesis was enrolling and following up study participants. During this period, the country experienced power outages due to increased demand for power due to high consumption; increased output in the mining sector as well as economic growth i.e. the demand outstripped the supply. Although the power outages which mostly affected the remote study sites subsequently improved over the study period, we initiated a study to examine to what extent QFT-GIT results were affected by power outages. We therefore aimed to determine the effect of power outages on IFN-γ responses during incubation.

4.3.1 Methods

a) Study subjects

9 people volunteered to take part in the study. These volunteers were healthy adults, most of whom were members of staff at ZAMBART project Head Office. The median age was 46 years (IQR: 31-54) and 6/9 were male. No other personal details were captured from the volunteers.

b) Ethical approval

This study was laboratory based and did not need ethical approval

c) Laboratory methods

To explore the effect of power outages during incubation of blood samples on IFN-γ response, simulated laboratory experiments were performed. Information on the pattern of power outages experienced in the different study sites was collected by a Quality Assurance team during routine monitoring visits. The patterns of power outages during incubation reported from the study sites were summarized into four categories as follows:

1) No power outage (standard incubation)
2) Early power outage
3) Late power outage
4) Early power outage with extension.

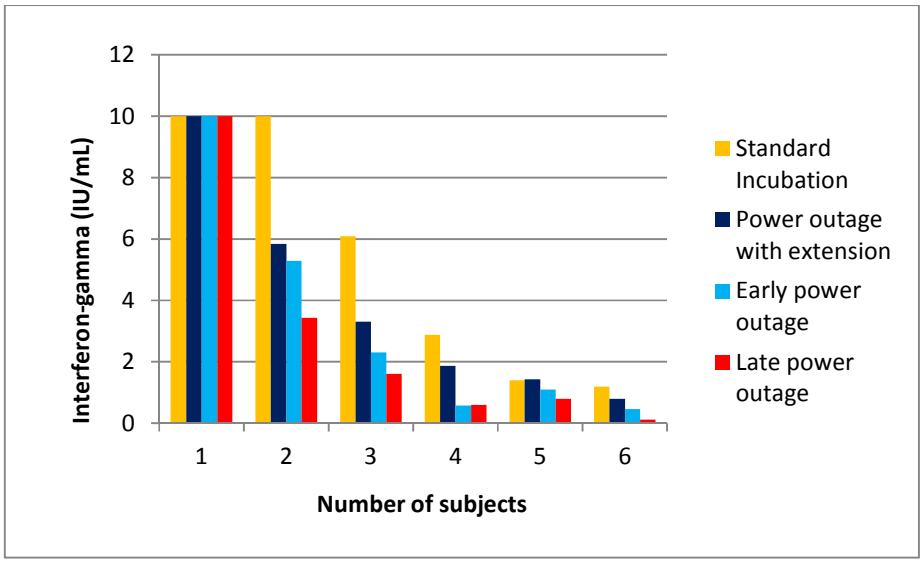
Four sets of QFT-GIT blood collection tubes were obtained from each of 9 volunteers and incubated according to the four power outage categories. The first set was incubated for 24 hours with no power interruption (standard incubation). The second set was incubated for 2 hours; power was turned off for 5 hours, and then switched back on for 17 hours (early power outage). The third set was incubated for 16 hours, power was switched off for 5 hours, and then power was turned back on for an additional 3 hours (late power outage). The fourth set was incubated for 2 hours, power was switched off for 5 hours, turned back on for 17 hours, and incubation extended by 5 hours to compensate for the time lost during the power outage (early power outage with extension).

The total incubation time for each experiment was 24 hours for standard incubation and early power outage with extension. However, the total incubation time for early and late power outages experiments was 19 hours each (excluding the 5 hours of power outage). After incubation, the rest of the samples were processed as per standard manufacturer recommendations. All concentrations greater than 10 IU/mL were reported as “10” in line with manufacturer recommendations.

4.3.2 Results

9 people volunteered to take part in the study. Power outages during incubation decreased IFN-γ responses to TB antigens and the effect was greatest during late power outages (**Figure 4.8**).

Figure 4.8: Effect of power outages on IFN-γ responses during incubation



Median IFN-γ responses to TB antigens obtained during the different power outages patterns were as follows: standard incubation 1.4 IU/ml (IQR 0.01-12.69); early power outage with extension 1.44 IU/ml (IQR 0.02-4.58); early power outage 0.57 IU/ml (IQR 0.005-3.8) and late power outage 0.6 IU/ml (IQR -0.03-2.52) IFN-γ responses. When compared to standard incubation, these differences in median IFN-γ responses were significant apart for the early power outage with extension group (Wilcoxon signed-rank test: early power outage, p=0.02; late power outage, p=0.02; early power outage with extension, p=0.08).

6 out of the 9 (66.67%) volunteers had positive QFT-GIT results and 3 (33.33%) were negative. Of the 6 with positive results, 5 (volunteers 1-5 in figure 4.8) remained positive in all the power outage experiments whilst 1 (volunteer 6) became negative (0.12 IU/ml) during the late power outage experiment .The 3 volunteers whose results remained negative during the different power outage experiments had median IFN-γ responses of zero.

4.4 Effect of storage temperature on IFN-γ response (Study 3)

Introduction

Conditions of storage of any test are important as there are defined storage temperatures for most tests. In hot or humid conditions, the selection of tests that are heat-stable is a priority. For most tests designed for use in resource limited tropical settings, the manufacturers' recommended room operating temperature is limited to a maximum of 30°C, not different from operating temperatures recommended for a wide range of other laboratory equipment. In settings where room temperature regularly exceeds 30˚C, air conditioning of the room where the assay is performed may be recommended.

According to the manufacturer, the unused tubes of QFT-GIT should be stored at room temperature i.e. 4-25°C. However, it is widely known that in some tropical settings like Zambia, the room temperature exceeds 25˚C and may even reach a maximum of 40˚C in certain areas. In

these places, laboratories at most primary health centers in such sites do not have air conditioning facilities. In this study we therefore aimed to determine the effect of increased storage temperature of unused blood collection tubes on IFN-γ responses.

4.4.1 Method

a) Study subjects

14 people volunteered to take part in the study. These volunteers were healthy adults, most of whom were members of staff at ZAMBART project Head Office. The median age was 46 years (IQR: 34-51) and 9/14 were male. No other personal details were captured from the volunteers.

A simulated laboratory experiment was done to determine the effect of increased storage temperature of unused blood collection tubes on IFN-γ response. From each of 14 volunteers, blood was collected in two sets of blood collection tubes. One set had been stored at 37°C for 3 months before blood was drawn and the other set had been stored in an air conditioned storage area where the temperature was maintained at less than 25 °C.

b) Ethical approval

This study was laboratory based and did not need ethical approval.

4.4.2 Results

There was no difference in IFN-γ responses between blood collected in tubes stored at <25°C and blood collected in tubes stored at 37°C for three months.

4.5 The financial cost of using QFT-GIT for the diagnosis of TB infection (Study 4)

Introduction

The evaluation of new diagnostic tools in any given setting should not only examine performance, feasibility and sustainability but should also consider costs and cost-effectiveness. This is because for resource-limited setting, cost can be a major barrier for the introduction of a test even if other performance factors are favourable.

Currently, a limited number of studies have been done looking at the cost or cost-effectiveness of QFT-GIT in resource-limited settings. We therefore conducted a small financial costing study. The aim of the study was to determine the financial cost of diagnosing TB infection using QFT-GIT in a field setting in Zambia.

4.5.1 Methods

We performed a study to look at the financial cost of diagnosing TB infection using QFT-GIT from the provider's perspective. Financial cost was defined as the accounting cost where costs were the monetary value of expenditures. Costs which patients incurred for seeking care for TB

infection were not included. The study was performed in six ZAMSTAR sites in Zambia; two sites from Kabwe and four from Lusaka. We collected costs using the ingredients approach (243).

We first identified all the ingredients required to perform QFT-GIT testing. In each site the health economist independently observed the entire process of performing QFT-GIT. This included all steps from sample collection to reading the results and final disposable of the waste. We recorded all processes, equipment and consumables used and time applied for a specific task. Time of staff spent on individual procedures and tasks was observed in detail for two weeks for all procedural steps separately. The amount of consumables used over time was observed and estimated with the help of laboratory staff. Other information was also collected through interviews with laboratory staff, project administrative staff and myself, as head of the QFT-GIT study.

We ascertained costs by reviewing expenditures, quotations and equipment and by consulting with laboratory staff, project administrative staff and me. I provided detailed information on the consumables needed to perform a QFT-GIT test i.e. where they were obtained from, at what price and the role of the funders. Laboratory staff provided information on the running costs of central laboratory including construction/maintenance of buildings, utilities (water, electricity, phone and internet), security and cleaning costs.

All costs were collected in June 2009 and compared to the average number of QFT-GIT tests done during the 1st follow-up visit of the main study under discussion in this thesis. The costs of transportation of samples from the point of collection to the central laboratory at Head office were included. All samples, regardless of test result were included.

Overhead costs were established by means of expenditure records in addition to staff costs. Overheads were apportioned according to the time proportion of personnel dedicated to QFT-GIT testing. Capital costs were depreciated and annualized over the estimated lifetime at a discount rate of 3% as applied in similar studies (244). The lifetime of equipment was estimated with the help of staff of the laboratory with consultation from staff from finance department.

Shared costs (for equipment and consumables) that were not only used for QFT-GIT procedures but also for other applications in the laboratory were apportioned by dividing the cost of each item by number of tasks. In order to obtain cost per QFT-GIT test, cost-per-step was then multiplied by the number of steps in which the item was used during the QFT-GIT procedure. All costs in local currency (Zambian kwacha) were converted into US Dollars (USD) at the official annual exchange rate for 2008.

4.5.2 Results

Our calculations were based on the average throughput of 2,942 individuals tested per year during SOCs visit 2 follow up. We differentiated between study-specific costs and overhead costs. The total cost per QFT-GIT test was estimated at US$ 22.29 (**Table 4.4**).

Table 4.4: Financial cost of performing one QFT-GIT test

Consumables	**(US$)**	**% of total cost**
Medical supplies	2.24	10%
3 QFT-GIT test tubes	3.50	16%
QFT-GIT ELISA kit	5.09	23%
Other (non-medical supplies & supervision)	2.74	12%
Subtotal	**13.58**	**61%**
Equipment (US$)		
Medical equipment	2.32	10%
Washer machine	0.74	3%
Elisa	0.74	3%
Other (non-medical equipment)	0.02	0%
Subtotal	**3.81**	**16%**
Personnel (study-specific) (US$)		
Nurses	1.327	6%
Laboratory Personnel	0.98	4%
Data personnel	0.90	4%
Study Manager	0.15	1%
Subtotal	**3.37**	**15%**
Overheads (US$)		
Recurrent	0.56	3%
Capital	0.41	2%
Personnel (support staff)	0.57	3%
Subtotal	**1.53**	**8%**
Total cost (US$)	**22.29**	**100%**

Under the current average rate of throughput, roughly two thirds (US$ 13.58; 61%) of the total cost per QFT-GIT test are made up of recurrent costs. This was followed by equipment costs at US$ 3.81 (17%) of the total cost per test. The study-specific costs comprised equipment costs (US$ 3.81), consumables costs (US$ 13.58) and staff costs (US$ 3.37) forming US$ 20.75/22.29 (93%) of the total costs. The overhead costs were estimated at US$ 1.53 forming 7% of the total cost per QFT-GIT test.

The most costly line items that drove the cost per QFT-GIT test high were consumables and medical equipment. QFT-GIT testing tubes and the QFT-GIT ELISA kit are proportional to the number of samples tested. All other line items are shared costs, and thus are subject to economies of scale.

4.5.3 Conclusion

The total cost of performing a QFT-GIT test was estimated at US$ 22.29 in our setting.

Summary

In this chapter I have illustrated the practical feasibility of using QFT-GIT in a poor resource setting like Zambia. I have shown that the sensitivity of QFT-GIT is greater than that of TST overall, at all the standard TST cut-offs and when stratified by HIV status. The sensitivity of QFT-GIT was 74.1% (95%CI: 65.0-81.9) (indeterminate results included) compared to that of TST at 51.6% (95% CI: 40.9-62.2) at a cut-off of ≥ 10 mm. I have showed that the test-retest reproducibility of QFT-GIT was high at 91.74% for both dichotomous (kappa=0.81 (95% CI: 0.78-0.85)) and continuous (ICC: 0.90; 95% CI 0.82- 0.97) data, even in a resource limited setting.

However, the studies I have presented here have demonstrated that there are some biological and operational factors that affect the performance of this test in this setting. HIV positivity, low CD4+ T-lymphocytes, delayed incubation of blood samples and power outages all affect test performance. With falling CD4 count, there was a trend towards an increase in both indeterminate and negative results overall (test for trend, $p<0.001$) and in HIV positive patients (test for trend, $p=0.03$). A 24-hour delay in incubation of blood samples decreased IFN-γ responses by 3.34 IU/ml and changed the results for 22.9% patients. Power outages, especially those that occurred during late incubation decreased IFN-γ responses to TB antigens by 0.8 IU/ml. Storage of blood collection tubes at 37°C had no effect on IFN-γ responses.

These studies emphasize the need for stringent sample collection and processing techniques to ensure the accuracy of QFT-GIT results. These factors may need to be taken into account in determining whether this test is appropriate for resource limited settings. While most of these factors can be controlled, the effect of HIV infection like TST may be a major limiting factor for the role of this test. Our financial costing study showed that the total cost of performing a QFT-GIT test was US$ 22.29 in our setting.

5.0 Baseline results

Introduction

In this chapter I will present the baseline results of the QFT-GIT cohort study which was nested within the SOCS study. After a descriptive analysis of the socio-demographic and clinical characteristics of the study population, I will explore the baseline quantitative QFT-GIT and TST results that will be linked to disease progression in the subsequent chapters. Due to the absence of a gold standard for the diagnosis of TB infection, I will evaluate the diagnostic performance of QFT-GIT and TST by examining the association between test positive results and risk factors for TBI. In addition I will determine concordance between the two tests.

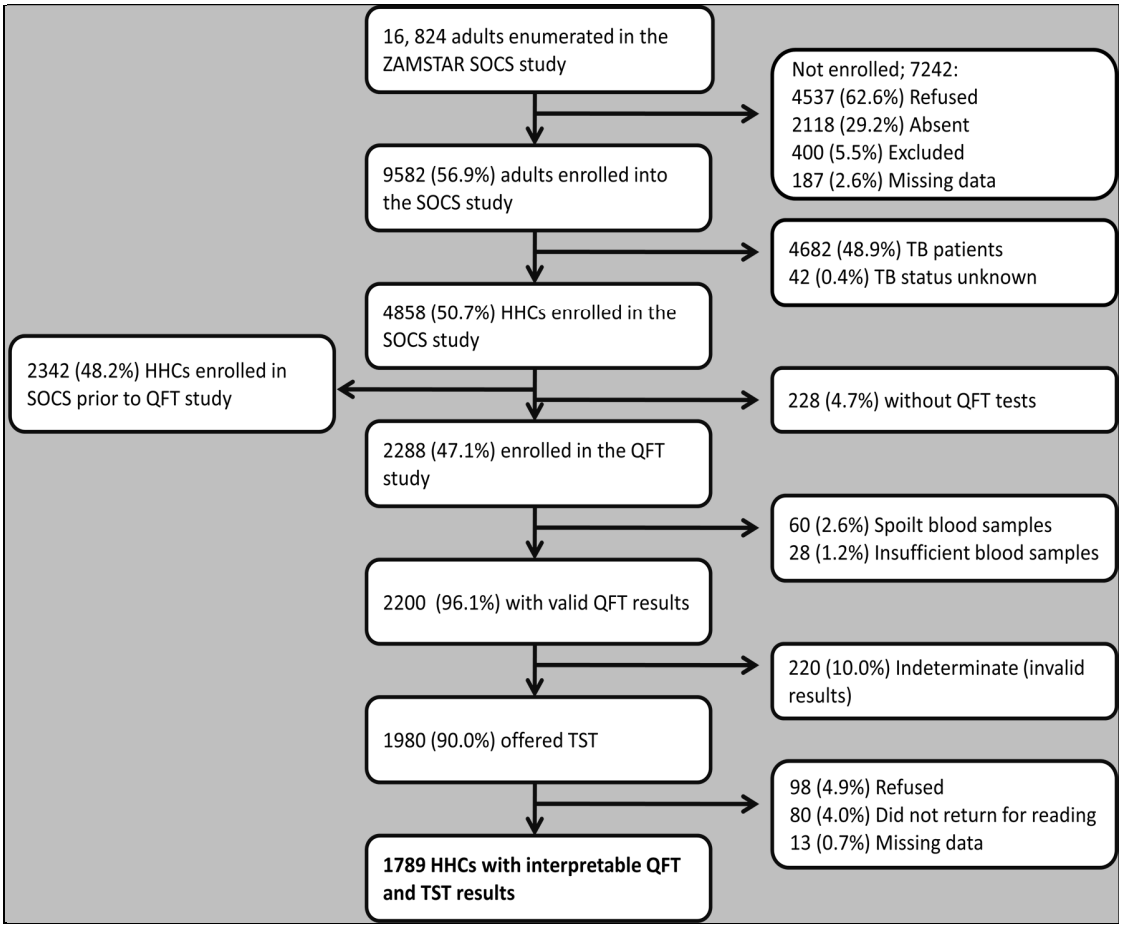
This chapter focuses on a population of household contacts (HHCs) of newly notified TB cases in whom QFT-GIT was performed at baseline. The specific objectives of this chapter are:

1) To describe the socio-demographic and clinical characteristics of household contacts (HHCs) in the baseline population.
2) To describe the baseline quantitative QFT-GIT and TST results.
3) To determine the prevalence of TB infection as measured by QFT-GIT and TST.
4) To determine risk factors associated with positive QFT-GIT and TST results.
5) To determine the level of agreement or concordance between QFT-GIT and TST results.

5.1 Study enrollment

For every household, once permission to enroll household members was given and the study fully explained; all members were then enumerated. Although all household members were enumerated, this study focuses on adult's ≥ 15 years invited to participate in the SOCS study. The SOCS study enumerated 16, 872 adults from 5,062 households. The total household size for adults, (as enumerated regardless of eligibility to the QFT-GIT cohort) varied from 1 to 25 with 11% having 1 adult household member (index case only), 32.4% having 2 adult household members, 19.1% having 3 adult household members and 37.5% having 4 or more adult household members. Of those enumerated, 9,582 (56.8%) agreed to take part in the SOCS study and were enrolled while 7,242 (42.9%) were not enrolled. Reasons for not enrolling into the study were refusal (4,537; 62.6%), absenteeism (2,118; 29.2%), exclusion (400; 5.5%) and missing data (187; 2.6%) (**Figure 5.1**).

Figure 5.1: Flow diagram of study participants at baseline



The SOCS study enrolled 9,582 adults from 4,948 households. The total household size, (adults enrolled in the SOCS study) varied from 1 to 14 with 46.3% having 1 household member (index case only), 31.6% having 2 household members, 12.1% having 3 household members and 10% having 4 or more household members. Out of 9,582 adults who accepted to take part in the SOCS study, 4,858 (50.7%) were HHCs and 4,682 (48.9%) were TB patients (index cases). TB patients and 42 individuals whose contact status (index or contact) was unspecified were excluded from the study.

Out of 4,858 HHCs enrolled in the SOCS study, 2,342 (48.2%) were excluded because they were enrolled before the QFT study began, hence QFT-GIT testing could not be offered to them. The 2,342 HHCs were determined by excluding all those enrolled into the SOCS study prior to the date of the first QFT-GIT test in each community. Most QFT-GIT tests were performed on the same day HHCs were enrolled into the SOCS study.

After HHCs were enrolled into the SOCS study, they were introduced to the QFT study. Of the 4,858 HHCs enrolled in the SOCS study, 2,288 accepted to be enrolled in the QFT study, resulting in an acceptance rate of 90.9 % (2,288/2,516), (2,342 HHCs not offered QFT-GIT were excluded from denominator). Those who accepted to take part in the QFT study had blood drawn for QFT-

GIT testing and a risk factor questionnaire administered to them. For 228 HHCs, QFT-GIT tests were not done and data on why these tests were not done was not collected.

A total of 2,200 (96.1%; 2,200/2,288) HHCs had valid QFT-GIT results. Reasons for invalid QFT-GIT result were (i) insufficient blood samples (1.2%; 28/2,288) , (ii) spoilt blood samples (2.6%; 60/2,288) defined as samples that had hemolysed or had been subjected to power outages during incubation, and (iii) indeterminate QFT-GIT results (9.6%; 220/2,288). All the invalid QFT-GIT results were excluded from analysis. Of the 2,200 HHCs with valid QFT-GIT results, 1,789 (90.0%) also had an interpretable TST result. Reasons for excluding TST results were (i) refusal (4.9%; 98/1,980), (ii) not returning for reading, (4.0%; 80/1,980) and (iii) missing data (0.7%; 13/1,980).

Therefore 1,789 HHCs with interpretable results for both QFT-GIT and TST form the baseline population of the QFT cohort study described in this chapter.

5.2 Socio-demographic and clinical characteristics of HHCs

The study population was predominantly women (71%) and the median age was 28 years (interquartile range [IQR], 21-43). Overall, there was approximately an equal number of single (45.4%) and married (44.8%) HHCs in this study population. However, when results were stratified by country, there were more single (58.2%; 438/752) and less widowed (3.6%; 27/752) or separated (2.8%; 21/752) HHCs in SA than Zambian communities (**Table 5.1**).

Table 5.1: Individual level demographic and clinical characteristics of HHCs at baseline

	HHCs (column %)		
	Zambia	**SA**	**Total**
Total	**1037**	**752**	**1789**
Sex			
Male	318 (30.7)	199 (26.7)	517 (29.0)
Female	719 (69.3)	547 (73.3)	1266 (71.0)
Missing	0	6	6
Age group (years)			
15-24	413 (39.8)	278 (37.0)	691 (38.6)
25-34	277 (26.7)	178 (23.7)	455 (25.4)
35-44	132 (12.7)	105 (14.0)	237 (13.3)
45-54	97 (9.3)	110 (14.6)	207 (11.6)
55-64	59 (5.7)	55 (7.3)	114 (6.4)
>65	59 (5.7)	25 (3.3)	84 (4.7)
Missing			1
Marital status			
Single	373 (36.0)	438 (58.2)	811 (45.4)
Married	534 (51.6)	266 (35.4)	800 (44.8)
Widow	82 (7.9)	27 (3.6)	109 (6.1)
Separated	46 (4.4)	21 (2.8)	67 (3.7)
Missing	2	0	2
Highest level of education			
Not attended school	74 (7.3)	42 (5.7)	116 (6.6)
Primary school	365 (36.2)	190 (25.6)	555 (31.7)
Secondary school	465 (46.1)	449 (60.5)	914 (52.2)
College or University	105 (10.4)	61 (8.2)	166 (9.5)
Missing	28	10	38
Economic activity			
Dependant	117 (11.4)	40 (5.3)	157 (8.8)
Unemployed -Not looking for employment	499 (48.5)	130 (17.4)	629 (35.4)
Unemployed-actively looking for employment	146 (14.2)	430 (57.5)	576 (32.4)
Employed	266 (25.9)	148 (19.8)	414 (23.3)
Missing	9	4	13
Smoking habits			
Never smoked	910 (88.0)	551 (73.3)	1461 (81.8)
Ex-smoker	47 (4.5)	29 (3.9)	76 (4.3)
Occasional smoker	28 (2.7)	32 (4.3)	60 (3.3)
Daily smoker	49 (4.7)	140 (18.6)	189 (10.6)
Missing	3	0	3
[1]Alcohol consumption			
No	782 (75.8)	555 (74.3)	1337 (75.2)
Yes	249 (24.1)	192 (25.7)	441 (24.8)
Missing	6	5	11
HIV status (laboratory reported)			
Negative	716 (69.8)	549 (75.4)	1265 (72.1)
Positive	310 (30.2)	179 (24.6)	489 (27.9)
Missing	11	24	35
Household level variables			
Smear status of the index			
Smear negative	355 (57.0)	325 (42.8)	680 (49.2)
Smear positive	268 (43.0)	435 (57.2)	703 (50.8)
Missing	144	88	232

Sleeping proximity to index			
Different house	102 (10.1)	22 (7.3)	124 (9.4)
Same house	403 (39.9)	150 (49.5)	553 (42.1)
Same room	58 (5.7)	5 (1.6)	63 (4.8)
Same bed	197 (19.5)	31 (10.2)	228 (17.3)
Unknown	251 (24.8)	95 (31.3)	346 (26.3)
Missing	26	449	475

[1]Defined as alcohol consumption four weeks prior to the interview.

Overall, most HHCs had attended secondary education (52.2%; 914/1,751), had no history of smoking (81.8%; 1461/1,786) or alcohol consumption (75.2%; 1,337/1,778). In addition, a greater proportion of them were unemployed (67.8%; 1,205/1,776) although only 32.4% (576/1,776) were actively looking for employment.

As expected in this setting, overall, the prevalence of HIV infection in HHCs was high at 27.9% (489/1,754). When stratified by country, a higher prevalence of HIV infection was observed in HHCs residing in Zambian communities (30.2 %; 310/1,026) compared to those residing in South African ones (24.6%; 179/728) (chi2; p=0.01).

HIV prevalence was also explored by sex and age group for each country. Overall HIV prevalence was higher among females, 31.7% (395/1,244) compared to males, 18.6% (94/505) (p<0.001) and the results were similar when stratified by country (chi2; p<0.001). When HIV prevalence was explored by age, it remained higher in the Zambian communities in all age groups apart from the 15-24 age group. In this age group, HIV prevalence was 20% (54/270) for HHCs residing in the SA communities compared to 18.6% (76/408) for those in the Zambian communities.

There were 43.8% (777/1,773) HHCs who self reported to have had an HIV test, of these 19.8% (148/746) were HIV positive and 80.2% (598/746) were HIV negative. Of the HHCs who self-reported to be HIV positive, 35.1% (52/148) were on antiretroviral therapy (ART) at baseline. The median time between ART initiation and study enrolment was 1.15 years (IQR: 0.53-1.82).

The uptake of IPT in this study population was low at 4.7% (7/148) among those who self-reported to be HIV positive. Data on the presence of a BCG scar was only available for 974 HHCs residing in Zambia. Of these, 26% (253) had a BCG scar whereas 74% (721) did not have a scar.

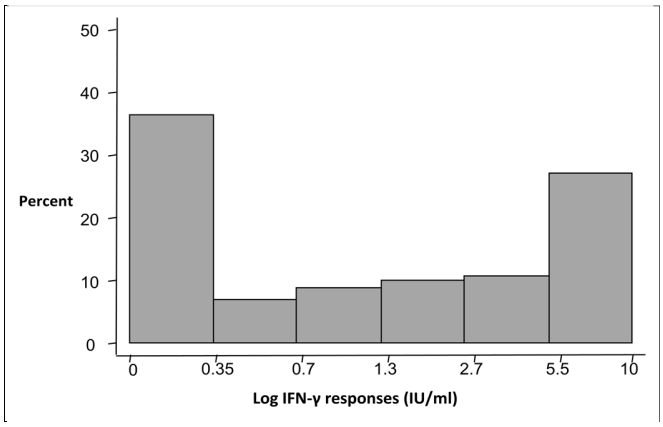
Overall, 50.8% (703/1,383) of HHCs lived with a smear positive TB index case. A greater proportion of HHCs who lived with smear positive TB index cases resided in SA (57.2%) than in Zambian (43.0%) communities. Index cases were mostly men (50.2%), with a median age of 33 years (IQR: 27, 40).

Out of 1,789 HHCs, 1,037 (58%) resided in Zambian communities and 752 (42%) in SA communities. The number of HHCs varied across the 24 communities from 18 to 140, with a mean of 74 HHCs per community. When the communities were stratified by urban and rural, there were only two rural communities, all of which were in Zambia. Therefore, overall the majority, 97.3% (1,740/1,789) of HHCs resided in urban communities in both countries. For the Zambian communities, 4.7% (49/1,037) of HHCs resided in rural areas while 95.3% (988/1,037) were in urban areas.

5.3 Quantitative QFT-GIT and TST responses

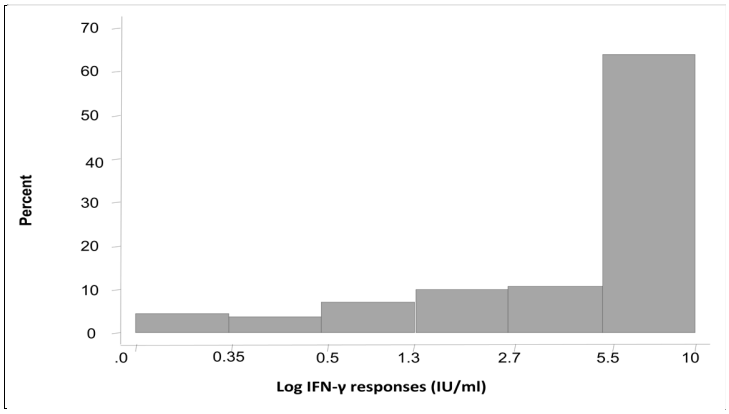
Overall, the median IFN-γ responses to TB antigens at baseline was 1.1 IU/ml (IQR; 0.1-5.7). **Figure 5.2** shows the distribution of TB antigen stimulated IFN-γ response at baseline. As shown in Figure 5.2, the majority of HHCs; 646 (36.1%) had IFN-γ responses below 0.35 IU/ml while 459 (25.7%) had IFN-γ levels between 5.5-10 IU/ml. There was a difference in IFN-γ response to TB antigens by country, with HHCs in SA having a larger response (median 1.5 IU/ml (IQR: 0.3-6.0)) than HHCs in Zambia (median 0.8 IU/ml (IQR: 0.02-5.4) (rank-sum; p<0.001). For both TB antigen and mitogen minus nil values, results ≥ 10 IU/ml are shown as 10 IU/ml as recommended by the manufacturer.

Figure 5.2: TB Antigen stimulated interferon-gamma responses at baseline



Overall, the median IFN-γ response to mitogen at baseline was 8.9 IU/ml (IQR: 2.55-10) (**Figure 5.3**). As shown in Figure 5.3, the majority of HHC, 1,109 (62%) had IFN-γ responses between 5.5-10 IU/ml.

Figure 5.3: Mitogen stimulated interferon-gamma responses at baseline



TB antigen stimulated IFN-γ responses were also stratified by HIV status **(Figure 5.4)** and responses were lower in HIV positive (median 0.4 IU/ml (IQR: 0.02-3.0)) compared to HIV negative HHCs (median 1.5 IU/ml (IQR: 0.13-7.0)) (rank-sum; p <0.001). Similar results were found when mitogen stimulated IFN-γ responses were stratified by HIV status (rank-sum; p=0.005) (**Figure 5.4**).

Figure 5.4: Box plots showing IFN-γ responses by HIV status at baseline

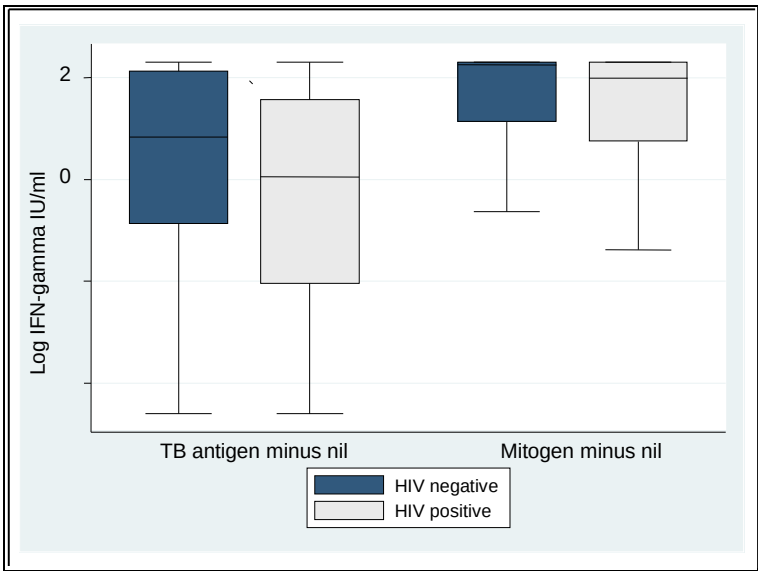
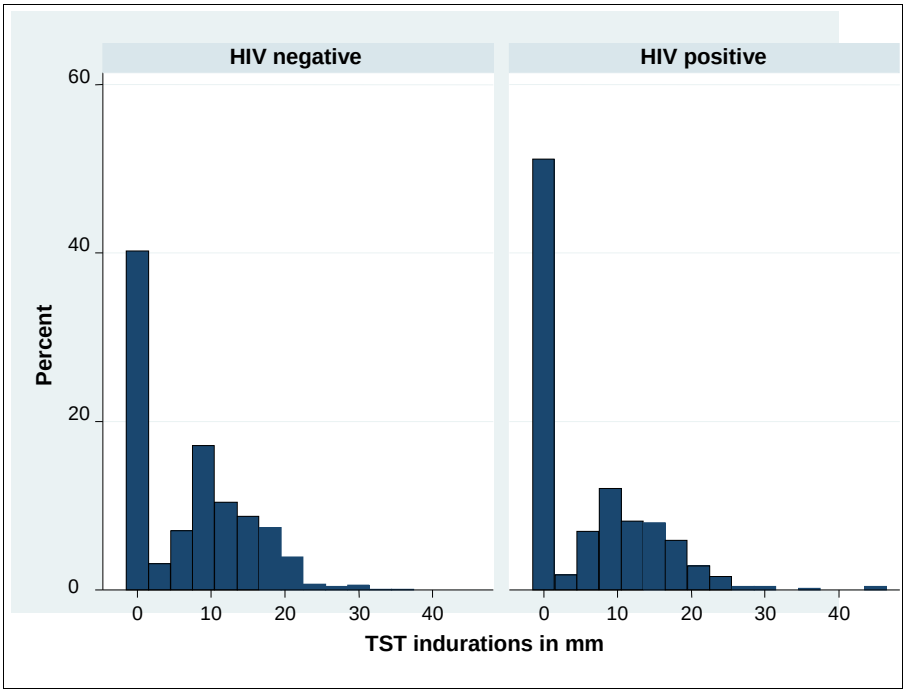


Figure 5.4: IFN-γ responses to *Mtb* antigens and mitogen. Box plots illustrate the median and distribution of log transformed IFN-γ results by HIV status.

Quantitative TST results at baseline showed that the mode of the TST distribution for non-zero indurations was 10 mm while the mean TST response was 7.1 mm (range 0-45). There was strong evidence that TST indurations differed by HIV status; mean induration in HIV positive was 6.3 mm (range 0-45) compared to 7.3 mm (range 0-35) in HIV negative HHCs (rank-sum; p=0.0005) (**Figure 5.5**). In addition, a greater proportion of HIV positives, 50.7% (248/489) had zero indurations compared to HIV negatives, 39.3% (497/1,265).

Figure 5.5: TST responses by HIV status at baseline



5.3.1 Effect of age on quantitative IFN-γ responses and TST reactivity

The median IFN-γ response to TB antigens ranged from 0.96 (IQR: 0.04-6.2)IU/ml in the youngest age group (15-24 years) to 1.28 (IQR: 0.12-7.5) IU/ml in the oldest age group (over 65 years).

There was a tendency of the TB antigen stimulated IFN-γ responses to peak between 55-64 years (median IFN-γ response: 2.08 (IQR: 0.35-7.6) and decline after 65 years of age (**Figure 5.6**). When this pattern was formally tested, there was a trend of increasing IFN-γ responses with increasing age group (test for trend; p =0.001). In all age groups the median IFN-γ responses were above the manufacturer's cut-off point of ≥ 0.35 IU/ml.

The mean TST reactions ranged from 6.6 mm (range 0-44) in the youngest age group (15-24 years) to 5.2 mm (range 0-22) in the oldest age group (over 65 years). Both quantitative QFT-GIT and TST responses were associated with increasing age (aOR for each 10 year increase for QFT-GIT 1.21; 95% CI: 1.08-1.36; p=0.02 for linear trend, and for TST aOR: 1.03; 95% CI: 0.95-1.12; p = 0.001 for linear trend).

Figure 5.6: Age-specific interferon-gamma responses to TB antigens

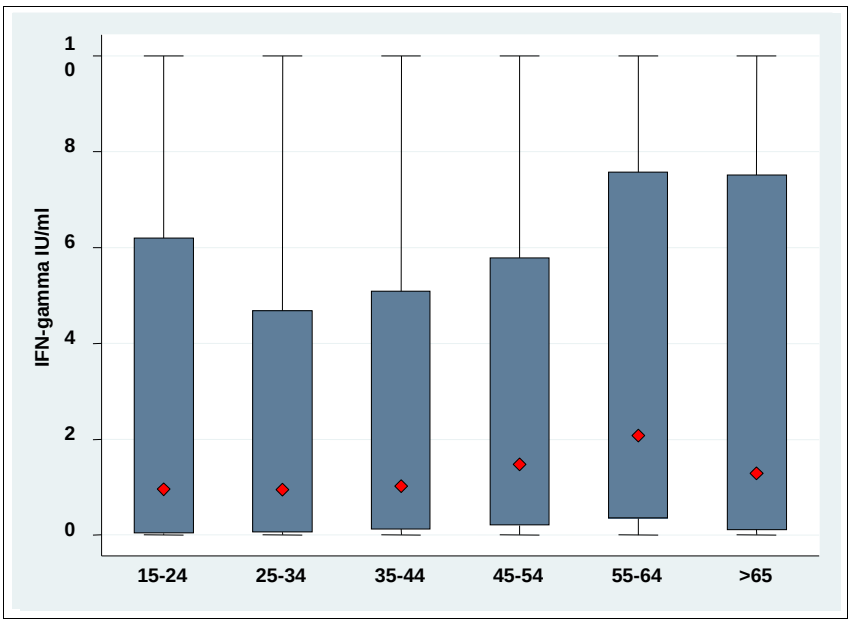


Figure 5.6: Box plots show the range, median (red diamonds), 25th and 75th percentiles of IFN-γ-responses to TB antigens in different age groups

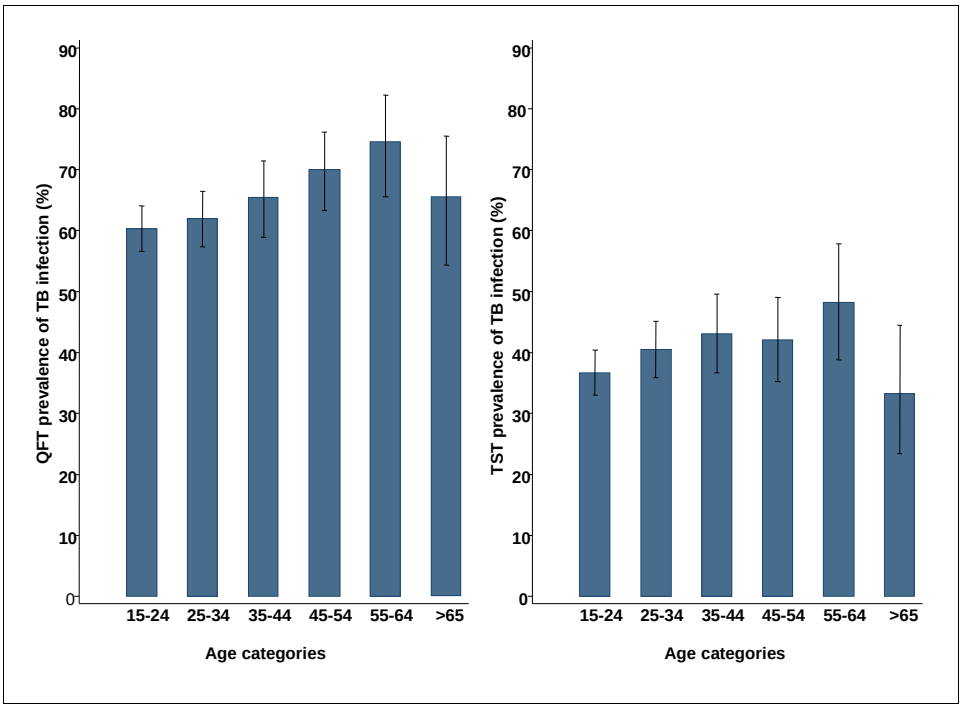
5.4 Prevalence of tuberculous infection (TBI)

The prevalence of TBI was 63.7% (1,140/1,789) using QFT-GIT at the manufacturer's cut-off point of ≥ 0.35 IU/ml and 39.6% (709/1,789) using TST at ≥10 mm cut-off. 31.6% (565/1,789) HHCs were positive on both QFT-GIT and TST (10 mm) while 40.2% (719/1,789) were positive on either QFT-GIT or TST. Overall, the prevalence of infection was higher in SA than in Zambian

communities using both QFT-GIT (SA: 71.1% vs. Zambia 58.3%; p<0.001) and TST (SA: 47.2.1% vs. Zambia 34.1%; p<0.001).

The prevalence of TBI was also explored by age group. For QFT-GIT, prevalence increased with age, peaked between 55-64 years and declined after 65 years of age. However, for TST prevalence, a trend with age was less clear (**Figure 5.7**).

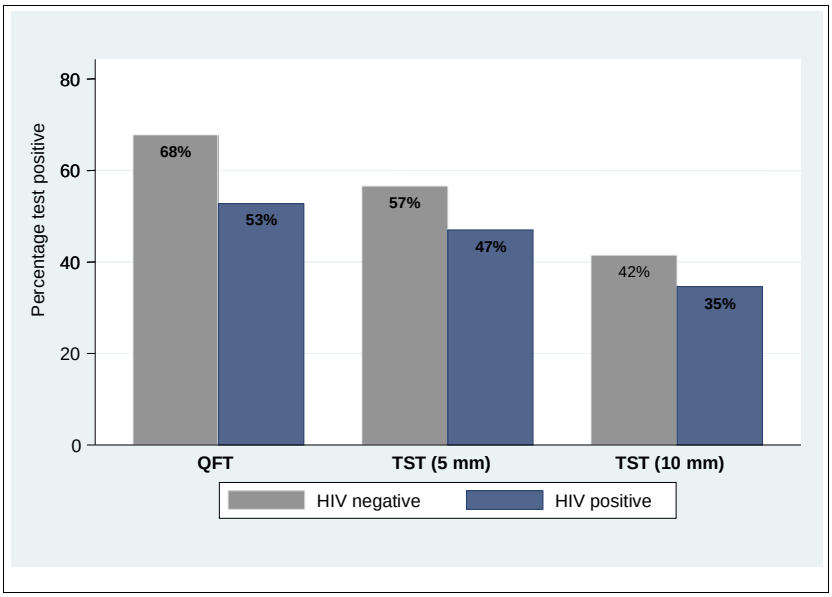
Figure 5.7: Prevalence of QFT-GIT and TST by age group



The prevalence of TBI stratified by HIV status showed a lower proportion of test positive results in HIV positive HHCs compared with HIV negative HHCs for both tests and regardless of TST cut-off. Using QFT-GIT, the prevalence of TBI was 52.8% (258/489) in HIV positive compared to 67.8% (857/1265) in HIV negatives HHCs (p<0.001). Using TST at ≥10 mm cut-off, the prevalence of TBI was 34.6% (169/489) in HIV positives compared to 41.5% (525/1265) in HIV negatives HHCs (p=0.008) (**Figure 5.8**).

Literature suggests that a lower TST cut-off point of 5 mm should be used in HIV positive individuals because of cutaneous anergy associated with progressive HIV immunosuppression resulting in high rates of test unresponsiveness (245). Using TST at ≥ 5mm cut-off, the prevalence of TBI was 47.0% (230/489) in HIV positives compared to 56.6% (716/1275) in HIV negatives HHCs (p<0.0003).

Figure 5.8: Proportion positive by each test stratified by HIV status



5.5 Risk factors associated with positive QFT-GIT and TST results

In unadjusted analysis, HIV (OR: 0.53; 95% CI: 0.43–0.66; p<0.001) and being an ex-smoker (OR:0.66; 95% CI:0.41-1.04) were negatively associated with QFT-GIT positivity, whereas daily smoking (OR: 1.56; 95% CI: 1.11-2.18; p=0.007), smear status of index (OR: 1.34; 95% CI: 1.06-1.69; p=0.01), residing in an urban area (OR: 2.03; 95% CI: 1.15–3.58; p<0.01) and country (OR: 1.76; 95% CI: 1.44–2.15; p<0.001) were positively associated with QFT-GIT positivity.

QFT-GIT positivity was also associated with increasing age (aOR for each 10 year increase for QFT-GIT 1.15; 95% CI: 1.06–1.25; p<0.001 for linear trend). In further analysis to test the null hypothesis that the effect of age group on QFT-GIT positivity was linear, there was no evidence of departure from linearity in this relationship (LR chi2 (1)1.52; p=0.22).

In multivariable analysis (adjusted for sex, age and community) results were similar as for the unadjusted analysis apart from smear status of the index case. In multivariable analysis, HIV (aOR: 0.48; 95% CI: 0.37-0.63; p<0.001) and being an ex-smoker (OR: 0.56; 95%CI: 0.32-1.00) were negatively associated with QFT-GIT positivity whereas, residing in an urban area (aOR: 2.37; 95% CI: 1.10-5.13; p<0.03) and country (aOR: 1.93; 95% CI: 1.48-2.51; p<0.001) were positively associated with QFT-GIT positivity. In unadjusted and multivariable analyses, data were consistent with no association of education, socioeconomic status, alcohol consumption and sleeping proximity to the index case with QFT-GIT positivity (**Table 5.2**). In addition, there was weak evidence of an association between presence of a BCG scar and QFT-GIT positivity (aOR: 0.71 (0.47-1.05; p=0.09).

Table 5.2: Univariable and multivariable odds ratios showing risk factors associated with positive QFT-GIT results

(n_{max} =1789).

	QFT-GIT positive n (row %)	Crude OR (95% CI)	[2]P value	Adjusted OR[1] (95% CI)	[2]P value
Sex					
Female	808 (63.8)	1		1	1
Male	329 (63.6)	0.99 (0.80-1.23)	0.94	1.08 (0.83-1.39)	0.57
Age group (years)					
15-24	417(60.3)	1	P<0.001	1	p<0.001
25-34	282(62.0)	1.07 (0.84-1.36)		1.04 (0.77-1.39)	
35-44	155(65.4)	1.24 (0.91-1.69)		1.27 (0.88-1.83)	
45-54	145 (70.0)	1.54 (1.10-2.15)		1.62 (1.09-2.42)	
55-64	85 (74.6)	1.93 (1.23-3.01)		2.34 (1.36-4.04)	
>65	55 (65.5)	1.25 (0.77-2.00)		1.78 (1.00-3.19)	
Highest level of education					
Not attended school	75 (64.7)	1	0.06	1	p<0.001
Primary school	374 (67.4)	1.13 (0.74-1.72)		1.41 (0.83-2.38)	
Secondary school	557(60.9)	0.85 (0.57-1.28)		1.03 (0.60-1.79)	
College or University	112 (67.5)	1.13 (0.69-1.87)		1.45 (0.76-2.77)	
Socioeconomic status					
Dependant	102 (65.0)	1	0.91	1	p<0.001
[3]Unemployed	768 (63.7)	1.03 (0.82-1.30)		1.11 (0.83-1.50)	
Employed	261 (63.0)	1.09 (0.74-1.59)		1.30 (0.80-2.11)	
Smoking habits					
Never smoked	918 (62.8)	1	0.007	1	p<0.001
Ex-smoker	40(52.6)	0.66 (0.41-1.04)		0.56 (0.32-1.00)	
Occasional smoker	42 (70.0)	1.38 (0.79-2.42)		1.10 (0.55-2.18)	
Daily smoker	137 (72.5)	1.56 (1.11-2.18)		1.10 (0.71-1.71)	
Alcohol consumption					
No	841 (62.9)	1		1	
Yes	289 (65.5)	1.12 (0.89-1.40)	0.32	1.06 (0.80-1.41)	0.67
HIV status					
Negative	857(67.7)	1			
Positive	258 (52.8)	0.53 (0.43-0.66)	p<0.001	0.48 (0.37-0.63)	p<0.001
Smear status of the index					
Smear negative	373 (61.0)	1		1	
Smear positive	424(67.7)	1.34 (1.06-1.69)	0.01	1.26(0.91-1.76)	0.15
Sleeping proximity to index					
Different house	71 (57.3)	1	0.63	1	0.0009
Same house	352 (63.6)	1.31 (0.88-1.94)		1.09 (0.62-1.89)	
Same room	36 (57.1)	0.99 (0.54-1.84)		1.20 (0.52-2.73)	
Same bed	142 (62.3)	1.23 (0.79-1.92)		1.15(0.62-2.14)	
Unknown	219 (63.3)	1.29 (0.85-1.95)		1.07 (0.60-1.93)	
Residence					
Rural	23 (46.9)	1		1	
Urban	1117 (64.2)	2.03 (1.15-3.58)	0.01	2.37 (1.10-5.13)	0.03
Country					
Zambia	605 (58.3)	1		1	
South Africa	535 (71.1)	1.76 (1.44-2.15)	p<0.001	1.93 (1.48-2.51)	p<0.001

[1]Odds ratios-adjusted for sex, age and community using random effects logistic regression. Missing: sex-6; age-1; education-38; socioeconomic-13; smoking habits-3; taken alcohol-11; HIV status-35; sleeping proximity to index-475. [2]P value from likelihood ratio test. [3] Total actively looking for employment and not looking for employment.

As a comparison to QFT-GIT, risk factors associated with TST positivity were determined. In unadjusted analysis, HIV (OR: 0.74; 95% CI: 0.60–0.92; p=0.008) was negatively associated with

TST positivity whereas having post-secondary education (OR: 1.76; 95% CI: 1.07-2.89; p=0.10), smear status of index (OR: 1.73; 95% CI: 1.21-2.48; p=0.003), residing in an urban area (OR: 2.62; 95% CI: 1.30-5.28; p=0.007) and country (OR: 1.72; 95% CI: 1.42-2.09; p<0.001) were positively associated with TST positivity. TST positivity was also associated with increasing age (aOR for each 10 year increase: 1.10; 95% CI 1.02-1.21; p <0.001 for linear trend).

In multivariable analysis, HIV (aOR: 0.65; 95% CI: 48-0.87; p=0.004) was negatively associated with TST positivity whereas smear status of index (OR: 1.44; 95% CI: 1.01-2.05; p=0.04), residing in an urban area (aOR: 3.17; 95% CI: 1.14-8.80; p=0.03) and country (aOR: 2.35; 95% CI: 1.71-3.24; p<0.001) were positively associated with TST positivity. In both unadjusted and multivariable analyses, data were consistent with no association of socioeconomic status, smoking, alcohol consumption and sleeping proximity to the index case with TST positivity (**Table 5.3**). In addition, there was weak evidence of an association between presence of a BCG scar and TST positivity (aOR: 0.83 (0.54-1.27; p=0.38).

Table 5.3: Univariable and multivariable odds ratios showing risk factors associated with positive TST results (n_{max} =1789).

	TST positive n (row %)	Crude OR (95% CI)	[2]P value	Adjusted OR[1] (95% CI)	[2]P value
Sex					
Female	510(40.3)	1		1	
Male	198 (38.3)	0.92 (0.74-1.13)	0.44	0.86 (0.65-1.13)	0.27
Age group (years)					
15-24	253 (36.6)	1	0.10	1	p<0.001
25-34	184 (40.4)	1.17 (0.92-1.50)		1.25 (0.91-1.72)	
35-44	102 (43.0)	1.31 (0.97-1.76)		1.66(1.12-2.46)	
45-54	87(42.0)	1.25 (0.91-1.72)		1.39(0.92-2.11)	
55-64	55 (48.2)	1.61(1.08-2.40)		2.01(1.18-3.40)	
>65	28 (33.3)	0.86 (0.53-1.40)		1.29(0.70-2.38)	
Highest level of education					
Not attended school	37 (31.9)	1	0.10	1	p<0.001
Primary school	232 (41.8)	1.53 (1.00-2.35)		1.67 (0.95-2.91)	
Secondary school	357(39.1)	1.37 (0.90-2.07)		1.34 (0.75-2.39)	
College or University	75(45.2)	1.76 (1.07-2.89)		1.28 (0.65-2.54)	
Socioeconomic status					
Dependant	155 (37.4)	1	0.18	1	p<0.001
[3]Unemployed	474 (39.3)	1.08(0.86-1.36)		1.01(0.73-1.39)	
Employed	72 (45.9)	1.41 (0.98-2.05)		1.41(0.86-2.32)	
Smoking habits					
Never smoked	563 (38.5)	1	0.13	1	p<0.001
Ex-smoker	31(40.8)	1.10 (0.69-1.76)		1.22 (0.66-2.27)	
Occasional smoker	30 (50)	1.59 (0.95-2.67)		1.27 (0.63-2.55)	
Daily smoker	85 (45.0)	1.30 (0.96-1.77)		1.16 (0.75-1.82)	
Alcohol consumption					
No	531(39.7)	1		1	
Yes	174 (39.5)	0.99 (0.79-1.23)	0.92	0.98(0.72-1.33)	0.88
HIV status					
Negative	525 (41.5)	1		1	
Positive	169(34.6)	0.74 (0.60-0.92)	0.008	0.65 (0.48-0.87)	0.004
Smear status of the index					
Smear negative	223 (36.5)	1		1	
Smear positive	286 (45.7)	1.73 (1.21-2.48)	0.003	1.44 (1.01-2.05)	0.04
Sleeping proximity to index					
Different house	42 (33.9)	1	0.67	1	p<0.001
Same house	196 (35.4)	1.07 (0.71-1.62)		0.78 (0.45-1.35)	
Same room	20 (31.7)	0.91 (0.47-1.73)		0.96 (0.42-2.21)	
Same bed	91 (39.9)	1.30 (0.82-2.05)		0.81 (0.44-1.49)	
Unknown	127 (36.7)	1.13 (0.73-1.74)		0.74 (0.41-1.34)	
Residence					
Rural	10 (20.4)	1		1	
Urban	699 (40.2)	2.62 (1.30-5.28)	0.007	3.17 (1.14-8.80)	0.03
Country					
Zambia	354 (34.1)	1			
South Africa	355 (47.2)	1.72 (1.42-2.09)	P<0.001	2.35 (1.71-3.24)	P<0.001

[1]Odds ratios-adjusted for sex, age and community using random effects logistic regression. Missing: sex-6; age-1; education-38; socioeconomic-13; smoking habits-3; taken alcohol-11; HIV status-35; sleeping proximity to index-475. [2]P value from likelihood ratio test. [3]Total actively looking for employment and not looking for employment.

5.6 Concordance between QFT-GIT and TST results

To determine the level of agreement or concordance between QFT-GIT and TST results, 1,789 individuals with valid results for both tests were examined (figure 5.1). Of these, 505 (28.2%) were concordantly negative, 565 (31.6%) were concordantly positive, while 719 (40.2%) were discordant (p<0.001) at TST ≥ 10 mm. Among TST positive HHCs, 20.3% (144/709) had a negative and 79.7% (565/709) a positive QFT-GIT result; whereas among the 1,080 TST negative HHCs, 46.8% (505/1080) had a negative and 53.2% (575/1080) a positive QFT-GIT result (TST ≥ 10 mm).

When stratified by HIV status, negative concordance was more common in HIV positives, 39.9% (195/489) compared to HIV negatives, 23.9% (303/1,265); positive concordance was more common in HIV negatives, 33.2% (420/1,265) compared to HIV positive, 27.2% (133/489); and discordance was more common in HIV negative, 42.8% (542/1,265) compared to HIV positive HHCs, 32.9% (161/489) (chi2; p<0.001).

Overall, there was a low level of agreement (% agreement=59.7%; kappa=0.24) between TST (≥ 10 mm) and QFT-GIT. Using different cut-off values for the TST did not improve overall test agreement (kappa: 0.26, 0.24 and 0.13 for TST cutoffs of 5, 10 and 15 mm respectively). However, overall agreement between the two tests was best (64.0%) with a TST cut-off of ≥ 5 mm and least good (49.2%) with a TST cut-off of ≥ 15 mm. When results were stratified by HIV status, agreement appeared slightly better in HIV positive (% agreement =67.1%; kappa 0.35) compared to HIV negative (%agreement= 57.1%; kappa 0.19) HHCs at a TST cut-off of ≥ 10 mm (**Table 5.4**).

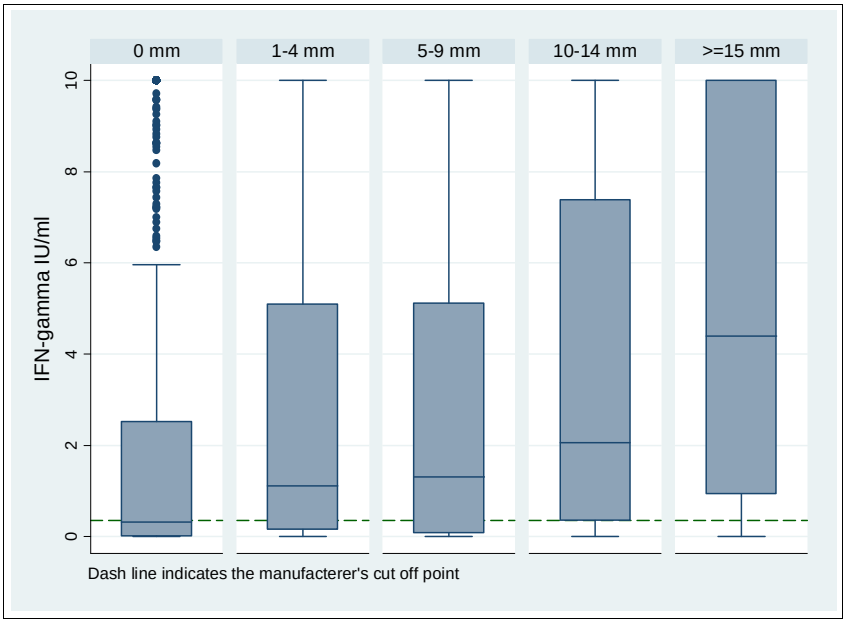
Table 5.4: Agreement between QFT-GIT and TST at different cut off levels, stratified by HIV status

	Overall			HIV negative			HIV positive		
	Agreement %	[1]Kappa (95% CI)	p-value	Agreement %	Kappa (95% CI)	p-value	Agreement %	[1]Kappa (95% CI)	p-value
QFT vs. TST 5 mm	64.0	0.26 (0.22-0.31)	<0.001	62.9	0.22 (0.17-0.27)	<0.001	67.3	0.35 (0.26-0.43)	<0.001
QFT vs. TST 10 mm	59.8	0.24 (0.2-0.28)	<0.001	57.1	0.19 (0.15-0.24)	<0.001	67.1	0.35 (0.28-0.43)	<0.001
QFT vs. TST 15 mm	49.2	0.13 (0.11-0.16)	<0.001	45.6	0.11 (0.08-0.14)	<0.001	58.7	0.20 (0.14-0.26)	<0.001

[1]**Cohen's k coefficient**

Agreement between QFT-GIT and TST was further analyzed using quantitative QFT-GIT and TST results. Overall, there was a weak positive correlation between TB antigen stimulated IFN-γ responses and TST indurations (Person's correlation coefficient=0.30; p<0.001). As the diameter of the TST induration increased there was an increase in the total amount of IFN-γ released (**Figure 5.9**). Similarly, there was an increase in the proportion of QFT-GIT positive results with increasing TST indurations ranging from 48.5% (369/760) at TST 0 mm, to 84.5% at TST ≥ 15 mm. In contrast, there was a decrease in the proportion of QFT-GIT negative results with increasing TST cut-offs ranging from 51.4% (391/760) at TST 0 mm to 15.5% (52/335) at TST ≥ 15 mm.

Figure 5.9: IFN-gamma responses at different TST cut offs



There were 719 HHCs with discordant results between the TST and QFT-GIT tests using TST at ≥ 10 mm. At this cut-off, QFT+/TST- discordance was found in 80% (575/719) of HHCs while 20% (144/719) had QFT-/TST+ discordance (chi2; p<0.001). QFT+/TST- discordance was more frequent than QFT-/TST+ discordance regardless of TST cut-off. Using TST at ≥ 5 mm, QFT+/TST- discordance was found in 63.5% (409/644) of HHCs while 36.5% (235/644) had QFT-/TST+ discordance (chi2; p<0.001). Using TST at ≥15 mm, QFT+/TST- discordance was found in 94.3% (857/909) of HHCs while 5.7% (52/909) had QFT-/TST+ discordance (chi2; p<0.001). Therefore as shown, QFT+/TST- discordance increased whereas QFT-/TST+ discordance decreased with increasing TST cut off points.

In order to account for key factors that could be responsible for discordant QFT/TST results multivariable analysis was performed adjusted for age, sex and household clustering. The discordant groups (TST+/IGRA-; TST-/IGRA+) were each compared with a concordant negative reference group (TST-/IGRA-). Compared to concordant negative results, QFT+/TST- discordance was positively associated with age group 55-64 years (aOR:3.09 (95% CI:1.45-6.61, p=0.003)), smear status of the index (aOR:1.45 (95% CI:1.18-1.78, p<0.001) and country (aOR:2.02 (95% CI:1.40-2.92, p<0.001). These results were similar for QFT-/TST+ discordance. However, while QFT+/TST- discordance was associated with increasing age (aOR for each 10 year increase: 1.18; 95% CI: 1.06-1.31; p <0.003 for linear trend) there was weak evidence for QFT-/TST+ discordance (aOR for each 10 year increase: 1.14; 95% CI: 0.95-1.37; p=0.15 for linear trend). Both types of discordance were negatively associated with HIV status (aOR for QFT+/TST-: 0.38; 95% CI: 0.26-0.56; p<0.001); aOR for QFT-/TST+: 0.41; 95% CI: 0.22-0.76; p=0.005). For each type of discordance, there was no evidence of an association with BCG scar (aOR for QFT+/TST-: 0.81; 95% CI: 0.49-1.35; p=0.43); aOR for QFT-/TST+: 0.98; 95% CI: 0.45-2.15; p=0.97).

Summary

In Chapter 5 of this thesis, I have presented the baseline results of the QFT study which forms the foundation of the survival analysis I will present in the subsequent two chapters. The study population at baseline consisted of 1,789 HHCs who were predominantly women (71%) and had a median age of 28 years (IQR: 21-43). Overall, the median IFN-γ responses to TB antigens was 1.1 IU/ml (IQR; 0.1-5.7). The majority of HHCs, 36.1% (646/1789) had IFN-γ responses below 0.35 IU/ml while 25.7% (459/1789) had IFN-γ responses between 5.5-10 IU/ml.

I have shown that as expected in this setting, overall, the prevalence of HIV infection was high at 27.9% and that of TBI was 63.7% as detected by QFT-GIT and 39.6% when TST was used. In addition, both tests showed that TBI prevalence was higher in SA communities (QFT-GIT: 71.7% and TST: 47.2%; p<0.001) compared to the Zambian ones (QFT-GIT: 58.3% and TST: 34.1%; p<0.001).

Both QFT-GIT and TST showed a significantly lower proportion of test positive results in HIV positive HHCs (QFT-GIT: 52.8%; TST: 34.6%; p<0.001) compared with HIV negative ones (QFT-GIT: 67.8% %; TST: 41.5%; p=0.008) regardless of TST cut-off. Similar results were obtained when TB antigen stimulated IFN-γ responses were stratified by HIV status (rank-sum; p <0.001).

In multivariable analysis adjusted for age, sex and community, HIV positivity (aOR: 0.48; 95% CI: 0.37–0.63; p<0.001) and being an ex-smoker (aOR:0.56; 95%CI:0.32-1.00; p<0.001) were negatively associated with QFT-GIT positivity while residing in an urban area (aOR: 2.37; 95% CI: 1.10–5.13; p<0.03), smear status of index (OR: 1.34; 95% CI: 1.06-1.69; p=0.01) and country (aOR: 1.93; 95% CI: 1.48–2.51; p<0.001) were negatively associated with QFT-GIT positivity. Similar results were obtained for TST. As expected, exploration of QFT-GIT (aOR:1.15; 95% CI: 1.06–1.25; p<0.001) and TST (aOR: 1.10; 95% CI 1.02-1.21; p <0.001) results by age revealed a trend to increased responses with increasing age suggesting cumulative exposure to *Mtb* and an increased likelihood of TB infection with age.

An assessment of agreement showed that out of 1,789 HHCs, 28.2% were concordantly negative, 31.6% were concordantly positive, while 40.2% were discordant (p<0.001) at TST ≥ 10 mm. There was a low level of agreement between the tests regardless of TST cut-off point (% agreement=59.7%; kappa=0.24). QFT+/TST- discordance (575/719; 80%) was more frequent than QFT-/TST+ discordance (144/719; 20%) at TST ≥10 mm and this was true of other TST cut-offs and regardless of HIV status.

6.0 Are IGRAs better than the TST at predicting HHCs at highest risk of progression to active TB?

Introduction

The aim of this chapter is to assess whether QFT-GIT can predict the development of active TB and whether its predictive ability is better than that of the TST in HIV positive and negative household contacts. I also report incidence rates of TB in these HHCs for both QFT-GIT and TST, stratified by HIV status. I define QFT-GIT results as a dichotomous exposure using the manufacturer definition (TB antigen response ≥0.35 IU/ml above the negative control). While incident TB is defined as self-reported TB confirmed in the patient treatment card, in a sensitivity analysis I explore other definitions of TB which include smear and culture results.

The specific objectives of this chapter are as follows:

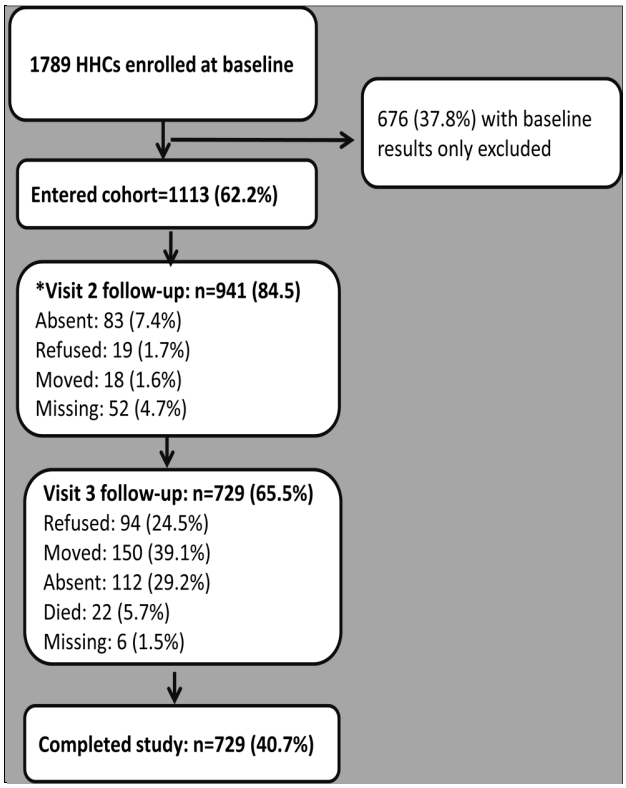
1. To determine the incidence rates of TB in HHCs with positive and negative QFT-GIT and TST results, stratified by country and HIV status.
2. To determine positive and negative predictive values for QFT-GIT and TST
3. To determine the association between QFT-GIT and TST positivity and the subsequent development of TB.
4. To determine risk factors associated with incident TB.
5. To conduct a sensitivity analysis exploring three other definitions of TB.

6.1 Participants' Characteristics and Follow-up

A total of 1,789 HHCs were recruited of whom 676 (37.8%) were excluded because they had no follow-up data leaving 1,113 (62.2%) HHCs available for this analysis. There were 1,113 HHCs available at the second follow-up visit, 18 months from enrolment. At Visit 2 (1st follow-up visit) of the study, 84.5% (941/1,113) agreed to continue with study, 7.4% (83/1,113) were absent, 1.6% (18/1,113) had moved out of the community, 1.7% (19/1,113) refused and 4.7% (52/1,113) had missing results. Apart from those who refused, all HHCs remained in the cohort and were followed up until visit 3; 36 months from enrolment.

At visit 3 (2nd follow-up visit), 729 (65.5%) HHCs were still in the cohort while 94 (24.5%) refused to continue with the study; 150 (39.1%) had moved out of the community; 112 (29.2%) were absent; 22 (5.7%) had died and 6 (1.5%) had missing results. Follow-up of HHCs at the scheduled visits is summarized in **Figure 6.1**. HHCs lost to follow-up were defined as those who were absent, had moved out of the community or had died by the time of the final follow-up visit, visit 3. Although 22 HHCs had date of deaths recorded, further analysis was not conducted among this group since it was not an outcome.

Figure 6.1: Study cohort from enrollment to completion



At visit 2, HHCs recorded as absent, moved, or those with missing results were followed up.
Visit 2=1st follow-up visit; Visit 3=2nd follow-up visit

The demographic and clinical characteristics of those who completed the study were compared to those who refused, were lost to follow-up and had missing data (**Table 6.1**). Generally these

groups did not differ with respect to demographic and clinical characteristics apart except for country (p<0.001).

Table 6.1: Comparison of HHCs lost to follow up with those completing the study, row % (N=1113)

	Completed study (n, row %)	**Refused (n, row %)**	**Lost to follow up at final visit (n, row %)**	**Missing results (n, row%)**	p value (chi-square)
Total	729	94	284	6	
Sex					
Male	193 (63.5)	28 (9.2)	81 (26.6)	2 (0.6)	
Female	534 (66.2)	66 (8.2)	203 (25.1)	4 (0.5)	0.84
Missing	2				
Age group (years)					
15-25	306 (62.2)	42 (8.5)	142 (28.9)	2 (0.4)	0.29
26-39	165 (67.6)	17 (7.0)	60 (24.6)	2 (0.8)	
40-87	258 (68.4)	35 (9.3)	82 (21.8)	2(0.5)	
Highest level of education					
Not attended school	47 (64.4)	12 (16.4)	13 (17.8)	1 (1.4)	0.14
Primary school	238 (67.8)	32 (9.1)	81 (23.1)	0 (0)	
Secondary school	356 (64.7)	42 (7.6)	148 (26.9)	4 (0.7)	
College or University	73 (64.0)	8 (7.0)	32 (28.1)	1 (0.9)	
Missing	15 (60.0)	0	10 (40.0)	0	
Socioeconomic status					
Dependant	158 (62.4)	24 (9.5)	70 (27.7)	1 (0.4)	0.59
Unemployed	483 (64.5)	65 (8.7)	196 (26.2)	4 (0.5)	
Employed	82 (79.6)	4 (3.9)	17 (16.5)	0	
Missing	6 (66.7)	1 (11.1)	1 (11.1)	1 (11.1)	
Smoking habits					
Never smoked	617 (66.5)	79 (8.5)	227 (24.5)	5 (0.5)	0.39
Ex-smoker	28 (63.6)	2 (4.5)	14. (31.8)	0	
Occasional smoker	19 (55.9)	2 (5.9)	13 (38.2)	0	
Daily smoker	63 (60.6)	10 (9.6)	30 (28.8)	1 (1.0)	
Missing	2 (100)				
Alcohol consumption					
No	549 (66.3)	69 (8.3)	206 (24.9)	4 (0.5)	
Yes	175 (62.9)	25 (9.0)	76 (27.3)	2 (0.7)	0.76
Missing	5 (71.4)	0	2 (28.6)		
HIV status					
Negative	506 (63.3)	69 (8.6)	219 (27.4)	5 (0.6)	
Positive	212 (71.4)	25 (8.4)	59 (19.9)	1 (0.3)	0.06
Missing	11 (64.7)	0	6 (35.3)	0	
Smear status of the index					
Smear negative	260 (67.5)	33 (8.6)	90 (23.4)	2 (0.5)	
Smear positive	253 (65.2)	28 (7.2)	105 (27.1)	2 (0.5)	0.65
Missing	216 (63.5)	33 (9.7)	89 (26.2)	2 (0.6)	
TST measured at enrolment					
<10mm	439 (63.9)	65 (9.5)	181 (26.3)	2 (0.3)	
≥10mm	290 (68.1)	29 (6.8)	103 (24.2)	4 (0.9)	0.14
QFT results *measured at enrolment*					
Negative	263 (64.6)	36 (8.8)	105 (25.8)	3 (0.7)	
Positive	466 (66.0)	58 (8.2)	179 (25.3)	3 (0.4)	0.88
Residence					
Urban	626 (65.7)	80 (8.4)	242 (25.4)	5 (0.5)	
Rural	103 (64.4)	14 (8.7)	42 (26.2)	1 (0.6)	0.99
Country					
Zambia	501 (68.8)	72 (9.9)	155 (21.3)	0	
South Africa	228 (59.2)	22 (5.7)	129 (33.5)	6 (1.6)	P<0.001

Lost to follow-up at final visit; 284=150 moved + 112 absent + 22 died

6.2 Summary of incident cases

Out of 1,113 HHCs who entered follow up, there were 51 incident cases. 44.0% (22/51) of incident cases were in the age range 26-39 years, 64% (32/51) were HIV positive, 42.5% (17/40; 11 had missing results) had a history of sleeping in the same bed as the index case, and 52.9% (27/51) resided in Zambia.

The total household size (as enumerated regardless of eligibility to the QFT cohort) of the incident cases varied from 1-9. Out of the 51 incident cases, 25 of them had records indicating disease site. The majority of incident cases, 88% (22/25) had pulmonary TB, while 12% (3/25) had extra-pulmonary disease. Out of those with pulmonary TB, 54.5% (12/22) were smear positive, 31.8% (7/22) smear negative and 13.6% (3/22) had missing data.

The median follow up period for incident cases was 0.99 years (IQR: 0.42-1.65). A description of incident cases by socio-demographic and clinical characteristics is shown in **Table 6.2**.

Table 6.2: Description of incident cases by socio-demographic and clinical characteristics

	Incident cases (col %)
Total	**N=51**
Sex	
Male	14 (27.4)
Female	37 (72.5)
Age group (years)	
15-25	14 (28.0)
26-39	22 (44.0)
40-87	14 (28.0)
Median age (years	29.5 (IQR:25-40)
Missing	1
Highest level of education	
Not attended school	3 (5.9)
Primary school	22 (43.1)
Secondary school	23 (45.1)
College or University	3 (5.9)
Socioeconomic status	
Dependant	15 (29.4)
Unemployed	32 (62.8)
Employed	4 (7.8)
Smoking habits	
Never smoked	37 (72.6)
Ex-smoker	2 (3.9)
Occasional smoker	4 (7.8)
Daily smoker	8 (15.7)
Alcohol consumption	
No	35 (68.6)
Yes	16 (31.4)
HIV status	
Negative	18 (36.0)
Positive	32 (64.0)
Missing	1
Smear status of the index	
Smear negative	15 (36.6)
Smear positive	26 (63.4)
Missing	10
Sleeping proximity to index	
Different house	4 (10.0)
Same house	13 (32.5)
Same room	2 (5.0)
Same bed	17 (42.5)
Unknown	4 (10.0)
Missing	11
Residence	
Urban	47 (92.2)
Rural	4 (7.8)
Country	
Zambia	27 (52.9)
South Africa	24 (47.1)

6.3 TB incidence rates stratified by baseline QFT-GIT and TST results

Out of the 1,113 HHC s that entered follow-up, 51 (4.6%) HHCs developed active TB (incident cases) over 2,433 person-years (pyrs) of follow-up, giving an incidence rate of 20.96/1000 pyrs. The median follow-up time was 2.43 years (IQR: 1.53-2.79).

Of the incident cases, approximately half, 52.9% (27/51) developed TB within a year of being enrolled into the study, 29.4% (15/51) between 1-1.9 years, 13.7% (7/51) between 2-2.9 years and 3.9% (2/51) between 3-4 years.

Out of 707 HHCs with QFT-GIT positive results at baseline, 38 developed active TB over 1555.3 pyrs of follow-up (incidence rate, 24.43 per 1000 pyrs). Out of 406 HHCs with QFT-GIT negative results at baseline, 13 developed active TB over 877.9 pyrs of follow-up (incidence rate, 14.81 per 1000 pyrs). Therefore, 74.5% (38/51) and 25.5% (13/51) of incident cases were QFT-GIT positive and negative at baseline respectively.

The (Incidence rate ratio (IRR) among incident cases with positive QFT-GIT results was 1.65 (95% CI 0.86-3.37; p=0.06) compared to those with negative results (**Table 6.3).**

Table 6.3: Overall incidence rate ratios (nmax=1113). All TB cases (n=51).

Variable	n (col %)	TB cases/[1]pyrs	Incidence rate/ 1000 pyrs (95% CI)	Incidence rate ratio (95% CI)	P value
Overall	**N=1113**	**51/2433**	**20.96 (15.93-27.58)**		
QFT-GIT (>=0.35 IU/ml)					
Negative	406	13/877.9	14.81 (8.60-25.50)	1	
Positive	707	38/1555.3	24.43 (17.78-33.58)	1.65 (0.86-3.37)	0.06
TST (>=10 mm)					
Negative	687	23/1476.0	15.58 (10.35-23.45)	1	
Positive	426	28/957.2	29.25 (20.20-42.36)	1.88 (1.04-3.41)	0.01
TST (mm) responses					
< 5	529	19/1128.7	16.83 (10.74-26.39)	1	0.03
5-9	158	4/347.3	11.52 (4.32-30.69)	0.68 (0.17-2.06)	
10-14	231	12/507.7	23.63 (13.42-41.62)	1.40 (0.62-3.05)	
≥ 15	195	16/449.5	35.59 (21.80-58.10)	2.11 (1.02-4.34)	
Concordance in TST and QFT-GIT results (N=1113)					
QFT -/TST -	323	10/693.2	14.42 (7.76-26.81)	1	0.04
QFT -/ TST+	84	3/184.7	16.24 (5.24-50.36)	1.13 (0.20-4.37)	
QFT +/ TST -	364	13/782.8	16.61 (9.64-28.60)	1.15 (0.47-2.93)	
QFT +/ TST +	342	25/772.5	32.36 (21.87-47.89)	2.24 (1.04-5.23)	

[1]pyrs =person-years; [2]TST (10 mm).

Out of 426 HHCs with TST positive results at baseline, 28 developed active TB over 957.2 pyrs of follow-up (incidence rate, 29.25 per 1000 pyrs). Out of 687 HHCs with TST negative results at baseline, 23 developed active TB over 1476 pyrs of follow-up (incidence rate, 15.58 per 1000 pyrs). Therefore, 54.90% (28/51) and 45.01% (23/51) of incident cases were TST positive and negative at baseline respectively.

The IRR among incident cases with TST positive results was 1.88 (95% CI 1.04-3.41; p=0.01) compared to those with negative results (table 6.3).

For TST, IRRs were 2.11 (95% CI: 1.02-4.34), 1.40 (95%CI: 0.62-3.05) and 0.68 (0.17-2.06) in HHCs with responses greater or equal to 15 mm, 10-14 mm, and 5-9 mm respectively compared to those less than 5 mm (table 6.3).

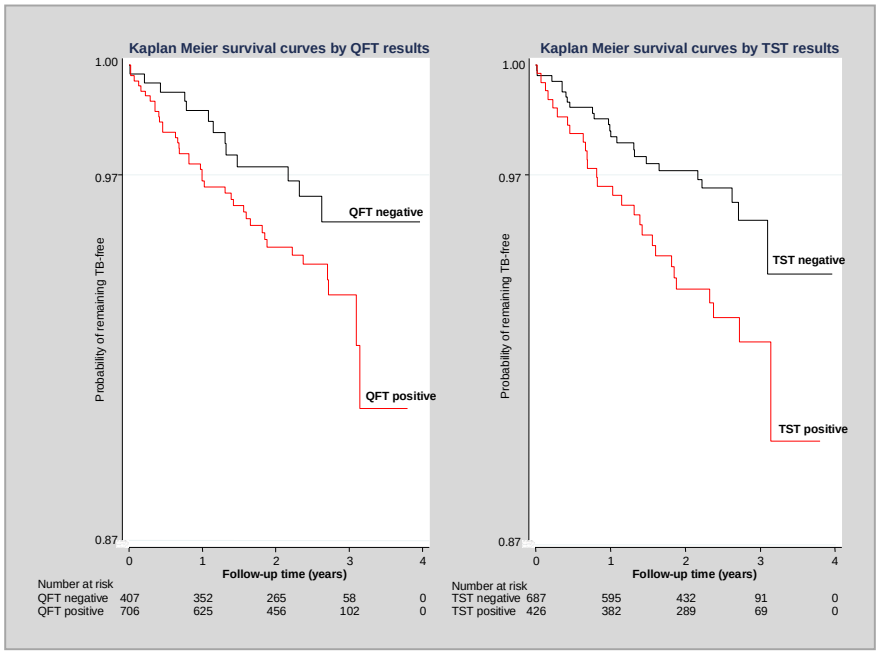
The incidence rate was highest among HHCs with concordant positive results compared to those with concordant negative results (IRR: 2.24, 95% CI: 1.04-5.23) (table 6.3). The incident rates among discordant pairs were comparable to each other (table 6.3).

6.4 Descriptive survival analysis

Kaplan-Meier was used to estimate TB-free survival probability as a function of time from entry into the study. Overall, the 4-year TB-free survival proportion (HHCs who did not develop TB at a given time) rate was 0.92 (95% CI: 87.5-94.8).TB-free survival proportion was lower in QFT-GIT positives, 0.90 (95%CI: 0.83-0.94) compared to QFT-GIT negatives, 0.95 (95%CI: 0.92-0.97). All incident cases developed TB before the median survival time (time with probability of survival equal to 0.5).

Kaplan Meier curves showing TB-free survival probabilities according to baseline QFT-GIT and TST results are shown in **Figure 6.2.**

Figure 6.2: Kaplan Meier curves showing probability of remaining TB-free according to baseline QFT and TST results

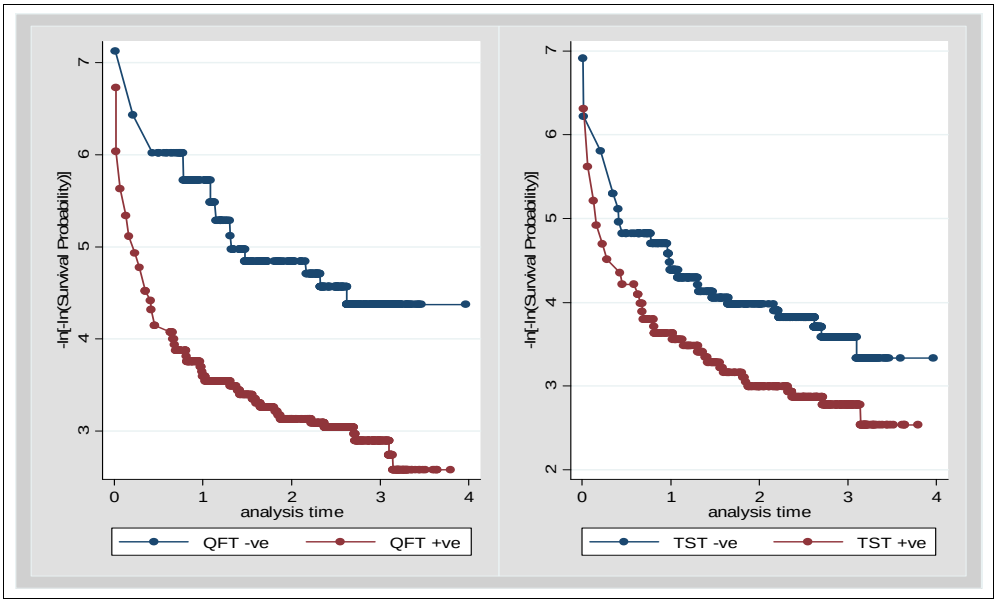


For both QFT-GIT and TST, the TB-free survival probability was lower among test-positive individuals compared test- negative ones. There was some evidence that survival curves differed between those who were tested positive and negative for TST (log-rank test, p=0.02) but not for QFT-GIT (log-rank test, p=0.11).

6.5 The Proportional Hazard Assumption (PHA) in the Cox model

The Kaplan-Meier curves presented in **Figure 6.3** showed that the proportional hazard assumption was met for both tests since there was an increasing difference between the two curves and the two curves did not cross. Furthermore, using log-minus-log survival plot (plots of Schoenfeld residuals) to test the proportional hazard assumption showed that the assumption was largely met since the curves for both tests were approximately linear.

Figure 6.3: Assessing the proportional hazards assumption: "log-log" plots



The proportional-hazards assumption is not violated when the curves are parallel.

Testing PHA by using the interaction test suggested no evidence against proportionality (LRT: p=0.70). The likelihood ratio test also showed no evidence that the effects of QFT-GIT (LRT: p=0.29) and TST (LRT: p=0.74) within time bands was not constant (time an effect modifier).

6.6 Risk factors for the development of TB

Incidence rates, unadjusted and adjusted analysis of the relationship between risk factors and TB incidence is shown in **Table 6.4.**

In unadjusted analysis , there was evidence that being between 26-39 years of age (HR:2.31; 95%CI: 1.18-4.52; p=0.04) HIV positivity (HR: 4.68; 95%CI: 2.62-8.35, p<0.001); smear status of the index (HR: 1.74; 95%CI: 0.92-3.28; p=0.09); sleeping in the same room and same bed as index (HR:1.88 ; 95%CI:0.64-5.53; p=0.02) and country (HR: 1.55; 95%CI:0.90-2.67; p=0.04) were associated with an increased risk of TB (table 6.4).

In multivariable analyses, adjusted for age, sex , HIV status and country there was evidence that occasional smoking, (HR: 4.07; 95%CI:1.31-12.63), HIV positivity (HR: 4.60; 95%CI:2.48-8.56), smear positivity of the index (HR: 2.00 ; 95%CI:1.04-3.87) and country (HR: 1.79 ; 95%1.02-3.15; p=0.04)) were associated with incidence of TB.

Data were consistent with no association of sex, education, socioeconomic status, residence, and sleeping proximity with increased risk of TB (**Table 6.4**).

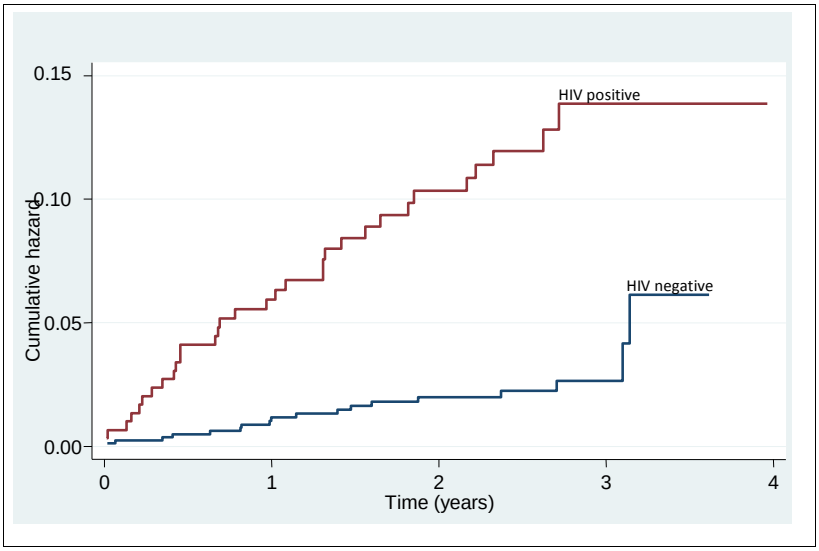
Table 6.4: TB incidence rates and hazard ratios stratified by baseline socio-demographic and clinical characteristics

	N (col %) N=1113	TB cases/pyrs	Incidence rate/ 1000 pyrs (95% CI)	Crude HR (95% CI)	P value	aHR (95% CI)	P value
Sex							
Male	304 (27.4%)	14/653.3	21.43 (12.69-36.18)	1		1	
Female	807 (72.6%)	37/1773.7	20.86 (15.11-28.79)	0.97 (0.53-1.80)	0.93	0.71 (0.38-1.34)	0.29
Age group (years)							
15-25	492 (44.2)	14/983.4	14.23 (8.43-24.04	1	0.04	1	p<0.001
26-39	244 (21.9)	22/665.4	33.06 (21.77-50.21	2.31 (1.18-4.52)		1.46 (0.72-2.93)	
40-87	377 (33.9)	14/770.9	18.16 (10.76-30.66	1.29 (0.61-2.70)		1.35 (0.64-2.85)	
Highest level of education							
Not attended school	73 (6.7)	3/155.3	19.31 (6.23-59.88)	1	0.41	1	p<0.001
Primary school	351 (32.3)	22/778.1	28.27 (18.62-42.94)	1.46 (0.44-4.89)		1.66 (0.39-7.13)	
Secondary school	550 (50.5)	23/1202.1	19.13 (12.71-28.79)	1.00 (0.30-3.33)		1.20 (0.27-5.34)	
College or University	114 (10.5)	3/246.1	12.19 (3.93-37.80)	0.64 (0.13-3.16)		0.74 (0.12-4.53)	
Socioeconomic status							
Dependant	253 (22.9)	15/538.4	27.86 (16.79-46.21)	1	0.48	1	p<0.001
Unemployed	748 (67.7)	32/1638.4	19.53 (13.81-27.62)	0.70 (0.38-1.30)		0.76 (0.39-1.49)	
Employed	103 (9.3)	4/236.4	16.92 (6.35-45.09)	0.62 (0.20-1.87)		0.83 (0.23-3.00)	
Smoking habits							
Never smoked	928 (83.4)	37/2039.2	18.14 (13.15-25.04)	1	0.11	1	p<0.001
Ex-smoker	44 (4.0)	2/99.4	20.12 (5.03-80.47)	1.10 (0.26-4.57)		0.79 (0.17-3.55)	
Occasional smoker	34 (3.1)	4/63.4	63.11 (23.69-168.15)	3.41 (1.22-9.59)		4.07 (1.31-12.63)	
Daily smoker	104 (9.4)	8/224.5	35.63 (17.82-71.25)	1.93 (0.90-4.16)		1.87 (0.77-4.56)	
Alcohol consumption							
No	828 (74.9)	35/1823.3	19.20 (13.78-26.74)	1		1	
Yes	278 (25.1)	16/594.5	26.91 (16.49-43.93)	1.39 (0.77-2.52)	0.27	1.31 (0.69-2.46)	0.41
HIV status							
Negative	799 (72.9)	18/1741.4	10.34 (6.51-16.40)	1		1	
Positive	297 (27.1)	32/655.0	48.86 (34.55-69.09)	4.68 (2.62-8.35)	P<0.001	4.60 (2.48-8.56)	p<0.001
Smear status of the index							
Smear negative	385 (49.8)	15/846.1	17.73 (10.69-29.41)	1		1	
Smear positive	388 (50.2)	26/836.9	31.07 (21.15-45.63)	1.74 (0.92-3.28)	0.09	2.00 (1.04-3.87)	0.04
Sleeping proximity to index							
Different house	98 (12.10)	4/202.4	19.76 (7.42-52.65)	1	0.02	1	P<0.001
Same house	470 (58.02)	13/987.3	13.17 (7.64-22.68)	0.68 (0.22-2.08)		0.52 (0.16-1.68)	
Same room and same bed	242 (29.88)	19/520.6	36.49 (23.28-57.21)	1.88 (0.64-5.53)		1.10 (0.36-3.35)	
Residence							
Rural	160 (14.4)	4/327.7	12.20 (4.58-32.52)	1		1	
Urban	953 (85.6)	47/2105.5	22.32 (16.77-29.71)	1.81 (0.65-5.03)	0.26	1.78 (0.63-5.04)	0.28
Country							
Zambia	728 (65.4)	27/1550.0	17.42 (11.95-25.40)	1		1	
South Africa	385 (34.6)	24/883.2	27.17 (18.21-40.54)	1.55 (0.90-2.67)	0.11	1.79 (1.02-3.15)	0.04

P value from likelihood test if > 2 sub-groups. Missing: sex-2; age-1; education-25; socioeconomic-9; smoking habits-3; taken alcohol-7; HIV status-17; index smear-340; sleeping proximity to index-152. [1]aHR: adjusted for age, sex, HIV and country

HIV was an important risk factor for the development of TB. **Figure 6.4** shows the Nelson-Aalen cumulative hazard estimate of TB incidence, by HIV status.

Figure 6.4: Nelson-Aalen cumulative hazard estimate of TB incidence, by HIV status.



Confounders and effect modifiers for the association between QFT-GIT positivity and TB incidence

Identification of confounders

HIV was an important confounder in the association between QFT-GIT positivity and TB incidence while age and sex were considered as priori confounders. There was no evidence that socioeconomic status, smoking habits, smear status of the index and sleeping proximity to the index were important confounders.

Identification of effect modifiers using likelihood ratio test

There was weak evidence that educational status (p=0.08 for interaction), smear status of the index (p=0.10) and HIV status (p=0.40) were effect modifiers. Country was considered as an important effect modifier (p=0.001). The rest of the risk factors showed no evidence of effect modification with the QFT-GIT positivity.

6.7 Overall univariable and multivariable analysis of the association between test positivity and TB incidence.

Four regression models were used in the univariable and multivariable analysis and results for each model are shown in **Table 6.5** as follows:

Table 6.5: Overall univariable and multivariable analysis of the association between test results, and TB incidence.

	QFT-GIT results		TST results	
Test result	**Hazard Ratio (95% CI)**	**P value**	**Hazard Ratio (95% CI)**	**P value**
Model 1				
Negative	1	0.11	1	
Positive	1.66 (0.88-3.11)		1.89 (1.09-3.28)	0.02
Model 2				
Negative	1		1	
Positive	1.60 (0.85-3.02)	0.15	1.82 (1.04-3.17)	0.03
Model 3				
Negative	1		1	
Positive	2.20 (1.14-4.25)	0.02	2.19 (1.24-3.86)	0.007
Model 4				
Negative	1		1	
Positive	6.38 (1.90-21.30)	0.003	2.32 (1.07-5.04)	0.03

Model 1: Unadjusted analysis
Model 2: Adjusted for age and sex as priori confounders.
Model 3: Adjusted for age and sex and HIV
Model 4: as in model 3 plus country as an effect modifier (adjusting for household clustering not included because it does not change results) (see results presented by country in next section).

In univariable analysis, the HR showing the association between QFT-GIT positivity and risk of TB was 1.66 (95%CI: 0.88-3.11; P=0.01). When results were adjusted for age and sex as priori confounders, HR was similar to the unadjusted one (table 6.5). In multivariable analysis, adjusted for sex, age and HIV (model 3), the adjusted HR was 2.20 (1.14-4.25; p=0.02). In model 4, the adjusted HR was 6.38 (95%CI: 1.90-21.30; p=0.003) after results were adjusted for sex, age, HIV plus an interaction between QFT-GIT positivity and country (p for interaction=0.001).

For TST, the HR remained similar across all models. In all models, there was strong evidence that TST positive HHCs had a two-fold increased risk of developing TB (table 6.5).

6.8 Analysis by Country

Incidence rates by country

Because there was interaction between country and QFT-GIT positivity (table 6.5), results were also reported separately by country.

6.8.1 Incidence rates in Zambia

Overall, 27 HHCs developed active TB over 1550 pyrs of follow-up, giving an incidence rate of 17.42/1000 pyrs (95%CI: 11.95- 25.40) for HHCs residing in Zambia. The median follow-up time was 2.44 years (IQR: 1.26-2.76).

Out of 412 HHCs with QFT-GIT positive results, 24 developed active TB over 936.2 pyrs of follow-up (incidence rate, 25.64 per 1000 pyrs). Out of 289 HHCs with QFT-GIT negative results, 3 developed active TB over 613.8 pyrs of follow-up (incidence rate, 4.89 per 1000 pyrs).

Therefore, 88.89% (24/27) and 11.11% (3/27) of incident cases were QFT-GIT positive and negative at baseline respectively.

The IRR among QFT-GIT positive HHCs was 5.24; (95% CI: 1.59-27.21; p=0.0007) compared to QFT-GIT negative ones (table 6.6).

Table 6.6: Zambia: Incidence rates and incidence rate ratios stratified by test (nmax=728). All TB cases (n=27)

Variable	n (col %)	TB cases/pyrs	Incidence rate/ 1000 pyrs (95% CI)	Incidence rate ratio (95% CI)	P value
Overall	**N=728**	**27/1550**	**17.42 (11.95- 25.40)**		
QFT (>=0.35 IU/ml)					
Negative	289	3/613.8	4.89 (1.58-15.15)	1	
Positive	412	24/936.2	25.64 (17.18-38.25)	5.24 (1.59-27.21)	0.0007
TST (10 mm)					
Negative	459	12/979.3	12.25 (6.96-21.58	1	0.02
Positive	242	15/570.7	26.28 (15.84-43.60	2.14 (0.94-5.02)	
TST (mm) responses					
< 5	360	11/737.2	14.92 (8.26-26.94)	1	0.02
5-9	111	1/242.1	4.13 (0.58-29.33)	0.28 (0.006-1.90)	
10-14	152	6/327.0	18.35 (8.24-40.84)	1.23 (0.37-3.63)	
≥ 15	105	9/243.7	36.93 (19.21-70.97)	2.47 (0.91-6.57)	
[2]Concordant results (N=728)					
QFT -/TST -	239	3/503.3	5.96 (1.92-18.48)	1	0.08
QFT -/ TST+	50	0/110.6	0	0 (0-11.01)	
QFT +/ TST -	220	9/476.0	18.91 (9.84-36.34)	3.17 (0.79-18.22)	
QFT +/ TST +	192	15/460.2	32.60 (19.65-54.07)	5.47 (1.55-29.47)	

[2]TST (10 mm) and QFT-GIT results.

For TST, 55.55% (15/27) and 44.44% (12/27) of incident cases were TST positive and negative at baseline respectively. At the standard TST cut off (10 mm), the IRR among TST positive HHCs was 2.14 (95% CI: 0.94-5.02). For other TST cut-offs in Zambia, IRRs were 0.28 (95% CI: 0.15-0.59),

1.23 (95%CI: 0.82-1.82) and 2.47 (1.80-3.26) (p=0.02) in HHCs for 5-9 mm, 10-14mm, greater or equal to 15 mm respectively compared to those less than 5 mm (table 6.6).

6.8.2 Incidence rates in SA

24 HHCs developed active TB over 883.2 pyrs of follow-up, giving an incidence rate of 27.17/1000 pyrs (95%CI: 18.21-40.54) for HHCs residing in SA. The median follow-up time was 2.41 years (IQR: 1.76-2.87).

Out of 256 HHCs with QFT-GIT positive results, 14 developed active TB over 619.1 pyrs of follow-up (incidence rate, 22.61 per 1000 pyrs). Of 105 HHCs with QFT negative results, 10 developed active TB over 264.1 pyrs of follow-up (incidence rate, 37.86 per 1000 pyrs). The IRR among HHCs with QFT-GIT positive results was 0.60; (95% CI: 0.25-1.50; p=0.11) compared to those with negative results (**Table 6.7**).

Table 6.7: South Africa: Incidence rates and incidence rate ratios stratified by test (nmax=358). All TB cases (n=24).

Variable	n (col %) N=361	TB cases/pyrs	Incidence rate/ 1000 pyrs (95% CI)	Incidence rate ratio (95% CI)	P value
Overall	**N=361**	**24/883.2**	**27.17 (18.21-40.54)**		
QFT (>=0.35 IU/ml)					
Negative	105	10/264.1	37.86 (20.37-70.37)	1	
Positive	256	14/619.1	22.61 (13.39-38.18)	0.60 (0.25-1.50)	0.11
TST (10 mm)					
Negative	205	11/496.7	22.14 (12.26-39.99)	1	
Positive	156	13/386.5	33.63 (19.53-57.92)	1.52 (0.63-3.74)	0.15
TST (mm) responses					
< 5	169	8/391.5	20.43 (10.21-40.86)	1	0.74
5-9	47	3/105.2	28.51 (9.20-88.41)	1.39 (0.24-5.81)	
10-14	79	6/180.7	33.20 (14.91-73.90)	1.62 (0.46-5.34)	
≥ 15	90	7/205.8	34.01 (16.22-71.35)	1.66 (0.51-5.25)	
[2]Concordant results (N=385)					
QFT -/TST -	74	7/190.0	36.85 (17.57-77.30	1	0.38
QFT +/ TST +	125	10/312.4	32.01 (17.23-59.50	0.87 (0.30-2.69)	
QFT -/ TST+	31	3/74.1	40.46 (13.05-125.44)	1.10 (0.18-4.81)	
QFT +/ TST -	131	4/306.8	13.04 (4.89-34.74)	0.35 (0.07-1.39)	

For TST, the IRR among TST positive HHCs was 1.52 (95% CI: 0.63-3.74) in comparison to those who were negative (Table 6.7). Furthermore, IRRs were 1.39 (95% CI: 0.99-1.92), 1.62 (95%CI: 1.19-2.21) and 1.66 (1.23-2.27) (p=0.74) in HHCs for 5-9 mm, 10-14mm, greater or equal to 15 mm respectively compared to those less than 5 mm (table 6.7).

6.8.2. Zambia: Univariable and multivariable analysis of the association between test positivity and TB incidence

Three regression models were used in the univariable and multivariable analysis and results for each model are shown in **table 6.8**:

Table 6.8: Zambia: Univariable and multivariable analysis of the association between test results, and TB incidence.

	QFT-GIT results		TST results	
	Hazard Ratio (95% CI)	**P value**	**Hazard Ratio (95% CI)**	**P value**
Model 1				
Negative	1		1	
Positive	5.29 (1.59-17.56)	0.007	2.15 (1.00-4.60)	0.05
Model 2				
Negative	1		1	
Positive	5.07 (1.52-16.94)	0.008	2.00 (0.92-4.33)	0.08
Model 3				
Negative	1		1	
Positive	6.57 (1.96-22.06)	0.002	2.37 (1.08-5.19)	0.03

Model 1: Unadjusted analysis
Model 2: Adjusted for age and sex as priori confounders.
Model 3: Adjusted for age and sex and HIV

In univariable analysis, the HR showing the association between QFT-GIT positivity and risk of TB was 5.29 (95%CI: 1.59-17.56; P=0.007). When results were adjusted for age and sex as priori confounders, HR was similar to the unadjusted one (table 6.8). In multivariable analysis, adjusted for sex, age and HIV (model 3), the adjusted HR was 6.57 (95%CI: 1.96-22.06; P=0.002).

For TST, the HR remained similar across all models; as in the overall results (table 6.8). In all models, there was some evidence that TST positive HHCs had a two-fold increased risk of developing TB, similar to the overall findings.

6.8.3 South Africa: Univariable and multivariable analysis of the association between test positivity and TB incidence

Three regression models were used in the univariable and multivariable analysis and results for each model are shown in **table 6.9** as follows:

Table 6.9: South Africa: Univariable and multivariable analysis of the association between test results, and TB incidence.

	QFT-GIT results		TST results	
	Hazard Ratio (95% CI)	**P value**	**Hazard Ratio (95% CI)**	**P value**
Model 1				
Negative	1		1	
Positive	0.60 (0.26-1.34)	0.21	1.57 (0.70-3.50)	0.27
Model 2				
Negative	1		1	
Positive	0.58 (0.25-1.30)	0.19	1.52 (0.68-3.39)	0.31
Model 3				
Negative	1		1	
Positive	0.84 (0.36-1.99)	0.70	1.98 (0.86-4.53)	0.10

Model 1: Unadjusted analysis
Model 2: Adjusted for age and sex as priori confounders.
Model 3: Adjusted for age and sex and HIV

The crude HR showing the association between QFT-GIT positivity and risk of TB for HHCs residing in SA was 0.60 (95% CI: 0.26-1.34; p=0.21). When results were adjusted for age and sex as priori confounders, HR was similar to the unadjusted one (table 6.9). In multivariable analysis, adjusted for sex, age and HIV (model 3), the adjusted HR was 0.84 (95%CI: 0.36-1.99; P=0.70) (table 6.9).

For TST results in SA, the HR remained similar across all models, similar to the overall results and results for Zambia. In all models, there was strong evidence that TST positive HHCs had a two-fold increased risk of developing TB, although results were not statistically significant.

6.9 Analysis by HIV Status

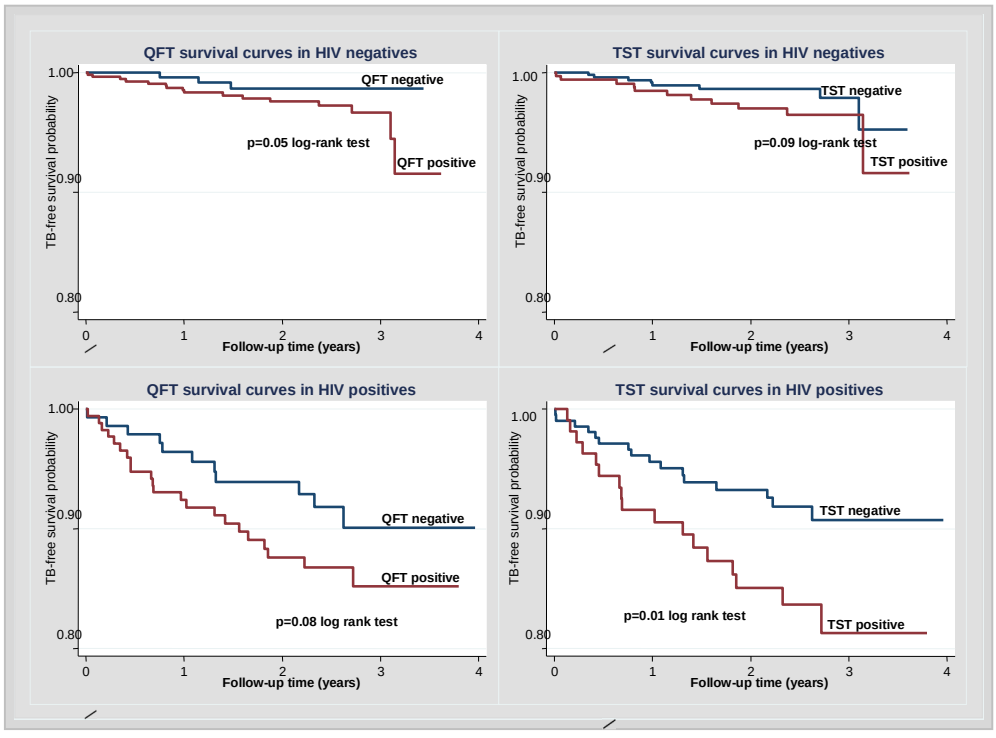
Effect of test results on TB incidence, stratified by HIV status

Kaplan Meier curves stratified by test results and HIV status are shown in **figure 6.5**. For HIV negative HHCs, there was strong evidence that the Kaplan-Meier survival curves differed between QFT-GIT positive and negatives (log rank test, p=0.05) but not for TST (log rank test, p=0.09) (Figure 6.5). For HIV positive HHCs, there was strong evidence that the survival curves differed for TST (log rank test, p=0.01) but evidence was modest for QFT-GIT (log rank test, p=0.08).

Overall, the 4-year TB-free survival proportion was lower in HIV positive HHCs, 0.86 (95%CI: 81.05-90.37) compared to HIV negatives, 0.94 (95%CI: 0.86-0.97) ones.

In both HIV negatives and positives TB-free survival proportion was lower in test positives HHCs compared to negative ones. In HIV negatives, TB-free survival proportion was 0.91 (95% CI: 0. 80-0.96) in QFT-GIT positives while it was 0.99 (95%CI: 0.96-0.99) in QFT-GIT negatives. Among HIV positive HHCs, TB-free survival proportion was 0.84 (95%CI: 0.76-0.89) in QFT-GIT positives while it was 0.90 (95%CI: 0.81-0.94) in QFT-GIT negatives. Results were similar for TST (**Figure 6.5**)

Figure 6.5: Kaplan Meier curves of TB-free survival probability according to baseline test results and stratified by HIV



The overall incidence rate among HIV negatives was 10.34 (18 cases/1741.4 pyrs) while for HIV positives it was 48.86 (32 cases/655 pyrs) per 1000 pyrs. Overall for QFT-GIT, the IRR was higher among HIV negative HHCs (IRR: 3.85; 95%CI: 0.90-34.51; p=0.07) compared to HIV positives (IRR; 1.93; 95%CI: 0.88-4.57; p=0.04). Overall for TST, the IRR for HIV negatives (IRR: 2.21; 95%CI: 0.78-6.72; p=0.05) was similar to that among HIV positives (IRR: 2.32; 95%CI: 1.09-5.00; p=0.009).

Table 6.10: Overall incidence rates, univariable and multivariable analysis of time to TB episode, stratified by HIV status (nmax=1113) All TB cases (n=51).

	Total	TB cases/pyrs	Incidence rate/ 1000 pyrs (95% CI)	Crude HR (95% CI)	P value	[1]Adjusted HR (95% CI)	P value
HIV negatives							
Overall	**799**	**18/1741.4**	**10.34 (6.13-16.34)**				
QFT							
Negative	265	2/565.7	3.53 (0.88-14.14)	1		1	
Positive	534	16/1175.7	13.61 (8.34-22.21)	3.88 (0.89-6.92)	0.07	[2]N/A	P<0.001
TST (10 mm)							
Negative	480	7/ 1017.3	6.88 (3.28- 14.43	1		1	
Positive	319	11/ 724.1	15.19 (8.41-27.43)	2.21 (0.86-5.72)	0.10	3.02 (0.75-12.10)	0.12
HIV positives							
Overall	**297**	**32/655.0**	**48.86 (33.42-68.97)**				
QFT							
Negative	138	10/306.2	32.66 (17.57-60.70)	1		1	
Positive	159	22/348.8	63.08 (41.53-95.80)	1.93 (0.91-4.07)	0.08	4.72 (1.35-16.46)	0.01
TST (10 mm)							
Negative	198	15/440.2	34.07 (20.54-56.52)	1		1	
Positive	99	17/214.7	79.17 (49.22-127.36)	2.32 (1.16-4.65)	0.02	2.13 (0.81-5.60)	0.12

[1]Adjusted for age, sex, and country as effect modifier (p value for interaction=0.5) [2]HR very large because all HIV negative QFT-GIT negative cases in SA.

In multivariable analysis adjusted for age, sex and country as an effect modifier, the HR for developing TB in HIV negative QFT-GIT positive was very large and clinically insignificant (all incident cases in this group were from SA) while in HIV positives QFT-GIT positives, the HR was 4.72 (95%CI: 1.35-16.46; p=0.01). There was therefore strong evidence of a five-fold increased risk of TB in HIV positive QFT-GIT positive HHCs compared to HIV positive QFT-GIT negative ones (table 6.10).

For TST, in multivariable analysis adjusted for age, sex and country as an effect modifier, the HR in HIV negative TST positive HHCs was 3.02 (95% CI: 0.75-12.10; p=0.12) while it was 2.13 (95%CI: 0.81-5.60; p=0.12) in HIV positives TST positive.

Effect of test results on TB incidence by country, stratified by HIV status

Zambia

For HHCs residing in Zambia, the IRR was 5.12 (95%CI: 1.45-27.57; p=0.002) in HIV positives while it was 0 among HIV negative for QFT-GIT (no one developed TB among the HIV negative QFT-GIT negative group). For TST, the IRR among HIV negatives was higher (IRR: 3.03; 95%CI: 0.65-18.72; p=0.06) than that of HIV positives (IRR: 2.36; 95%CI: 0.83-6.71; p=0.04).

For QFT-GIT, in multivariable analysis adjusted for age and sex, there was strong evidence of a five-fold increased risk of TB in HIV positives (aHR: 4.77; 95%CI: 1.36-16.66; p=0.01), as in the overall analyses (table 6.10).

For TST, in multivariable analysis adjusted for age and sex, the HR in HIV negative HHCs was 3.02 (95% CI: 0.75-12.09; p=0.12) while it was 2.21 (95%CI: 0.84-5.85; p=0.11) in HIV positives (table 6.11).

Table 6.11: Zambia: Univariable and multivariable analysis of time to TB episode; stratified by HIV status (nmax=728) All TB cases (n=27).

Variable	Total	TB cases/pyrs	Incidence rate/ 1000 pyrs (95% CI)	Crude HR (95% CI)	P value	[2]Adjusted HR (95% CI)	P value
HIV negatives							
QFT							
Negative	187	0/383.6	0	1		1	
Positive	327	9/696.5	12.92 (6.72-24.83)	0 (0-0.92)		[2] N/A	
TST (10 mm)							
Negative	321	3/650.6	4.61 (1.49-14.30)	1		1	
Positive	193	6/429.5	13.97 (6.27-31.09)	3.06 (0.76-12.27)	0.11	3.02 (0.75-12.09)	0.12
HIV positives							
QFT							
Negative	103	3/226.8	13.23 (4.27-41.01)	1		1	
Positive	102	15/221.6	67.69 (40.80-112.27)	5.03 (1.46-17.38)	0.01	4.77 (1.36-16.66)	0.01
TST (10 mm)							
Negative	144	9/314.9	28.58 (14.87-54.92)	1		1	
Positive	61	9/133.5	67.43 (35.08-129.60)	2.36 (0.94-5.95)	0.07	2.21 (0.84-5.85)	0.11

[2] No one developed TB in the HIV negative QFT negative group. [2]HR very large as all HIV negative QFT-GIT negative cases in SA.

South Africa

For test positive HHCs residing in SA, the IRR was 0.62 (95%CI: 0.19-2.09; p=0.19) in HIV positives while it was 1.33 (95%CI: 0.25-13.12; p=0.39) among HIV negative for QFT-GIT. For TST, the IRR was 1.55 (0.33-7.84; p=0.26) among HIV negatives compared to 2.06 (0.62-7.19; p=0.09) among HIV positives. Crude HRs were similar to the IRR (**Table 6.12**).

For QFT-GIT, in multivariable analysis adjusted for age and sex, there was no evidence of an increased risk of TB in HIV negatives (aHR: 1.09; 95%CI: 0.22-5.38; p=0.91), while in HIV positives adjusted HR was 0.67 (95%CI: 0.23-1.96; P=0.47).

For TST, in multivariable analysis adjusted for age and sex, the HR in HIV negative HHCs was 1.46 (95% CI: 0.39-5.52; p=0.57) while it was 2.16 (95%CI: 0.74-6.32; p=0.16) in HIV positives (Table 6.12).

Table 6.12: South Africa: Univariable and multivariable analysis of time to TB episode; stratified by HIV status (nmax=385) All TB cases (n=24).

Variable	Total	TB cases/pyrs	Incidence rate/ 1000 pyrs (95% CI)	Crude HR (95% CI)	P value	[1]Adjusted HR (95% CI)	P value
HIV negatives							
QFT							
Negative	78	2/182.1	10.99 (2.75-43.93)	1		1	
Positive	207	7/479.2	14.61 (6.96-30.64)	1.35 (0.28-6.50)	0.71	1.09(0.22-5.38)	0.91
TST (10 mm)							
Negative	159	4/366.7	10.91 (4.09-29.06)	1		1	
Positive	126	5/294.6	16.97(7.06-40.78)	1.63 (0.44-6.09)	0.47	1.46 (0.39-5.52)	0.57
HIV positives							
QFT							
Negative	35	7/79.4	88.14 (42.02-184.88)	1		1	
Positive	57	7/127.1	55.05 (26.25-115-48)	0.64 (0.22-1.83)	0.41	0.67 (0.23-1.96)	0.47
TST (10 mm)							
Negative	54	6/125.3	47.88 (21.51-106.57)	1		1	
Positive	38	8/81.2	98.46 (49.24-196.89)	2.06 (0.71-5.94)	0.18	2.16 (0.74-6.32)	0.16

[1]Adjusted for sex and age

6.10 Positive and Negative Predictive Values

Overall, the PPV for QFT-GIT was 5.38% (95%CI: 3.84-7.31), this was slightly lower than that of TST, 6.57% (95% CI: 4.41- 9.36) even when results were stratified by country (**Table 6.13)** especially for SA. In addition, overall both tests had comparable NPV estimated at 97% (Table 6.13). Overall PPV and NPV by country are shown in table 6.13.

Table 6.13: Negative and Positive values by test and country

	Overall	Zambia	SA
QFT-GIT			
PPV % (95%CI)	5.38 (3.84-7.31)	5.50 (3.56-8.08)	5.19 (2.86-8.55)
NPV % (95%CI)	96.81 (94.60-98.29)	98.97 (97.03 -99.79)	91.30 (84.59-95.75)
TST			
PPV % (95%CI)	6.57 (4.41- 9.36)	5.84 (3.30-9.44)	7.69 (4.16-12.79)
NPV% (95%CI)	96.65 (95.02-97.87)	97.40 (95.59-98.68)	94.91 (91.07- 97.43)

Positive and negative predictive values, stratified by HIV status

Overall, among HIV negatives, the PPV and NPV of QFT-GIT was comparable to that of TST (**Table 6.14).**

Overall, in HIV positives, while the NPV of both tests remained comparable (but lower than that in HIV negatives), the PPV for QFT-GIT lower at 13.8% (95% CI: 8.88-29.22) compared to that of TST at 17.2% (95% CI: 10.33-26.06)(table 6.15). Results by country are shown in **Tables 6.14** and **6.15.**

Table 6.14: Negative and positive predictive values by test in HIV negative HHCs, overall and by country

	Overall	**Zambia**	**SA**
QFT-GIT			
PPV % (95%CI)	3.00 (1.72-4.82)	2.75 (1.27-5.16)	3.38 (1.37-6.84)
NPV % (95%CI)	99.25 (97.30-99.9)	100 (98.05-100.0)	97.44 (91.04-99.69)
TST			
PPV % (95%CI)	3.45 (1.73-6.09)	3.11(1.15-6.64)	3.97 (1.30- 9.02)
NPV% (95%CI)	98.54 (97.02-99.41)	99.07 (97.29- 99.81)	97.48 (93.68-99.31)

Table 6.15: Negative and Positive predictive values by test in HIV positive HHCs, overall and by country

	Overall	**Zambia**	**SA**
QFT-GIT			
PPV % (95%CI)	13.84 (8.88-20.22)	14.71 (8.47-23.09)	12.3 (5.08-23.68)
NPV % (95%CI)	92.75 (87.08-96.47)	97.1 (91.72-99.40)	80.0 (63.06-91.56)
TST			
PPV % (95%CI)	17.17 (10.33-26.06)	14.75 (6.98-26.17)	21.05 (9.55-37.32)
NPV% (95%CI)	92.42 (87.81- 95.70)	93.75(88.47-97.10)	88.89 (77.37- 95.81)

6.11 Sensitivity Analysis for Incident TB

Previously in this chapter, incident TB was defined as self-reported TB confirmed in the clinic TB register. In this section a sensitivity analysis aimed at exploring other definitions of incident TB is presented. The sensitivity analysis is for overall estimates. Three definitions were used to define incident TB as follows:

- Self-reported TB confirmed in the TB treatment register but excluding co-prevalent TB cases.
- Bacteriologically confirmed TB (presence of one or more sputum smears positive for acid fast bacilli).
- Bacteriologically confirmed TB **plus** one or more positive cultures for *M.tuberculosis* **plus** positive on MPB64 antigen test.

6.11.1 Self-reported TB confirmed in the TB treatment register but excluding co-prevalent TB cases

Out of a total of 51 incident cases, 6 were defined as co-prevalent TB cases, leaving 45 incident cases. Some selected characteristics of the 6 co-prevalent cases were as follows: 4/6 (66.7%) were female; 4/6 (66.7%) were HIV positive; 3/5 (60.0%) were smear positive and all 6 lived in urban areas in Zambia. There were no differences between co-prevalent cases and incident cases with regard to the following characteristics: sex (p=0.66); age (p=0.35); socioeconomic status (p=0.62); education (p=0.65); smoking (p=0.46); Alcohol (p=0.65); HIV (p=0.63); smear status (p=0.61) and residence (p=0.44).

Excluding co-prevalent cases, the overall incidence rate was 18.50 (95%CI: 13.81-24.77) per 1000 pyrs. For both tests, TB IR was higher among test positive HHCs compared to test negative ones (IRR for QFT-GIT 1.55; 95% CI: 0.78-3.30; p=0.09 and for TST: 1.76; 95%CI: 0.94-3.33, p=0.03).

Overall for QFT-GIT, the IRR was higher among HIV negative HHCs (IRR: 3.37; 95%CI: 0.77-30.53; p=0.04) compared to HIV positives (IRR; 1.85; 95%CI: 0.80-4.65; p=0.06). These results were similar as for the main analysis. For TST, the IRR for HIV negatives (IRR: 1.81; 95%CI: 0.60-5.71; p=0.12) was similar as for HIV positives (IRR: 2.37; 95%CI: 1.05-5.41; p=0.01).

Crude analysis showed similar HRs for developing TB for QFT-GIT (HR: 1.56; 95% CI: 0.80-3.02; p=0.19) and TST (HR: 1.72; 95%CI: 0.95-3.12; p=0.07) as for the main analysis. In multivariable analysis adjusted for sex, age, HIV status, and country as effect modifier (p for interaction=0.09), adjusted HR for QFT-GIT was 7.79 (95%CI: 1.81-33.55; p=0.006) while that for TST was 2.13 (95%CI: 0.90-5.05, p=0.08) (table 6.16).

Table 6.16: Univariable and multivariable analysis; TB defined excluding co-prevalent TB (nmax=45) All TB cases (n=45).

Variable	n (col %)	TB cases/pyrs	Incidence rate/ 1000 pyrs (95% CI)	Crude HR (95% CI)	P value	[2]Adjusted HR (95% CI)	P value
Overall	N=1107	45/2433	18.50 (13.81-24.77)				
QFT							
Negative	406	12/877.9	13.67 (7.76-24.07)	1		1	
Positive	701	33/1554.9	21.22 (15.09-29.85)	1.56 (0.80-3.02)	0.19	7.79 (1.81-33.55)	0.006
TST (10 mm)							
Negative	685	21/1476	14.23 (9.28-21.82)	1		1	
Positive	422	24/956.9	25.08 (16.81-37.42)	1.77 (0.98-3.17)	0.06	2.13 (0.90-5.05)	0.08

[2]Adjusted for sex, age, HIV status, and country as effect modifier (p=0.09)

6.11.2 Bacteriologically confirmed TB (presence of one or more sputum smears positive for acid fast bacilli).

Out of the 51 incident cases, 34 (66.67%) had smear status recorded and 17 (33.33%) had missing results. Of the 34 incident cases with smear status recorded, 52.94 (18/34) were smear positive while 47.06% (16/34) were smear negative.

TB incidence rates were higher among test positive HHCs compared to those with negative results for both QFT (IRR: 1.97; 95% CI 0.62-8.24; p=0.11) and TST (IRR: 2.42; 95% CI: 0.86-7.37; p=0.03) (table 6.17).

For QFT-GIT, the IRR was higher among HIV negative HHCs (IRR: 4.33; 95%CI: 0.60-189.79; p=0.07) compared to HIV positives (IRR: 2.19; 95%CI: 0.36-23.05; p=0.18). These results were similar as for the main analysis. For TST, the IRR among HIV negatives was 5.62 (95%CI: 1.12-54.32; p=0.009) compared to HIV positives at 1.54 (95%CI: 0.22-9.09; p=0.29).

Crude analysis showed slightly lower HR for developing TB for QFT-GIT (HR: 2.00; 95% CI: 0.66-6.09; p=0.22) than for TST (HR: 2.49; 95%CI: 0.96-6.42; p=0.06). In multivariable analysis adjusted for sex, age, HIV status, and country as effect modifier, adjusted HR for QFT-GIT was 7.69 (95%CI: 0.98-60.43; p=0.05) while that for TST it was 3.40 (95%CI: 0.99-11.70; p=0.05) (table 6.17).

Table 6.17: Univariable and multivariable analysis; TB defined by smear (nmax=34) All TB cases (n=18).

Variable	n (col %)	TB cases/pyrs	Incidence rate/ 1000 pyrs (95% CI)	Crude HR (95% CI)	P value	[1]Adjusted HR (95% CI)	P value
Overall	N=34	18/2433	7.40 (4.66-11.74)				
QFT							
Negative	9	4/877.9	4.56 (1.71-12.14	1		1	
Positive	25	14/1555.3	9.00 (5.33-15.20	2.00 (0.66-6.09)	0.22	7.69 (0.98-60.43)	0.05
TST (10 mm)							
Negative	15	7/1476.0	4.74 (2.26-9.95)	1		1	
Positive	19	11/957.2	11.49 (6.36-20.75)	2.49 (0.96-6.42)	0.06	3.40 (0.99-11.70)	0.05

[1]Adjusted for sex, age, HIV status and country as effect modifier (p=0.59)

6.11.3 Bacteriologically confirmed TB plus one or more positive cultures for *M.tuberculosis* plus positive on MPB64 antigen test.

Using this **strict** criterion, overall there were 8 incident cases of TB that occurred over 1653.4 pyrs, giving an IR of 4.84 (95%CI: 2.42-9.67) per 1000 pyrs. *Due to the small number of incident cases these results were unreliable and further analysis was not possible*. However briefly, TB incidence rate was 3.23 (95%CI: 0.81-12.92) (2 cases/618.9 pyrs) and 5.80 (95%CI: 2.60-12.91) (6 cases/1034.6 pyrs) per 1000 pyrs among those with negative and positive QFT-GIT results respectively. For QFT-GIT the IRR was 1.79 (95%CI: 0.32-18.18; p=0.25). For TST, TB incidence rate was 4.87 (95%CI: 2.02-11.69) (5 cases/1027.4 pyrs) and 4.79 (95%CI: 1.54-14.86) (3 cases/626 pyrs) per 1000 pyrs among those with negative and positive results respectively. For TST the IRR was 0.98 (95%CI: 0.15-5.06; p=0.5).

6.12 Summary of incidence rate ratios and adjusted HR for the main and sensitivity analyses stratified by country

IRRs are summarized in **Table 6.18**. Overall for QFT-GIT, the IRR ranged from 1.55 to 1.97 with TB defined using smear having the highest estimates. Overall for TST, the IRR ranged from 0.98 to 2.42 with TB defined using smear having the highest estimates. However, for all definitions for both tests, confidence intervals were overlapping. For HHCs residing in Zambia, there was strong evidence when QFT-GIT was used that the IRR ranged from 5.24 to 6.56 for all definitions. Similarly for TST, there was some moderate to strong evidence that IRR ranged from 1.89 to 3.00 for all definitions. For HHCs residing in SA, IRR were similar across all definitions for both tests.

A summary of adjusted HR for overall estimates and stratified by country is also presented in **table 6.19.**

Table 6.18: Summary of incidence rate ratios and 95% CI for the main and sensitivity analyses, overall and stratified by country

Variable	Main analysis	P value	Exclude Co-prevalent TB[1]	P value	Smear	P value
Overall						
QFT (>=0.35 IU/ml)						
Negative	1		1		1	
Positive	1.65 (0.86-3.37)	0.06	1.55 (0.78-3.30)	0.09	1.97 (0.62-8.24)	0.11
TST (10 mm)						
Negative	1		1			
Positive	1.88 (1.04-3.41)	0.01	1.76 (0.94-3.33)	0.03	2.42 (0.86-7.37)	0.03
Zambia						
QFT (>=0.35 IU/ml)						
Negative	1		1		1	
Positive	5.24 (1.59-27.21)	0.0007	6.23 (1.50-55.17)	<0.001	6.56 (0.93-284.56)	0.02
TST (10 mm)						
Negative	1		1		1	
Positive	2.14 (0.94-5.02)	0.02	1.89 (0.73-4.96)	0.08	3.00 (0.76-14.00)	0.04
South Africa						
QFT (>=0.35 IU/ml)						
Negative	1		1		1	
Positive	0.60 (0.25-1.50)	0.11	0.60 (0.25-1.50)	0.11	0.57 (0.10-3.88)	0.24
TST (10 mm)						
Negative	1		1		1	
Positive	1.52 (0.63-3.74)	0.15	1.52 (0.63-3.74)	0.15	1.71 (0.29-11.70)	0.25

[1]In the main analysis, TB is defined as self-reported TB confirmed in the clinic register. [2]All co-prevalent cases were residents in Zambia so results remained unchanged for SA.

Table 6.19: Summary of adjusted HR and 95% CI for the main and sensitivity analyses

	1Main analysis	P value	Exclude Co-prevalent TB[1]	P value	Smear	P value
Overall						
QFT (>=0.35 IU/ml)						
Negative	1		1		1	
Positive	6.38 (1.90-21.30)	0.003	7.79 (1.81-33.55)	0.006	7.69 (0.98-60.43)	0.05
TST (10 mm)						
Negative	1		1		1	
Positive	2.32 (1.07-5.04)	0.03	2.13 (0.90-5.05)	0.08	3.40 (0.99-11.70)	0.05

[1]In the main analysis, TB is defined as self-reported TB confirmed in the patient treatment card.; adjusted for sex, age, HIV status and country as an effect modifier

Summary

From a total of 1,789 HHCs seen at baseline, 1,113 (62.2%) HHCs entered follow-up and were included in the main analysis. Overall, the demographic and clinical characteristics of those who completed the study did not differ significantly to those who refused, were lost to follow-up or had missing data.

The overall incidence rate of TB was 20.96 cases (95% CI: 15.93-27.58) per 1000 pyrs. TB incidence rate was higher among test positive HHCs compared to those who were negative (IRR for QFT: 1.65; 95% CI: 0.86-3.37; p=0.06) and for TST (IRR: 1.88; 95%CI: 1.04-3.41; p=0.01).

The IR was highest among HHCs with concordant positive results compared to those with concordant negative results (IRR: 2.24, 95% CI: 1.04-5.23; p=0.01) (table 6.3). As expected IRR was highest when both tests were positive, 2.24 (95%CI: 1.04-5.23) compared to discordant or concordant negative results.

HIV was an important confounder in the association between QFT-GIT positivity and TB incidence while age and sex were considered as priori confounders. Country was considered as an important effect modifier (p=0.001). In univariable analysis, the HR showing the association between QFT-GIT positivity and risk of TB was 1.66 (95%CI: 0.88-3.11; P=0.01). In multivariable analysis adjusted for sex, age, HIV plus country as an effect modifier the HR for QFT-GIT was 6.38 (95%CI: 1.90-21.30; p=0.003) while that for TST was 2.32 (1.07-5.04; p=0.03).

For Zambia for QFT-GIT, the IRR among QFT-GIT positive HHCs was 5.24; (95% CI: 1.59-27.21; p=0.0007) compared to 2.14 (95% CI: 0.94-5.02) among TST positive HHCs. In SA the IRR among HHCs with QFT-GIT positive results was 0.60; (95% CI: 0.25-1.50; p=0.11) compared to 1.52 (95% CI: 0.63-3.74) among TST positive HHCs.

Overall for QFT-GIT, the IRR was higher among HIV negative HHCs (IRR: 3.85; 95%CI: 0.90-34.51; p=0.07) compared to HIV positives (IRR; 1.93; 95%CI: 0.88-4.57; p=0.04). Overall for TST, the IRR for HIV negatives (IRR: 2.21; 95%CI: 0.78-6.72; p=0.05) was similar to that among HIV positives (IRR: 2.32; 95%CI: 1.09-5.00; p=0.009). In multivariable analysis, there was therefore strong evidence of a five-fold increased risk of TB in HIV positive QFT-GIT positive HHCs compared to HIV positive QFT-GIT negative ones (HR : 4.72; 95%CI: 1.35-16.46; P=0.01). For TST, in multivariable analysis adjusted for age, sex and country as an effect modifier, the HR in HIV negative TST positive HHCs was 3.02 (95% CI: 0.75-12.10; p=0.12) while it was 2.13 (95%CI: 0.81-5.60; p=0.12) in HIV positives TST positive.

Overall, among HIV negatives, the PPV and NPV of QFT-GIT was comparable to that of TST (NPV range 98.5%-99.2% and PPV of approximately 3.00% for both tests) although the confidence intervals for PPV were overlapping. Overall, in HIV positives, the NPV of both tests remained comparable but the PPV for QFT-GIT was slightly lower at 13.8% (95% CI: 8.88-29.22) compared to that of TST at 17.2% (95% CI: 10.33-26.06).

In a sensitivity analysis, for QFT-GIT, there was strong evidence that the adjusted HR was as reported for the main analysis (aHR: 6.38; 95%CI: 1.90-21.30; p=0.003). HRs were similar when TB was defined excluding co-prevalent cases and smear although the confidence interval was overlapping for smear. Overall for TST, all definitions provided some evidence that the adjusted HR ranged from 2.32 in the main analysis to 3.40 when defined by smear. Sensitivity analysis results where TB was defined as the presence of one or more positive cultures for *Mtb* that were also positive on ZN staining and MPB64 antigen test were unreliable due to the small number of incident cases.

Risk factors for TB were also explored. In unadjusted analysis , there was strong evidence that 26-39 years (HR:2.31; 95%CI: 1.18-4.52; p=0.04); HIV positivity (HR: 4.68; 95%CI: 2.62-8.35, p<0.001); smear status of the index (HR: 1.74; 95%CI: 0.92-3.28; p=0.09); sleeping in the same room and same bed as index (HR:1.88 ; 95%CI:0.64-5.53; p=0.02) and country (HR: 1.55; 95%CI:0.90-2.67; p=0.04) were associated with an increased risk of TB.

In multivariable analyses, adjusted for age, sex , HIV status and country there was strong evidence that occasional smoking, (HR: 4.07; 95%CI:1.31-12.63), HIV positivity (HR: 4.60; 95%CI:2.48-8.56), smear positivity of the index (HR: 2.00 ; 95%CI:1.04-3.87) and country (HR: 1.79 ; 95%1.02-3.15; p=0.04)) were associated with incidence of TB.

7.0 Are household contacts with high levels of IFN-γ in response to *Mtb* specific antigen in the QFT-GIT assay at higher risk of developing TB?

Introduction

In this chapter I will answer the main research question of the thesis: Are HHCs with high levels (≥10 IU/ml) of IFN-γ in response to *Mtb* specific antigens (ESAT-6, CFP-10 or TB 7.7) in the QFT-GIT assay at higher risk of developing TB compared to those with low levels (≥ 0.35 <10 IU/ml)?

Specific objectives of this chapter are:

1. To determine the incidence rates of TB in HHCs with low and high IFN-γ levels in the QFT-GIT assay, also stratified by country and HIV status.
2. To determine the association between high IFN-γ levels in the QFT-GIT assay and the risk of developing TB?

7.1 The cohort for this analysis

Out of the 1,113 HHCs who entered follow-up, 406 HHCs had IFN-γ levels <0.35 IU/ml at baseline and were therefore excluded from analysis. Therefore 707 HHCs were present for analysis in this chapter. Out of these 536 (75.8%) had IFN-gamma levels ≥ 0.35 and <10 IU/ml (low IFN-γ levels) while 171 (24.2%) HHCs had ≥ 10 IU/ml (high IFN-γ levels).

7.2 Socio-demographic and clinical characteristics of HHCs with low versus high IFN-γ levels

Of the 171 HHCs, 74.8% were female, median age was 28 years (IQR:21-45), 58.8% had attended secondary school or higher education, 88.9% had no history of smoking and 54.7% had lived with a smear positive index case (**Table 7.1.**).

There was no difference between HHCs with low and high IFN-γ levels with regard to the following characteristics: sex (p=0.55), age (p=0.37), level of education (p=0.93), socioeconomic status (p=0.99), smoking habits (p=0.20), alcohol consumption (p=0.90), smear status of the index (p=0.73), proximity to the index case (p=0.28).

However, there were differences between HHCs with low and high IFN-γ levels with respect to the following characteristics: HIV status (p=0.04), TST results at the 10 mm cut-off (p=0.02) and country (p=0.01) (table 7.1).

17.1% of HHCs with high IFN-γ levels were HIV positive compared to 24.8% with low levels. A greater proportion of HHCs with high levels (70.2%) resided in Zambia than with low levels (59.0%) (Table 7.1).

Table 7.1: Socio-demographic and clinical characteristics of HHCs with low versus high IFN-γ levels

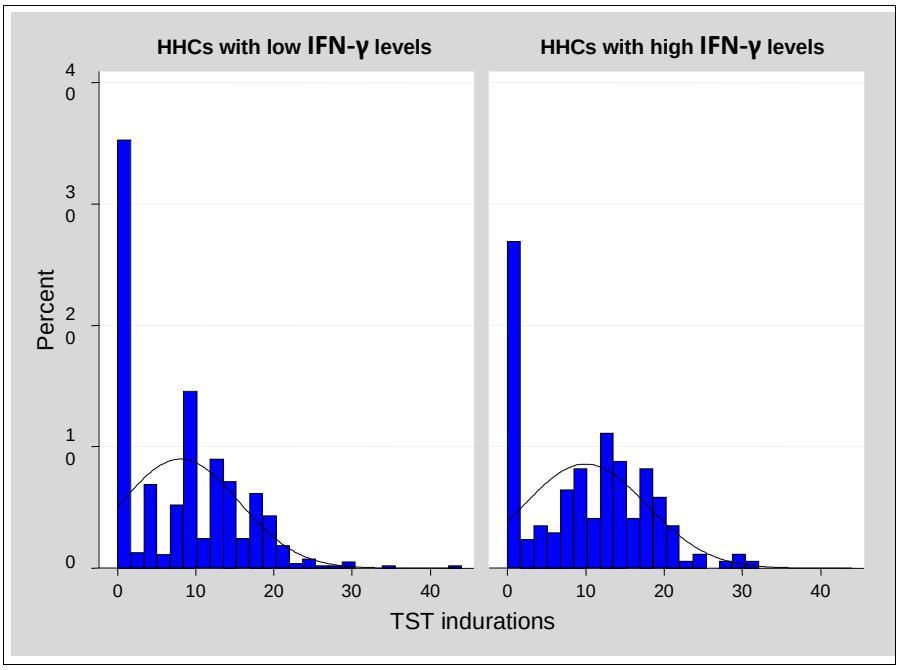
	Low IFN-γ levels	High IFN-γ levels	p value (chi-square)*
Total	**536 (column %)**	**171 (column %)**	
Sex			
Male	147 (27.5)	43 (25.1)	0.55
Female	388 (72.5)	128 (74.8)	
Missing	1	0	
Age group (years)			
15-25	198 (37.1)	73 (43.2)	0.37
26-39	149 (27.9)	42 (24.8)	
40-86	186 (34.9)	54 (31.9)	
Median age (IQR)	31 (22; 47)	28 (21; 45)	
Highest level of education			
Primary school and below	217 (41.6)	70 (41.2)	0.93
Secondary school and above	305 (58.4)	100 (58.8)	
Missing	14	1	
Socioeconomic status			
Unemployed & dependent	478 (90)	154 (90.1)	
Employed	53 (10.0)	17 (9.9)	0.99
Missing	5	0	
Smoking habits			
Never smoked or ex-smoker	453(85.0)	152 (88.9)	
Smoker (occasional or daily)	80 (15.0)	19 (11.1)	0.20
Missing	3	0	
Alcohol consumption			
No	393 (74.1)	126 (73.7)	0.90
Yes	137 (25.8)	45 (26.3)	
Missing	6	0	
HIV status			
Negative	394 (75.2)	141 (82.9)	0.04
Positive	130 (24.8)	29 (17.1)	
Missing	12	1	
TST results (10 mm)			
Negative	290 (54.1)	75 (43.9)	0.02
Positive	246 (45.9)	96 (56.1)	
Smear status of the index			
Smear negative	180 (47.1)	53 (45.3)	0.73
Smear positive	202 (52.9)	64 (54.7)	
Missing		54	
Sleeping proximity to index			
Different house	38 (9.9)	19 (14.7)	0.28
Same house	232 (60.7)	71 (55.0)	
Same room and same bed	112 (29.3)	39 (30.2)	
Missing	154	42	
Residence			
Rural	75 (14.0)	26 (15.2)	
Urban	461 (86.0)	145 (84.4)	0.69
Country			
Zambia	316 (59.0)	120 (70.2)	0.01
South Africa	220 (41.0)	51 (29.8)	

Fisher's exact used when the expected numbers were less than 5.

Using TST at the 10 mm cut-off, 56.1% (96/171) of HHCs with high IFN-γ levels and 246 (45.9%) of those with low levels were TST positive (table 7.1).

TST responses (TST>0 mm) were higher in HHCs with high IFN-γ levels (mean: 9.9 mm; standard deviation (SD): 7.9 mm) compared to those with low IFN-γ levels (mean: 6.2 mm; SD: 7.1 mm) (figure 7.1). In addition, 26.31% (45/171) of HHCs with high IFN-γ levels had TST indurations of 0 mm compared to 34.14 % (183/536) with low IFN-γ levels.

Figure 7.1: Distribution of TST responses in low and high IFN-γ levels



7.3 Overall TB incidence rates

Out of the 707 HHCs that entered follow-up, 38 (5.4%) HHCs developed active TB over 1558.0 person-years (pyrs) of follow-up, giving an incidence rate of 24.39 (95%CI: 17.75-33.52) per 1000 pyrs. The median follow-up time was 2.44 years (IQR: 1.59-2.82).

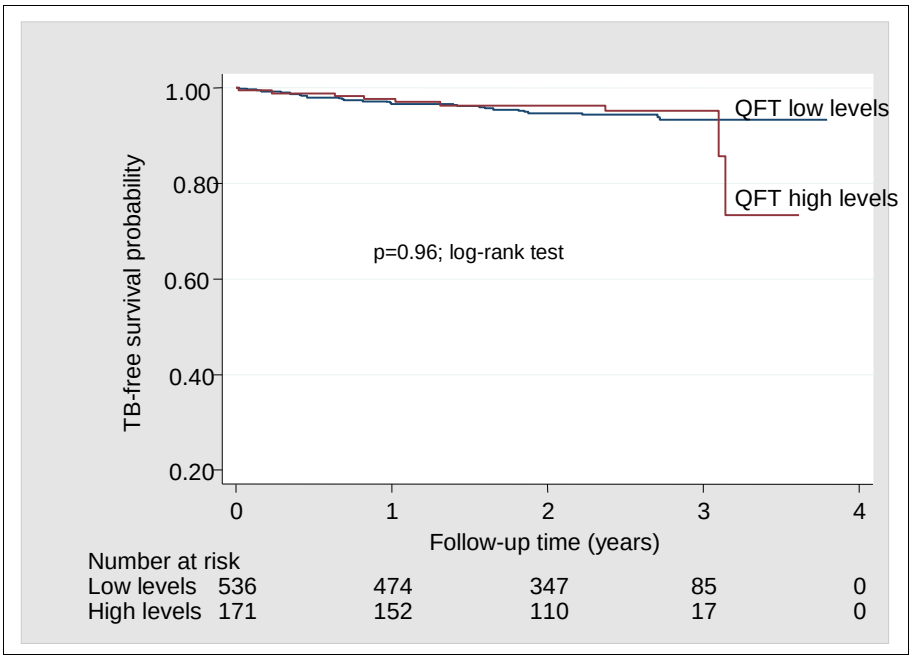
TB incidence rates were 24.51/1000 pyrs (9 cases/367.2 pyrs) in HHCs with high levels and 24.35 (29 cases/1190.7 pyrs) among those with low levels of IFN-γ, giving an IRR of 1.0 (95% CI: 0.42-2.18). (p=0.48).

7.4 Kaplan Meier TB-free survival probability for low and high levels

Kaplan Meier curves showing TB-free survival probabilities in HHCs with high and low IFN-γ levels are shown in **Figure 7.2**. Overall, the two-year (actual last observed exit was at 3.8 years) TB-free survival probability at 0.96 (95%CI: 0.91-0.98) among HHCs with high IFN-γ levels was

similar to those with low IFN-γ levels at 0.95 (95% CI: 0.92-0.96) (figure 7.2). There was no evidence that survival curves differed between those with low and high IFN-γ levels for unadjusted analysis (log rank test, p=0.96) (**figure 7.2**).

Figure 7.2: Kaplan Meier survival curves for high and low IFN-γ levels

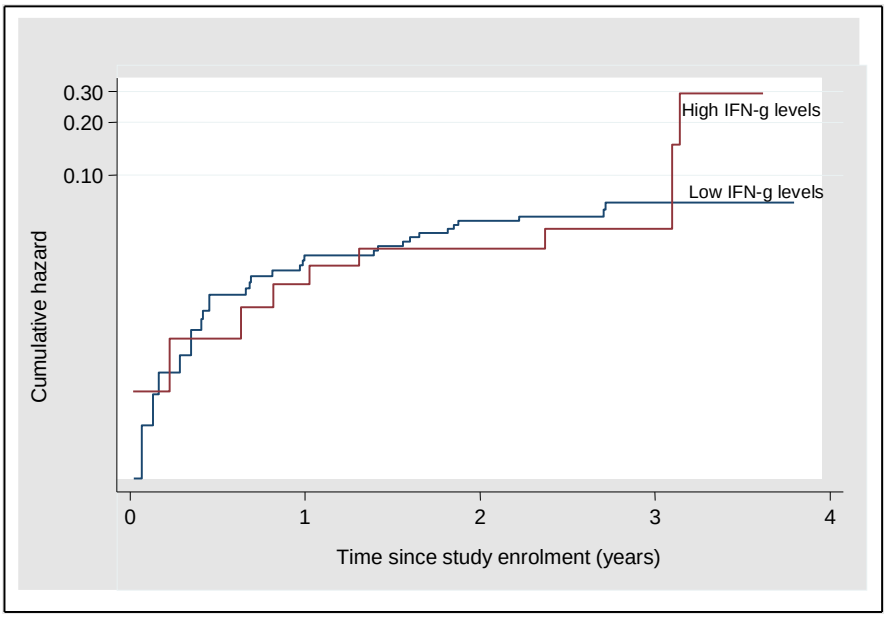


Note: Kaplan-Meier curves: survival curves ends in a plateau; represents some HHC without disease after the study's maximum follow-up time

7.5 The Proportional Hazard Assumption in the Cox model

Figure 7.3 shows the cumulative hazard against time since study enrolment for HHCs with low and high levels. It suggests that there that the proportional hazard assumption was violated since the lines appear not to be reasonably parallel. Mathematically, this assumption was tested by adding interaction terms to the proportional hazards model, the result of the interaction test suggested no evidence against proportionality (LRT: p=0.60).

Figure 7.3: Nelson-Aalen Cumulative hazard against time for HHCs with low and high levels, in order to check for the proportional hazards assumption



7.6 Risk factors for the development of TB

Incidence rates, unadjusted and adjusted HRs of risk factors for incident TB for the primary objective is shown in **Table 7.2.**

In unadjusted analysis, age group 26-39 years (HR: 1.83; 95%CI: 0.83-4.03; P=0.30); smoking (HR: 1.93; 95%CI: 0.91-4.08; p=0.09), HIV positivity (HR: 4.55; 95%CI: 2.38-8.68, p<0.001); sleeping in the same room and same bed as index (HR:2.01 ; 95%CI:0.58-6.97; p=0.05) and being in an urban area (HR:2.76; 95%CI:0.66-11.47; p=0.16) were positively associated with an increased risk of TB (table 7.2).

Higher education was negatively associated with risk for TB (HR: 0.52; 95%CI: 0.27-0.99; p=0.05) (table 7.2).

Table 7.2: TB incidence rates and hazard ratios stratified by baseline socio-demographic and clinical characteristics

	N (col %) N=707	TB cases/pyrs	Incidence rate/ 1000 pyrs (95% CI)	Crude HR (95% CI)	P value	aHR (95% CI)	P value
Sex							
Male	190 (26.91%)	10/414.6	24.11 (12.98-44.83	1		1	
Female	516 (73.06%)	28/1140.3	24.55 (16.95-35.56	1.03 (0.50-2.12)	0.94	0.84 (0.40-1.76)	0.64
Age group (years)							
15-25	271 (38.60	11/589.7	18.65 (10.33-33.68	1		1	
26-39	191 (27.21	14/410.4	34.11 (20.20-57.59	1.83 (0.83-4.03)		1.19 (0.53-2.67)	0.007
40-87	240 (34.19	12/544.3	22.05 (12.52-38.82	1.19 (0.52-2.69)	0.30	1.29 (0.57-2.93)	
Highest level of education							
Primary school and below	287 (41.47	22/633.0	34.75 (22.88-52.78	1		1	
Secondary school and above	405 (58.53	16/891.0	17.96 (11.00-29.31	0.52 (0.27-0.99)	0.05	0.54 (0.28-1.03)	0.06
Socioeconomic status							
Unemployed & dependent	632 (90.03	34/1387.8	24.50 (17.50-34.29	1		1	
Employed	70 (9.97	4/156.8	25.50 (9.57-67.95	1.07 (0.38-3.02)	0.89	0.94 (0.28-3.13)	0.92
Smoking habits							
Never smoked or ex-smoker	605 (85.94)	29/1341.2	21.62 (15.02-31.11	1		1	
Smoker (occasional or daily)	99 (14.06)	9/210	42.86 (22.30-82.37	1.93 (0.91-4.08)	0.09	2.27 (1.04-4.94)	0.04
Alcohol consumption							
No	519 (74.04)	26/1152.6	22.56 (15.36-33.13	1		1	
Yes	182 (25.96	12/392.4	30.59 (17.37-53.85	1.35 (0.68-2.68)	0.39	1.28 (0.64-2.56)	0.49
HIV status							
Negative	535 (77.09)	16/1178.4	13.58 (8.32-22.16	1		1	
Positive	159 (22.91	22/348.8	63.09 (41.53-95.80	4.55 (2.38-8.68)	P<0.001	4.30 (2.15-8.60)	P<0.001
Smear status of the index							
Smear negative	233 (46.69	13/513.1	25.33 (14.71-43.63	1		1	
Smear positive	266 (53.31	18/570.5	31.55 (19.88-50.08	1.23 (0.60-2.51)	0.57	1.38 (0.66-2.90)	0.39
Sleeping proximity to index							
Different house	57 (11.15	3/128.3	23.38 (7.54-72.49	1		1	
Same house	303 (59.30	12/645.6	18.59 (10.55-32.73	0.80 (0.22-2.83)		0.61 (0.16-2.27)	
Same room and same bed	151 (29.55	15/318.6	47.08 (28.38-78.10	2.01 (0.58-6.97)	0.05	1.13 (0.31-4.07)	0.0007
Residence							
Rural	101 (14.29	2/206.6	9.68 (2.42-38.71	1		1	
Urban	606 (85.71	36/1351.4	26.64 (19.21-36.93	2.76 (0.66-11.47)	0.16	2.22 (0.52-9.38)	0.28
Country							
Zambia	436 (61.67	24/936.2	25.64 (17.18-38.25	1		1	
South Africa	271 (38.33	14/621.8	22.52 (13.33-38.02	0.87 (0.45-1.68)	0.68	0.98 (0.50-1.90)	0.95

P value from likelihood test if > 2 sub-groups. Missing: sex-1; age-5; education-15; socioeconomic-5; smoking habits-1; taken alcohol-6; HIV status-13; index smear-208; sleeping proximity to index-196. [1]Adjusted HR: adjusted for age, HIV and country .

In multivariable analyses, adjusted for age, HIV status and country there was strong evidence that smoking, (HR: 2.27; 95%CI: 1.04-4.94; p=0.04) and HIV positivity (HR: 4.30; 95%CI: 2.15-8.60; p<0.001), were associated with increased risk of TB (table 7.2).

Data were consistent with no association of sex, education, socioeconomic status, alcohol use, and smear status with increased risk of TB in both univariable and multivariable analysis (table 7.2).

7.7 Incidence rates, stratified by country

In Zambia, out of the 436 HHCs that entered follow-up, 24 (5.5%) HHCs developed active TB over 936.16 pyrs of follow-up, giving an incidence rate of 25.64/1000 pyrs (table 7.3). The median follow-up time was 2.46 years (IQR: 1.32-2.77).

Table 7.3: Incidence rates for Zambia; All TB cases (N=24).

Variable	N (col %)	TB cases/pyrs	Incidence rate/ 1000 pyrs (95% CI)	Incidence rate ratio (95%CI)	P value
Overall	**N=436**	**24/936.16**	**25.64 (17.18-38.24)**		
Low IFN-γ levels	316	18/688.5	26.14 (16.47-41.49)	1	
High IFN-γ levels	120	6/247.6	24.23 (10.88-53.93)	0.93 (0.30-2.44)	0.45

In SA, out of the 271 HHCs that entered follow-up, 14 (5.2%) HHCs developed active TB over 621.79 pyrs of follow-up, giving an incidence rate of 22.52/1000 pyrs (**Table 7.4**). The median follow-up time was 2.39 years (IQR: 1.76-2.91).

Table 7.4: Incidence rates for South Africa; All TB cases (N=14).

Variable	N (col %)	TB cases/pyrs	Incidence rate/ 1000 pyrs (95% CI)	Incidence rate ratio (95%CI)	P value
Overall	**N=271**	**14/621.79**	**22.52 (13.33-38.02)**		
Low IFN-γ levels	220	11/502.2	21.90 (12.13-39.55)	1	
High IFN-γ levels	51	3/119.6	25.08 (8.09-77.78)	1.14 (0.20-4.33)	0.40

By country, the IRR for those residing in Zambia was 0.93 (95%CI: 0.30-2.44; p=0.45) and it was 1.14 (95%CI: 0.20-4.33; p=0.40) for those residing in SA. Hence there was no difference in incidence rates between those with low and high levels, overall and by country.

7.8 Incidence rates, stratified by HIV status

HIV Negatives

Out of the 535 HIV negative HHCs that entered follow-up, 16 (3.0%) HHCs developed active TB over 1178 pyrs of follow-up, giving an overall incidence rate of 13.58/1000 pyrs. The median follow-up time was 2.44 years (IQR: 1.59-2.81).

TB incidence rates were 19.56/1000 pyrs (6 cases/306.7 pyrs) in HHCs with high levels and 11.47 (10 cases/871.7 pyrs) among those with low levels of IFN-γ, giving an IRR of 1.70 (95%CI: 0.51-5.18) **(Table 7.5**). However, results were not statistically significant.

Table 7.5: Overall incidence rates, stratified by HIV status; All TB cases (N=38).

Variable	N (col %)	TB cases/pyrs	Incidence rate/ 1000 pyrs (95% CI)	Incidence rate ratio (95%CI)	P value
HIV negatives	**N=535**	**16/1178**	**13.58 (8.32-22.16)**		
Low IFN-γ levels	394 (73.6)	10/871.7	11.47 (6.17-21.32)	1	
High IFN-γ levels	141 (26.3)	6/306.7	19.56 (8.79-43.54)	1.70 (0.51-5.18)	0.16
HIV positives	**N=159**	**22/348.8**	**63.08 (41.54-95.80)**		
Low IFN-γ levels	130 (81.8)	19/291.0	65.29 (41.65-102.36)	1	
High IFN-γ levels	29 (18.2)	3/57.8	51.94 (16.75-161.04)	0.79 (0.15-2.70)	0.38

*13 HHCs with missing HIV test results; col=column

HIV positives

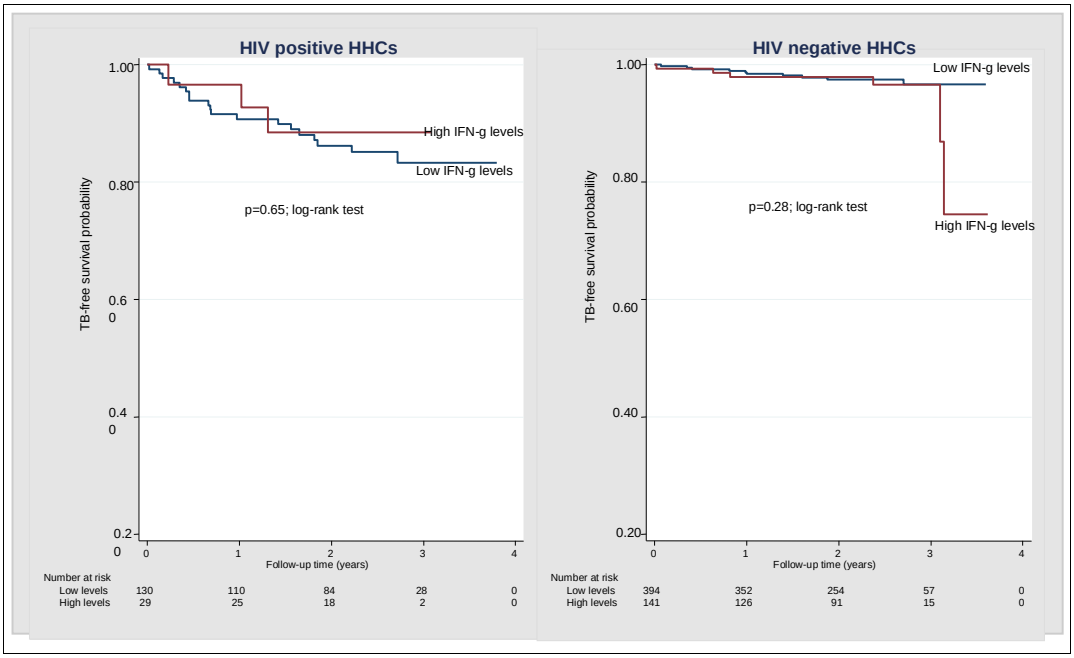
Out of the 159 HIV positive HHCs that entered follow-up, 22 (13.8%) HHCs developed active TB over 348.8 pyrs of follow-up, giving an overall incidence rate of 63.08/1000 pyrs. The median follow-up time was 2.43 years (IQR: 1.56-2.87).

TB incidence rates in HIV positives was 51.94/1000 pyrs (3 cases/57.8 pyrs) in HHCs with high levels and 65.29/1000 pyrs (19 cases/291.0 pyrs) among those with low levels of IFN-γ, giving an IRR of 0.79 (95%CI: 0.15-2.70; p=0.38) (table 7.3). However, results were not statistically significant.

7.9 TB-free survival probability for low and high levels, stratified by HIV status

Kaplan Meier curves showing TB-free survival probabilities according to low/high levels and stratified by HIV are shown in **Figure 7.4**. In HIV negatives, the 2-year (actual last observed exit was at 3.6 years) TB-free survival probability was 0.98 (95%CI: 0.93-0.99) in those with high IFN-γ levels and similar to those with low IFN-γ levels at 0.97 (95%CI: 0.95-0.99). In HIV positives, the 2-year (actual last observed exit was at 3.8 years) TB-free survival probability was 0.88 (95%CI: 0.67-0.96) in those with high IFN-γ levels compared to those with low levels at 0.86 (95%CI: 0.78-0.91) (Figure 7.4).

Figure 7.4: Kaplan Meier curves of TB-free survival probability according to IFN-γ levels and stratified by HIV



In HIV negatives, there was no evidence (log-rank test; p=0.28) that the survival curves differed between HHCs with low and high IFN-γ levels (figure 7.4). Similarly, in HIV positives, there was no evidence that survival curves differed between HHCs with low and high IFN-γ levels (log-rank test; p=0.65)(Figure 7.4).

7.10 Confounders and effect modifiers for the association between high IFN-γ levels and TB incidence

Identification of confounders

Age and sex were considered as priori confounders in the association between high levels and risk of developing TB. Country was identified as important confounder since it was associated with both incidence of TB and high levels and was not in the causal pathway.

Identification of effect modifiers using likelihood ratio test

HIV was identified as important effect modifier priori even if the statistical evidence was weak (LRT, p=0.36). There was no evidence for interaction for the rest of the risk factors that age (p=0.65), sex (p=0.50), socioeconomic status (p=0.34), smoking habits (p=0.82), smear status of the index (p=0.54), sleeping proximity to the index (p=0.95) and country (p=0.80) (table 7.4). However, there was weak evidence that education was an effect modifier (p=0.13).

7.11 Overall univariable and multivariable analysis

Four regression models were used in the univariable and multivariable analysis and results for each model are shown in table 7.6 as follows:

Table 7.6: Overall univariable and multivariable analyses

	Adjusted Hazard Ratio (95% CI)	P value
Model 1		
Low IFN-γ levels	1	
High IFN-γ levels	1.02 (0.48-2.15)	0.96
Model 2		
Low IFN-γ levels	1	
High IFN-γ levels	1.06 (0.50-2.26)	0.87
Model 3		
Low IFN-γ levels	1	
High IFN-γ levels	1.73 (0.63-4.78)	0.29
Model 4		
Low IFN-γ levels	1	
High IFN-γ levels	1.74 (0.63-4.79)	0.29

Model 1: Unadjusted analysis
Model 2: Adjusted for age and sex as priori confounders.
Model 3: Adjusted for age and sex plus interaction between high IFN-γ levels and HIV (separate results for HIV are shown in table 7.8)
Model 4: as in model 3 plus adjusted for country

In univariable analysis, there was no evidence that high IFN-γ levels were associated with an increased risk of TB (HR: 1.02 (95% CI: 0.48-2.15; p=0.96). When results were adjusted for age and sex as priori confounders, HR was similar to the unadjusted one (**Table 7.6).** In multivariable analysis, adjusted for sex, age with interaction between HIV and high levels (model 3), the adjusted HR was 1.73 (95% CI: 0.63-4.78; p=0.29) (p value for interaction for HIV, p=0.36). Adjusting for the effect of country (model 4), gave similar results as in model 3. However, for all models the results were not statistically significant (table 7.6).

7.12 Univariable and multivariable analysis by country

Three regression models were used in the univariable and multivariable analysis that was stratified by country and results for each model are shown in **Table 7.7**.

Table 7.7: Univariable and multivariable analysis, stratified by country.

	Zambia		SA	
High/low IFN-γ levels	**Hazard Ratio (95% CI)**	**P value**	**Hazard Ratio (95% CI)**	**P value**
Model 1				
Low IFN-γ levels	1		1	
High IFN-γ levels	0.94 (0.37-2.37)	0.90	1.13 (0.32-4.07)	0.85
Model 2				
Low IFN-γ levels	1		1	
High IFN-γ levels	0.98 (0.38-2.50)	0.97	1.09 (0.30-3.92)	0.89
Model 3				
Low IFN-γ levels	1		1	
High IFN-γ levels	1.91 (0.51-7.15)	0.33	1.37 (0.27-7.09)	0.70

Model 1: Unadjusted analysis
Model 2: Adjusted for age and sex as priori confounders.
Model 3: Adjusted for age and sex plus interaction between high IFN-γ levels and HIV (p value for interaction with HIV, p=0.6)

In univariable analysis, there was no evidence that high IFN-γ levels were associated with an increased risk of TB for both Zambia (HR: 0.94 (95% CI: 0.37-2.37; p=0.90) and SA (HR: 1.13 (95% CI: 0.32-4.07; p=0.85). when results were adjusted for age and sex as priori confounders, results were similar to the unadjusted analysis for both countries (table 7.7).

In multivariable analysis, adjusted for sex, age with interaction between HIV and high levels (model 3), the adjusted HR was 1.91 (95% CI: 0.51-7.15; p=0.33) for Zambia and 1.37 (95%CI: 0.27-7.09; p=0.70) for SA (table 7.7). For all models results were not statistically significant (table 7.7).

7.13 Univariable and multivariable analysis stratified by HIV status

Three regression models were used in the analysis exploring the association between high IFN-γ levels and TB incidence stratified by HIV status. For both HIV positives and negatives, HRs were similar from model 1 to model 3 within the group (**Table 7.8.**) In HIV negatives, there was some evidence from these three models that high IFN-γ levels were associated with an almost two-fold increased risk of developing TB although results were not statistically significant (Table 7.8).

Table 7.8: Overall univariable and multivariable analysis, stratified by HIV status.

Models	**HR in HIV negatives (95% CI)**	**P value**	**HR in HIV positives (95% CI)**	**P value**
Model 1				
Low IFN-γ levels	1		1	
High IFN-γ levels	1.73 (0.63-4.77)	0.29	0.75 (0.22-2.55)	0.65
Model 2				
Low IFN-γ levels	1		1	
High IFN-γ levels	1.74 (0.63-4.80)	0.28	0.81 (0.24-2.77)	0.74
Model 3				
Low IFN-γ levels	1		1	
High IFN-γ levels	1.75 (0.63-4.84)	0.28	0.79 (0.23-2.74)	0.72

Model 1: Unadjusted analysis
Model 2: Adjusted for age and sex as priori confounders.
Model 3: as in model 2 plus adjusted for country

In HIV positives evidence from models 1-3 suggested a protective effect of high levels; there was an almost twenty percent reduction in the risk of TB among HHCs with high IFN levels although results were not statistically significant (table 7.8).

Summary

Out of the 1,113 HHCs who entered follow-up, 406 HHCs had IFN-γ levels <0.35 IU/ml and were therefore excluded from analysis. Therefore 707 HHCs were present for analysis in this chapter. Out of these 536 (75.8%) had IFN-gamma levels ≥ 0.35 and <10 IU/ml (low IFN-γ levels) while 171 (24.2%) HHCs had high IFN-γ levels (≥ 10 IU/ml).

Out of the 707 HHCs available for analysis, 38 (5.4%) HHCs developed active TB over 1558.0 person-years (pyrs) of follow-up, giving an incidence rate of 24.39/1000 pyrs. The median follow-up time was 2.44 years (IQR: 1.59-2.82). There was no difference in incidence rates between HHCs with low and high levels, by country and overall (overall IRR: 1.0 (95% CI: 0.42-2.18)).

In HIV negatives, out of the 535 HHCs that entered follow-up, 16 (3.0%) HHCs developed active TB over 1178 pyrs of follow-up, giving an overall incidence rate of 13.58/1000 pyrs. The IRR in HIV negatives was 1.70 (95%CI: 0.51-5.18; p=0.16). In HIV positives, out of the 159 HHCs that entered follow-up, 22 (13.8%) HHCs developed active TB over 348.8 pyrs of follow-up, giving an overall incidence rate of 63.08/1000 pyrs. The IRR in HIV positives was 0.79 (95%CI: 0.15-2.70; p=0.38).

Therefore in HIV negatives, incidence rates were higher in HHCs with high levels compared to those with low IFN-γ levels, although statistical evidence was weak (IRR: 1.70; 95%CI: 0.51-5.18; p=0.16). In HIV positives, incidence rates were lower in HHCs with high levels compared to those with low IFN-γ levels (IRR: 0.79), although statistical evidence was equally weak (IRR: 0.79; 95%CI: 0.15-2.70; p=0.38).

Country was an important confounder in the association between high IFN-γ levels and TB incidence while age and sex were considered as priori confounders. HIV positivity was an effect modifier (p for interaction=0.36).

Overall, unadjusted HR in HHCs with high IFN-γ levels was 1.02 (95%CI: 0.48-2.15; p=0.96) Hence there was equally no evidence of an association between high IFN-γ levels and TB incidence overall. Results were consistent when stratified by country.

Overall, in multivariable analysis adjusted for age, sex, country and HIV as an effect modifier, HR was 1.74 (95%CI: 0.63-4.79; p=0.29). Unadjusted HR among HIV negative HHCs was 1.73 (95%CI: 0.63-4.77; p=0.29) and 0.75 (95%0.22-2.55; p=0.65) among HIV positives ones respectively. In a multivariable analysis adjusted for age, sex and country, the HR remained similar as unadjusted analysis for both HIV negatives and positives.

Overall, there was no evidence to suggest that HHCs with high levels of IFN-γ in response to *Mtb* specific antigens in the QFT-GIT assay were at a higher risk of developing TB than those with low levels.

8.0 Exploring quantitative QFT-GIT cut-offs

Introduction

In this chapter I aim to explore other quantitative QFT-GIT cut-offs to determine whether there is an association between high IFN-γ levels and risk of progression to active TB. In answering the main question (presented in chapter 7) I restricted the analysis to a binary exposure (high IFN-γ levels versus low levels). However, in this chapter I will answer the main question by using three to six QFT-GIT subgroups defined by different methods.

Specific objectives of this chapter are to conduct a sensitivity analysis with the following aims:

1. To explore other quantitative QFT-GIT cut-offs as a comparison to the ones used to answer the main research question (high IFN-γ levels versus low levels).
2. To determine incidence rates and incidence rate ratios for each group, stratified by HIV status.
3. For each exploratory group, to determine whether HHCs with high levels of IFN-γ in response to *Mtb* specific antigens in the QFT-GIT assay are at higher risk of developing TB compared to those with low levels.

8.1 Exploratory analysis results to define QFT-GIT and TST groups used in sensitivity analysis

The cohort for this analysis includes all the 1,113 HHCs who entered follow-up.

Three QFT-GIT groups and one TST group were defined after exploratory analysis **(Table 8.1)**. These groups were used for sensitivity analysis.

Table 8.1: QFT-GIT and TST definitions used for sensitivity analysis (n=1113)

Group definition by IFN-γ (IU/ml) and TST cut-offs (mm)	N=1113 for each group	Median IFN-γ response for each sub-group (95% CI)	Method used
Group 3 group (IU/ml)			
<0.27	377	0.01 (0-0.07)	Data divided into 3 equal groups.
0.28 to 3.12	365	1.03 (0.54-1.94)	
3.13 to 10	371	9.19 (5.55-10)	
Group 4 (IU/ml)			
<0.35	406	0.02 (0-0.1)	Choice based on distribution of baseline IFN-γ responses of this data and literature review (246)
0.35 to <1.0	147	0.6 (0.43-0.83)	
1.0 to 5.0	261	2.4 (1.57-3.25)	
>5.0 to 10	299	10 (7.58-10)	
Group 6 (IU/ml)			
<0.35	406	0.02 (0-0.1)	Based on literature review but modified by author (166)
0.35 to 0.49	53	0.42 (0.39-0.45)	
0.5 to 0.99	94	0.77 (0.62-0.89)	
1.0 to 3.99	228	2.13 (1.52-2.86)	
4.0 to 9.99	161	6.76 (5.21-8.02)	
10	171	10 (10-10)	
TST group (mm)			
<5	529	0.3 (0.01-2.49)	Literature review-commonly used approaches(169, 246)
5 to 9	158	1.5 (0.15-6.19)	
10 to 14	231	2.18 (0.42-7.89)	
>15	195	3.88 (0.93-10)	

Kwallis test for median, p-value=0.0001 for all groups. Overall median is 1.01 (95% CI: 0.07-5.55).

8.2 Overall TB incidence rates and incidence rate ratios

Incidence rate and incidence rate ratios (IRRs) by group and sub-group are shown in table 8.2.

For all the groups, HHCs in sub-groups with the highest IFN-γ levels had increased IRRs ranging from 1.5 to 2-fold compared to the reference sub-group (**Table 8.2**). The IRRs of HHCs in sub-groups with the highest IFN-γ levels were 1.85 (95%CI: 0.90-3.83), 1.46 (95%CI: 0.69-3.10), 1.65 (95%CI: and 2.11 (95%CI: 1.10-4.05) for groups 3, 4, 6 and the TST group respectively (table 8.2).

For group 3 and the TST group, HHCs in sub-groups with the highest IFN-γ levels had the highest IRRs compared to the reference sub-group. For group 4 and 6, IRRs were highest in HHCs with IFN-γ levels between 1.0-5.0 IU/ml (IRR: 2.08; 95%CI: 1.02-4.24) and 1.0-3.99 IU/ml (IRR: 1.83; 95%CI: 0.86-3.90) respectively (Table 8.2).

Table 8.2: Overall incidence rates and incidence rate ratios by group. All TB cases (n=51).

Group (IU/Ml)	N (col %) N=1113	TB cases/pyrs	Incidence rate/ 1000 pyrs (95% CI)	IRR (95% CI)	P value
Group 3					
<0.27	377	11/812	13.55 (7.50-24.46)	1	0.05
0.28 to 3.12	365	20/823.8	24.28 (15.66-37.63)	1.79 (0.87-3.70)	
3.13 to 10	371	20/797.4	25.08 (16.18-38.88)	1.85 (0.90-3.82)	
*Test for trend, p=0.10					
Group 4					
<0.35	406	13/875.3	14.85 (8.62-25.58)	1	0.17
0.35 to <1.0	147	6/330.8	18.14 (8.15-40.37)	1.22 (0.46-3.21)	
1.0 to 5.0	261	18/583.8	30.83 (19.43-48.94)	2.07 (1.03-4.17)	
>5.0 to 10	299	14/643.4	21.76 (12.89-36.74)	1.46 (0.69-3.10)	
*Test for trend, p=0.15					
Group 6					
<0.35	406	13/875.3	14.85 (8.62-25.58)	1	0.16
0.35 to 0.49	53	1/120.7	8.29 (1.17-58.84)	0.56 (0.07-4.14)	
0.5 to 0.99	94	5/210.2	23.79 (9.90-57.16)	1.60 (0.58-4.45)	
1.0 to 3.99	228	14/514.1	27.23 (16.13-45.98)	1.83 (0.87-3.86)	
4.0 to 9.99	161	9/345.8	26.03 (13.54-50.02)	1.75 (0.76-4.05)	
10	171	9/367.2	24.51 (12.75-47.10)	1.65 (0.71-3.83)	
*Test for trend, p=0.08					
TST group (mm)					
<5	529	19/1128.7	16.83 (10.74-26.39)	1	0.15
5 to 9	158	4/347.3	11.52 (4.32-30.69)	0.68 (0.23-2.00)	
10 to 14	231	12/507.7	23.63 (13.42-41.62)	1.40 (0.68-2.88)	
>15	195	16/449.5	35.59 (21.80-58.10)	2.11 (1.10-4.05)	
*Test for trend, p=0.01					

*** Score test for trend of odds, chi2 (1)**

A test for trend for each group showed no evidence of a trend for groups 3, 4 and 6 while there was strong evidence that there was a trend for the TST group (test for trend, p=0.01) (**Table 8.2**).

8.2.1 TB incidence rates and incidence rate ratios by country

Zambia

For group 3, although the IRR was highest in HHCs with IFN-γ levels between 0.28-3.12 IU/ml (IRR: 4.98; 95%CI: 1.60-15.57) (**Table 8.3**). For groups 4 and 6, IRRs were highest in those with IFN-γ levels between 1.0-5.10 IU/ml (IRR: 8.05; 95%CI: 2.81-23.10) and 1.0-3.99 IU/ml (IRR: 6.93; 95%CI: 2.28-20.99) respectively. These results for group 4 and 6 were similar to those in the overall analysis (table 8.2). For the TST group, IRR was highest in HHCs with the largest TST indurations (IRR: 2.50; 95%CI: 1.03-6.04) (**Table 8.3**).

Table 8.3: Zambia: incidence rates of TB and incidence rate ratios, by group (nmax=728). All TB cases (n=27).

Group (IU/Ml)	N (col %) N=728	TB cases/pyrs	Incidence rate/ 1000 pyrs (95% CI)	IRR (95% CI)	P value
Group 3					
<0.27	278	3/582.5	5.15 (1.66-15.97)	1	0.004
0.28 to 3.12	211	12/467.4	25.67 (14.58-45.20)	4.98 (1.60-15.57)	
3.13 to 10	239	12/500	24.00 (13.63-42.26)	4.66 (1.48-14.69)	
*Test for trend, p=0.41					
Group 4					
<0.35	292	3/613.8	4.89 (1.57-15.15)	1	0.04
0.35 to <1.0	84	3/189.1	15.86 (5.11-49.19)	3.24(0.71- 14.71)	
1.0 to 5.0	153	13/330.2	39.37 (22.86-67.81)	8.05(2.81-23.10)	
>5.0 to 10	199	8/416.9	19.19 (9.60-38.37)	3.93 (1.15-13.42)	
*Test for trend, p=0.01					
Group 6					
<0.35	292	3/613.8	4.89 (1.58-15.15)	1	0.07
0.35 to 0.49	28	1/65.3	15.31 (2.16-108.69)	3.13 (0.37-26.76)	
0.5 to 0.99	56	2/123.8	16.16 (4.04-64.60)	3.30 (0.61-17.86)	
1.0 to 3.99	135	10/295.4	33.86 (18.22-62.92)	6.93 (2.28-20.99)	
4.0 to 9.99	97	5/204	24.50 (10.20-58.87)	5.01 (1.38-18.17)	
10	120	6/247.6	24.23(10.88-53.93)	4.96(1.42-17.27)	
*Test for trend, p=0.005					
TST group (mm)					
<5 mm	360	11/737.2	14.92 (8.26-26.94	1	0.16
5 to 9 mm	111	1/242.1	4.13 (0.58-29.33	0.28 (0.04-1.87)	
10 to 14 mm	152	6/327	18.35 (8.24-40.84	1.23 (0.45-3.32)	
>15 mm	105	9/243.7	36.93 (19.21-70.97	2.47 (1.06-5.80)	
*Test for trend, p=0.03					

*** Score test for trend of odds, chi2 (1)**

A test for trend in Zambia showed evidence of a trend for all groups apart from group 3 (p=0.41) (table 8.3)

South Africa

For HHCs residing in SA, all QFT-GIT groups showed that highest levels had a protective effect when compared to the reference sub-group although results were not statistically significant. The IRRs of HHCs in sub-groups with the highest IFN-γ levels were 0.77 (95%CI: 0.29-2.05), 0.69 (95%CI: 0.25-1.89) and 0.65 (95%CI: 0.18-2.36) for groups 3, 4 and 6 respectively (**Table 8.4**).

Table 8.4: South Africa: Incidence rates of TB and incidence rate ratios, by group (nmax=385). All TB cases (n=24).

Group (IU/Ml)	N (col %) N=385	TB cases/pyrs	Incidence rate/ 1000 pyrs (95% CI)	IRR (95% CI)	P value
Group 3					
<0.27	99	8/229.4	34.86 (17.44-69.72)	1	0.24
0.28 to 3.12	154	8/356.4	22.45 (11.22-44.88)	0.64 (0.24-1.70)	
3.13 to 10	132	8/297.4	26.90 (13.45-53.80)	0.77 (0.29-2.05)	
*Test for trend, p=0.61					
Group 4					
<0.35	114	10/261.4	38.25 (20.58-71.09)	1	0.18
0.35 to <1.0	63	3/141.7	21.17 (6.83-65.64)	0.55 (0.15-1.97)	
1.0 to 5.0	108	5/253.6	19.72 (8.21-47.37)	0.51 (0.18-1.48)	
>5.0 to 10	100	6/226.5	26.49 (11.90-58.96)	0.69 (0.25-1.89)	
*Test for trend, p=0.38					
Group 6					
<0.35	114	10/261.4	38.25 (20.58-71.09)	1	0.29
0.35 to 0.49	25	0/55.3	0	n/a	
0.5 to 0.99	38	3/86.4	34.74 (11.20-107.70)	0.91 (0.25-3.29)	
1.0 to 3.99	93	4/218.8	18.28 (6.86-48.72)	0.48 (0.15-1.48)	
4.0 to 9.99	64	4/141.7	28.22 (10.59-75.20)	0.74 (0.23-2.34)	
10	51	3/119.6	25.08 (8.09-77.78)	0.65 (0.18-2.36)	
*Test for trend, p=0.47					
TST group (mm)					
<5 mm	169	8/391.5	20.43 (10.22-40.86)	1	0.34
5 to 9 mm	47	3/105.2	28.51 (9.20-88.41)	1.39 (0.37-5.23)	
10 to 14 mm	79	6/180.7	33.20 (14.91-73.90)	1.62 (0.57-4.63)	
>15 mm	90	7/205.8	34.01 (16.22-71.35)	1.66 (0.61-4.54)	
*Test for trend, p=0.26					

***** Score test for trend of odds, chi2 (1)

TST results showed that IRRs were highest in HHCs with the highest IFN-γ levels (IRR: 1.66; 95%CI: 0.61-4.54; p=0.17) although results were not statistically significant. A test for trend in SA showed no evidence of a trend for all the groups (table 8.4).

8.2.2 TB incidence rates, stratified by HIV status

For HIV negatives, HHCs with the highest IFN-γ levels had the highest incidence rates in all groups apart from group 6 (table 8.5). The IRRs of HHCs with the highest IFN-γ levels were 4.02 (95%CI: 0.99-16.34), 4.38 (95%CI: 1.06-18.06), and 5.51 (95%CI: 1.33-22.82) for groups 3, 4 and 6 respectively. For the TST group, the IRR of HHCs with the highest IFN-γ levels was 2.76 (95%CI: 0.88-8.61) (**Table 8.5**).

There was a tendency of increasing IRRs with increasing IFN-γ production levels. A test for trend in HIV negatives showed strong evidence of a trend across all groups (table 8.5). For all the QFT-GIT groups,

HIV negative HHCs with the highest IFN-γ levels had increased IRRs ranging from 4 to 5-fold compared to the reference sub-group (table 8.5). For the TST group, there was an almost 3-fold increase in IRR in HHCs with the highest IFN-γ level compared to the reference sub-group although results were not statistically significant.

Table 8.5: Overall incidence rates of TB and incidence rate ratios in HIV negatives, by group (nmax=799). All TB cases (n=18).

Group (IU/Ml)	N (col %) N=799	TB cases/pyrs	Incidence rate/ 1000 pyrs (95% CI)	IRR (95% CI)	P value
Group 3					
<0.27	244	2/517.49	3.86 (0.97-15.45)	1	0.07
0.28 to 3.12	260	6/580.26	10.34 (4.65-23.02)	2.67 (0.57-12.45)	
3.13 to 10	295	10/643.67	15.54 (8.36-28.87)	4.02 (0.99-16.34)	
*Test for trend, p=0.05					
Group 4					
<0.35	264	2/563.02	3.55 (0.89-14.20)	1	0.10
0.35 to <1.0	105	3/229.66	13.06 (4.21-40.50)	3.68 (0.69-19.51)	
1.0 to 5.0	194	5/434.42	11.51 (4.79-27.65)	3.24 (0.69-15.25)	
>5.0 to 10	236	8/514.32	15.55 (7.78-31.10)	4.38 (1.06-18.06)	
*Test for trend, p=0.05					
Group 6					
<0.35	264	2/563.0	3.55 (0.89-14.20)	1	0.18
0.35 to 0.49	39	0/85.0	0	0	
0.5 to 0.99	66	3/144.6	20.74 (6.69-64.31)	5.84 (1.21-28.25)	
1.0 to 3.99	168	4/377.1	10.60 (3.98-28.26)	2.99 (0.59-15.02)	
4.0 to 9.99	121	3/264.9	11.33 (3.65-35.12)	3.19 (0.59-17.32)	
10	141	6/306.7	19.56 (8.79-43.54)	5.51 (1.33-22.82)	
*Test for trend, p=0.02					
TST group (mm)					
<5 mm	362	5/760.1	6.58 (2.74-15.80)	1	0.20
5 to 9 mm	118	2/257.2	7.78 (1.94-31.10)	1.18 (0.23-6.08)	
10 to 14 mm	178	5/393.8	12.70(5.28-30.50)	1.93 (0.57-6.52)	
>15 mm	141	6/330.3	18.16 (8.16-40.43)	2.76 (0.88-8.61)	
*Test for trend, p=0.05					

*** Score test for trend of odds, chi2 (1)**

In HIV positives, IRs were highest in HHCs with the highest IFN-γ levels for group 3 and the TST group but this was less obvious for groups 4 and 6 (**Table 8.6**). There was also a clear tendency of increasing IRRs with increasing IFN-γ levels in groups 3 and the TST group. A test for trend in HIV positives showed strong evidence of a trend in these groups while for other groups it was less obvious (Table 8.6).

For all the QFT-GIT groups, HIV positive HHCs with the highest IFN-γ levels had increased IRRs ranging from 1.6 to 2.6 compared to the reference sub-group (table 8.6). For the TST group, results were similar as in HIV negatives with an almost 3-fold increase in IRR in HHCs with the highest IFN-γ level compared to the reference sub-group.

Table 8.6: Overall incidence rates of TB and incidence rate ratios in HIV positives, by group (nmax=297) All TB cases (n=32).

Variable	N (col %) N=297	TB cases/pyrs	Incidence rate/ 1000 pyrs (95% CI)	IRR (95% CI)	P value
Group 3					
<0.27	130	8/291	27.49 (13.75-54.98	1	0.02
0.28 to 3.12	98	14/225.9	61.99 (36.71-104.66	2.26 (0.97-5.27)	
3.13 to 10	69	10/138.1	72.39 (38.95-134-53	2.63 (1.08-6.44)	
*Test for trend, p=0.04					
Group 4					
<0.35	138	10/306.2	32.66 (17.57-60.70	1	0.23
0.35 to <1.0	39	3/93.3	32.15(10.37-99.70	0.98 (0.27-3.57)	
1.0 to 5.0	63	13/139.6	93.10(54.06-160-33	2.85 (1.29-6.26)	
>5.0 to 10	57	6/115.8	51.81(23.27-115.31)	1.58 (0.58-4.32)	
*Test for trend, p=0.09					
Group 6					
<0.35	138	10/306.2	32.66 (17.57-60.70	1	0.25
0.35 to 0.49	14	1/35.6	28.07(3.95-199.29	0.86 (0.11-6.70)	
0.5 to 0.99	25	2/57.7	34.67(8.67-138.65	1.06 (0.23-4.84)	
1.0 to 3.99	56	10/127.3	78.56(42.27-146.00	2.40 (1.03-5.62)	
4.0 to 9.99	35	6/70.40	85.22(38.29-189.69	2.61 (0.98-6.91)	
10	29	3/57.8	51.94(16.75-161.04	1.59 (0.44-5.70)	
*Test for trend, p=0.06					
TST group (mm)					
<5 mm	161	13/356.3	36.48 (21.18-62.83	1	0.10
5 to 9 mm	37	2/83.9	23.82 (5.96-95.27	0.65 (0.15-2.86)	
10 to 14 mm	51	7/110.1	63.58 (30.31-133.37	1.74 (0.70-4.32)	
>15 mm	48	10/104.6	95.58(51.43-177.64	2.62 (1.18-5.79)	
*Test for trend, p=0.01					

***** Score test for trend of odds, chi2 (1)

In both HIV positives and negatives, there was a clear trend of a tendency of increasing IRRs with increasing IFN-g levels for groups 3 and the TST group while this was less clear for groups 4 and 6 (**Figure 8.1 and 8.2**).

Figure 8.1: Incidence rates in groups 3 and 6 stratified by HIV status

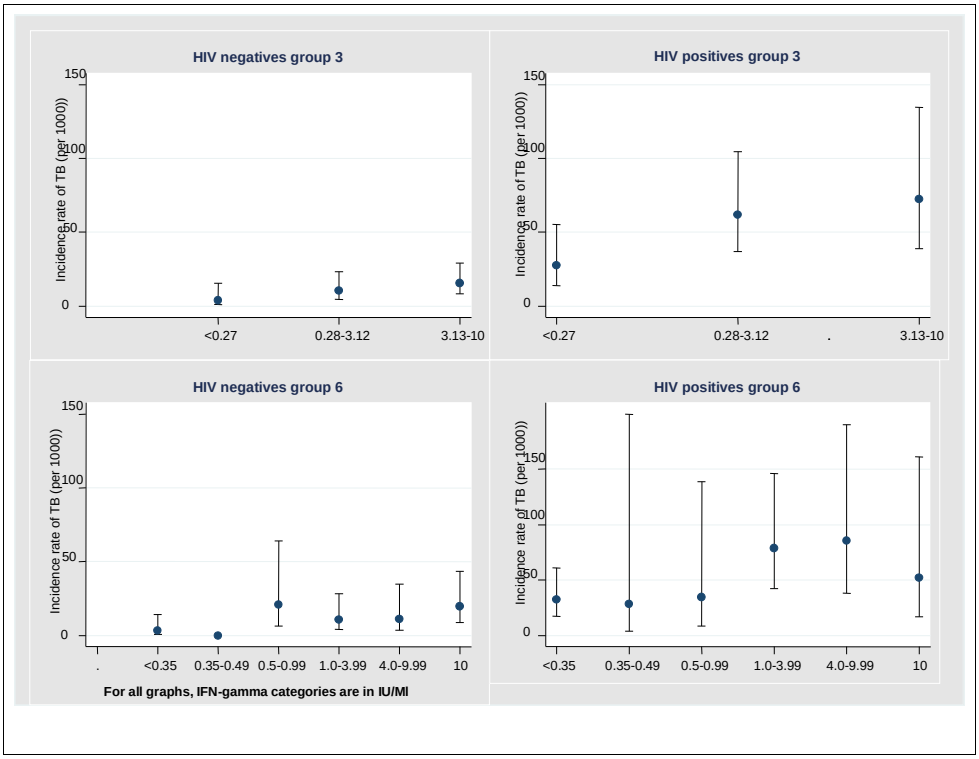
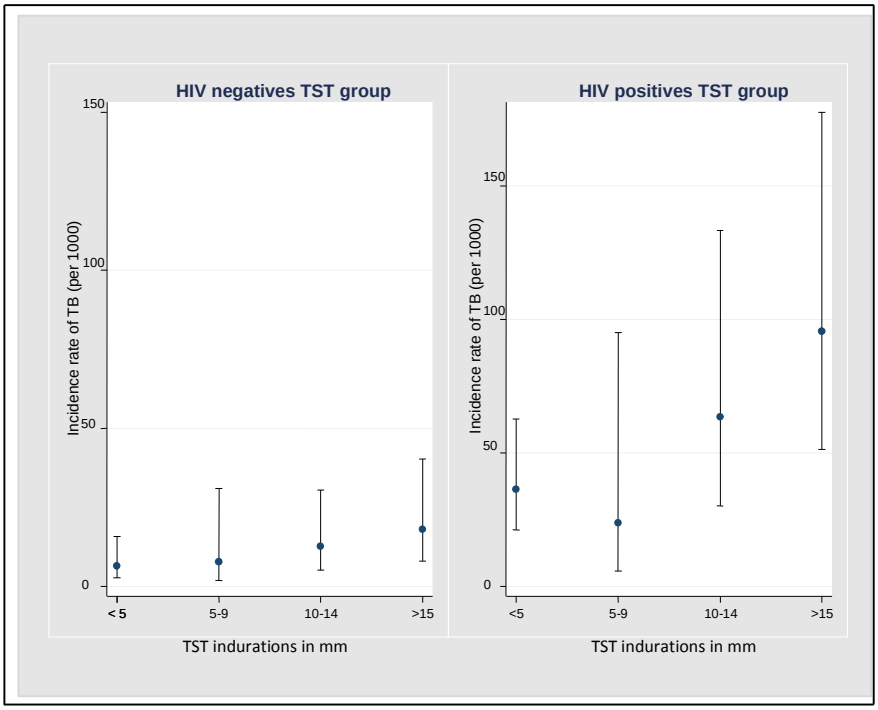


Figure 8.2: Incidence rates in the TST Group



8.2.3 Examining linear effects between magnitude of IFN-γ response and TB incidence by group

Assessing departure from linearity using LRT showed that overall there was no evidence that the relationship between IFN-γ levels and log odds of disease was not linear for all the groups i.e. a linear trend adequately described the relationship (**Table 8.7)** The linear trend was observed for both HIV negatives and positives and for all groups in SA and half the groups in Zambia (table 8.7).

Table 8.7: Testing for departure from linearity

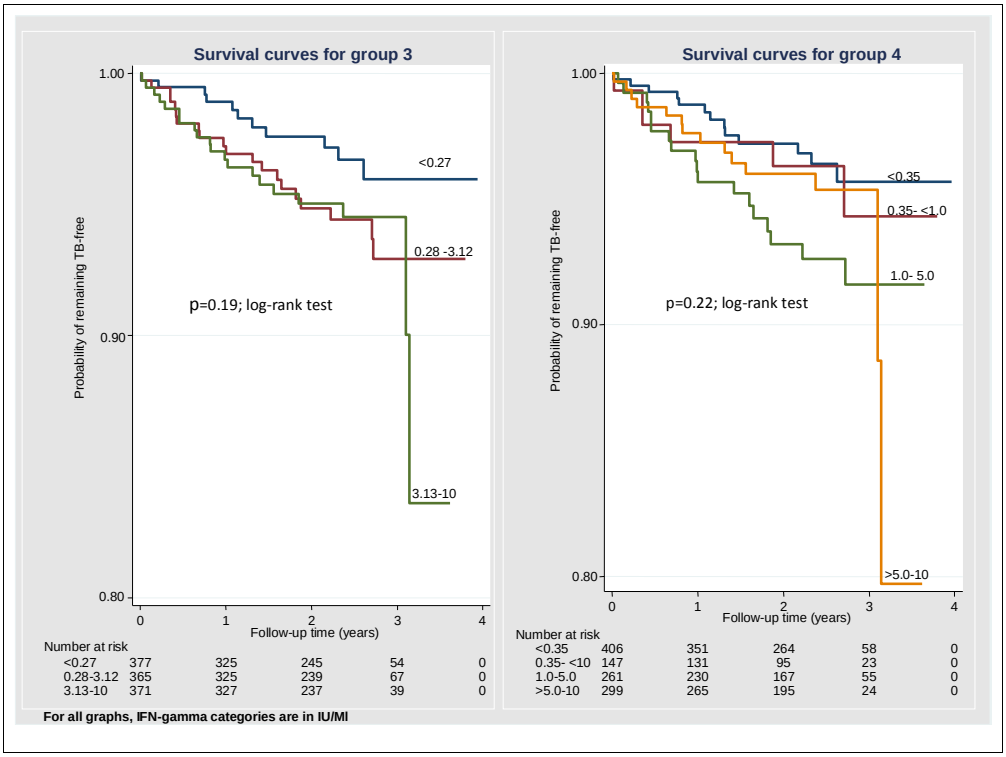
	LRT chi(2)	p-value
Overall		
Group 3	0.83	0.36
Group 4	2.21	0.33
Group 6	1.59	0.81
TST group	1.71	0.42
Overall HIV negative		
Group 3	0.18	0.67
Group 4	0.89	0.64
Group 6	2.93	0.40
TST group	0.05	0.97
Overall HIV positives		
Group 3	0.99	0.32
Group 4	4.05	0.13
Group 6	2.37	0.67
TST group	1.26	0.53
Zambia		
Group 3	3.77	0.05
Group 4	7.99	0.02
Group 6	4.45	0.35
TST group	4.17	0.12
SA		
Group 3	0.53	0.47
Group 4	1.07	0.58
Group 6	4.41	0.35
TST group	0.13	0.94

8.3 TB-free survival probability using Kaplan Meier estimates

Figure 8.3 shows Kaplan Meier survival curves for groups 3 and 4. For both groups, HHCs with the highest IFN-γ categories had the lowest TB-free survival probability. For group 3, the two-year TB-free survival probability was at 0.95 (95%CI: 0.92-0.97) among HHCs with the highest IFN-γ levels compared to 0.97 (95% CI: 0.95-0.99) among those in the reference group (figure 8.3). For group 4, the TB-free survival probability was lower at 0.96 (95%CI: 0.93-0.98) among HHCs with the highest IFN-γ levels compared to 0.97 (95% CI: 0.95-0.98) among those in the reference group (figure 8.3).

However, overall there was no evidence that survival curves differed for these groups (log rank test: group 3, p=0.19 and group 4, p=0.22) (figure 8.3).

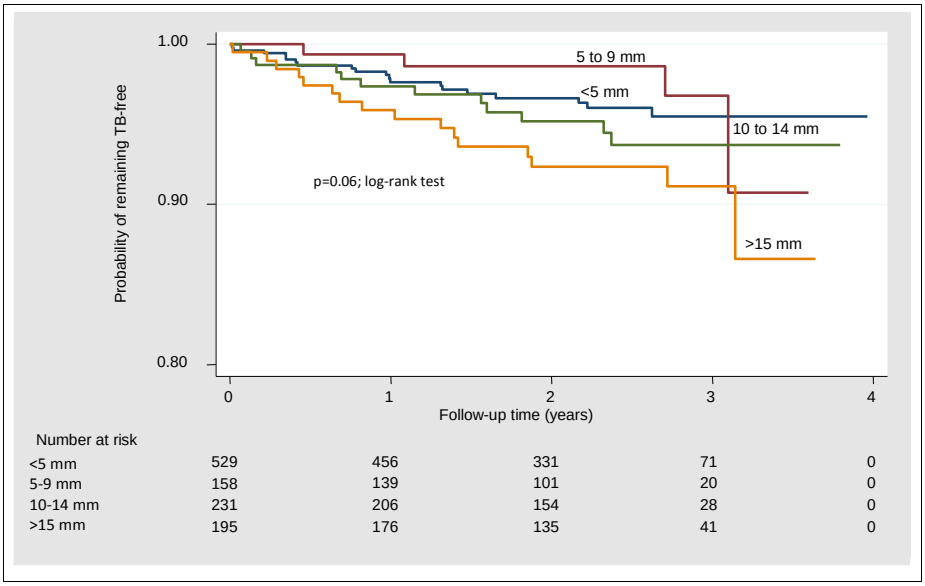
Figure 8.3: Kaplan Meier survival curves for groups 3 and 4



Note: Kaplan-Meier curves: survival curves ends in a plateau; represents some HHC without disease after the study's maximum follow-up time

A Kaplan-Meier curve for the TST group is shown in figure 8.4. Overall, the two-year TB-free survival probability was 0.97 (95%CI: 0.94-0.98) in HHCs with TST indurations <5 mm while those with TST indurations > 15 mm it was at 0.92 (95%CI: 0.87-0.95) (figure 8.4). There was some evidence that survival curves differed for the TST group (log rank test, p=0.06).

Figure 8.4: Kaplan Meier survival curves by TST group



TB-free survival probability stratified by HIV status.

When TB-free survival probabilities were stratified by HIV, results were similar to the overall results for all groups; see examples in **Figure 8.5 and Figure 8.6.**

Figure 8.5: Kaplan Meier curves for group 3 stratified by HIV

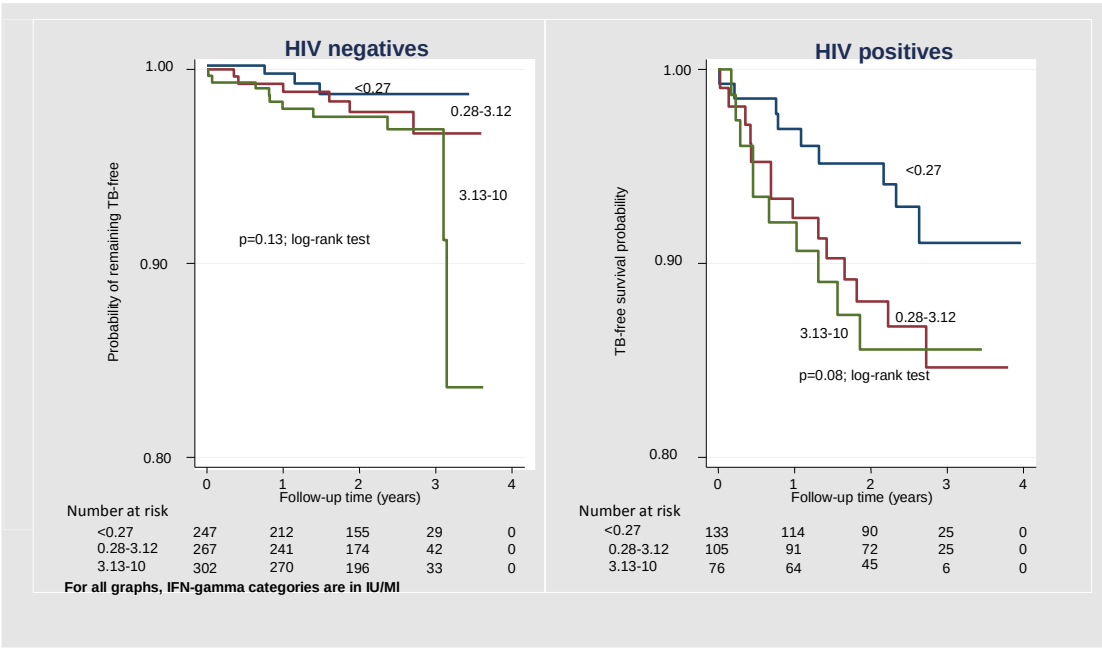
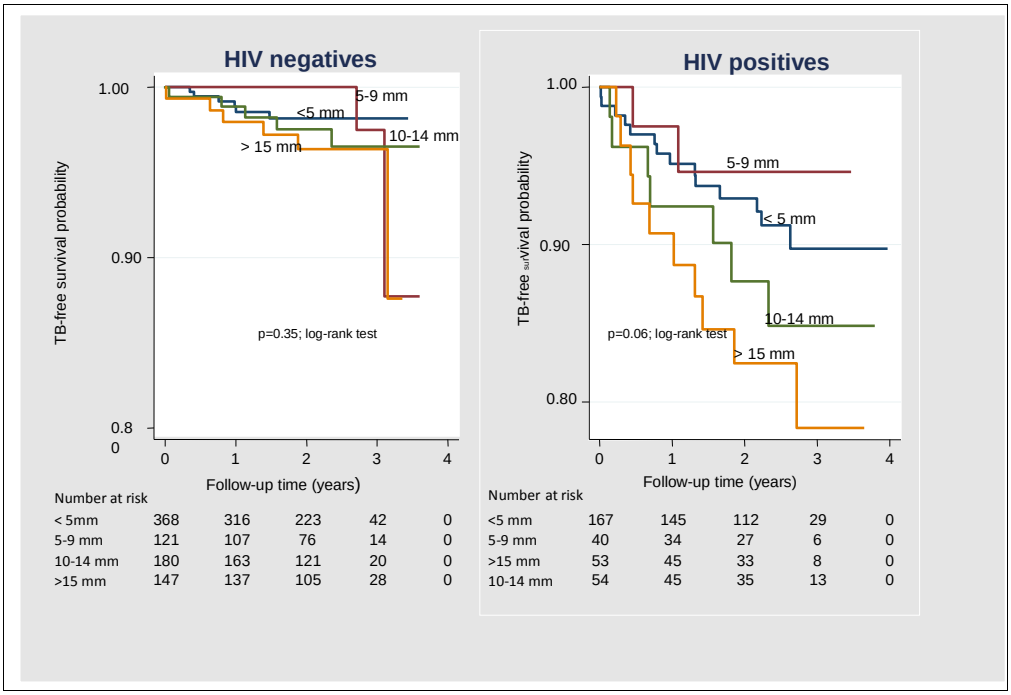


Figure 8.6: Kaplan Meier curves for the TST group stratified by HIV



8.4 Overall univariable and multivariable analyses

The results for the overall univariable and multivariable analyses are presented in table 8.8.

In crude analyses there was some evidence in all groups that HHCs with the highest IFN-γ levels had 1.5-2- fold increased risk of developing TB although results were not statistically significant (table 7.13, model 1). HHCs with the highest IFN-γ levels had the highest risk of TB for group 3 (HR: 1.88; 95%CI: 0.90-3.93; p=0.17) and the TST group (HR: 2.12; 95%CI: 1.09-4.13; p=0.08). However, for groups 4 and 6 the risk of TB was highest in those with IFN-γ levels between 1.0 to 5.0 IU/ml (HR 2.08; 95%CI: 1.02-4.24; p=0.23) and 1.0 to 3.99 IU/ml (aHR: 1.83; 95% CI: 0.86-3.90, p=0.47 respectively.

When age and sex was added as priori confounders (model 2), the results were similar as in crude analyses across all groups except in group 6. In group 6, the risk of TB was highest in HHCs with IFN-γ levels between 4.0 to 9.99 IU/ml (aHR: 1.85; 95% CI: 0.79-4.34; p=0.20) (table 7.13, model 2). In multivariable analysis adjusted for sex, age and HIV as an effect modifier (as in analysis for primary question), there was strong evidence across all QFT-GIT groups that HHCs with the highest IFN-γ levels had an approximately 4-5-fold increased risk of developing TB in follow-up compared to the reference sub-group (table 8.8, model 3). For the TST group the HR in those with TST indurations > 15 mm was 2.77 (95%CI: 0.85-9.10).

When results were further controlled by country (model 4), results were similar as for model 3 for all groups (table 8.8). A test for trend showed strong evidence of a trend in the TST group (test for trend,

p=0.01) while there was no evidence for the rest of the groups (test for trend: p=0.10 for group 3; p=0.15 for group 4; p=0.08 for group 6) (**Table 8.8**).

Table 8.8: Overall univariable and multivariable analysis of time to TB episode; hazard ratios; 95% CI and P-values

	Model 1		Model 2		Model 3		Model 4	
Variable	**Crude HR (95% CI)**	**P value**	**Adjusted HR (95% CI)**	**P value**	**Adjusted HR (95% CI)**	**P value**	**Adjusted HR (95% CI)**	**P value**
Group 3								
<0.27	1	0.17	1	0.44	1	P<0.001	1	P<0.001
0.28 to 3.12	1.79 (0.86-3.74)		1.68 (0.80-3.55)		2.65 (0.53-13.18)		2.42 (0.48-12.08)	
3.13 to 10	1.88 (0.90-3.93)		1.86 (0.89-3.90)		4.07(0.89-18.62)		3.83(0.83-17.58)	
[1]Test for trend, p value LRT	0.36		0.48					
Group 4								
<0.35	1	0.23	1	0.57	1	P<0.001	1	P<0.001
0.35 to <1.0	1.22 (0.46-3.20)		1.23 (0.47-3.24)		3.70 (0.62-22.22		3.40 (0.56-20.46	
1.0 to 5.0	2.08 (1.02-4.24)		1.92 (0.93-3.96		3.15 (0.61-16.31		2.93 (0.56-15.22	
>5.0 to 10	1.49 (0.70-3.17)		1.48 (0.70-3.16)		4.45 (0.94-21.01		4.35 (0.92-20.51	
Test for trend, p value	0.33		0.48					
Group 6								
<0.35	1	0.47	1	0.71	1	P<0.001	1	P<0.001
0.35 to 0.49	0.56 (0.07-4.28)		0.55 (0.72-4.24		[2]n/a		n/a	
0.5 to 0.99	1.59 (0.57-4.46)		1.62 (0.58-4.55)		6.08 (1.01-36.49		5.68 (0.94-34.18	
1.0 to 3.99	1.83(0.86-3.90)		1.65 (0.76-3.59)		2.89 (0.53-15.84		2.70 (0.49-14.84	
4.0 to 9.99	1.79 (0.76-4.19)		1.77 (0.76-4.16)		3.26 (0.54-19.54		3.09 (0.51-18.55	
10	1.67 (0.71-3.91)		1.67 (0.71-3.91)		5.60 (1.12-27.79		5.48 (1.10-27.19	
Test for trend, p value	0.81		0.87					
TST group (mm)								
<5 mm	1	0.08	1	0.21	1	P<0.001	1	P<0.001
5 to 9 mm	0.68 (0.23-2.01)		0.68 (0.23-2.01)		1.16 (0.22-6.00		1.20 (0.23-6.21	
10 to 14 mm	1.42 (0.69-2.92)		1.30 (0.62-2.74)		1.97 (0.57-6.81		2.02 (0.58-6.97	
>15 mm	2.12 (1.09-4.13)		2.11 (1.08-4.11)		2.77 (0.85-9.10		2.58 (0.78-8.49	
Test for trend, p value	0.42		0.39					

Model 1: Unadjusted analysis
Model 2: Model 1 adjusted by a priori confounding factors: age, sex
Model 3: as in model 2 plus HIV as an effect modifier
Model 4: as in model 3 plus controlled for country
[1]Test for trend in the unadjusted model
[2]HR: $1.5x10^{-9}$($1.9X10^{-10}$)

Summary

Three QFT-GIT groups and one TST group were used for sensitivity analysis after exploratory analysis (table 8.1). Overall for all the groups, HHCs with the highest IFN-γ levels had increased IRRs ranging from 1.5 to 2 compared to the reference sub-group (table 8.2). The IRRs of HHCs with the highest IFN-γ levels were 1.85 (95%CI: 0.90-3.83), 1.46 (95%CI: 0.69-3.10), 1.65 (95%CI: and 2.11 (95%CI: 1.10-4.05) for groups 3, 4, 6 and the TST group respectively (table 8.2). A test for trend for each group showed no evidence of a trend for groups 3, 4 and 6 while there was strong evidence for the TST group (test for trend, p=0.01) (table 8.2).

For group 3 and the TST group, HHCs with the highest IFN-γ levels had the highest IRRs compared to the reference sub-group. For group 4 and 6, IRRs were highest in HHCs with IFN-γ levels between 1.0-5.0 IU/ml (IRR: 2.08; 95%CI: 1.02-4.24) and 1.0-3.99 IU/ml (IRR: 1.83; 95%CI: 0.86-3.90) respectively (table 8.2).

Overall, HIV negative HHCs with the highest IFN-γ levels had increased IRRs ranging from 4 to 5-fold compared to the reference sub-group (table 8.5). For the TST group, there was an almost 3-fold increase in IRR in HHCs in the highest IFN-γ levels compared to the reference sub-group.

Overall for the QF-GIT groups, HIV positive HHCs with the highest IFN-γ levels had increased IRRs ranging from 1.6 to 2.6 compared to the reference sub-group (table 8.6). For the TST group, results were similar as in HIV negatives with an almost 3-fold increase in IRR in HHCs with the highest IFN-γ level compared to the reference sub-group. In HIV positives, IRRs were highest in HHCs with the highest IFN-γ levels for group 3 and the TST group but this was less obvious for groups 4 and 6 (table 8.6). There was a tendency of increasing IRRs with increasing IFN-γ levels in groups 3 and the TST group (test for trend: group 3 p=0.04; group 4, p=0.09; group 6, p=0.06; TST group p=0.01) (table 8.6).

Therefore in both HIV positives and negatives, there was a trend towards increasing IRRs with increasing IFN-g levels for groups 3 and the TST group while this was less clear for groups 4 and 6.

In crude analyses there was some evidence in all groups that HHCs with the highest IFN-γ levels had 1.5-2 increased risk of developing TB although results were not statistically significant (table 7.13, model 1). In multivariable analysis adjusted for sex, age and HIV as an effect modifier, there was strong evidence across all QFT-GIT groups that HHCs with the highest IFN-γ levels had approximately 4-5-fold increased risk of developing TB in follow-up (table 8.8, model 3). For the TST group the HR in those with TST indurations > 15 mm was 2.77 (95%CI: 0.85-9.10). Apart from group 6, HHCs with the highest IFN-γ levels had the highest HRs (table 8.8, model 3).

9.0 Discussion

Introduction

In this chapter, I will discuss the results of this research project. Although the results of each chapter will be discussed separately, these discussions are not independent of each other. The objectives of the discussion are as follows:

1. To give an overview of the key study findings for each objective.
2. To describe what each finding adds to our understanding of the main research question.
3. To discuss results in light of previous research in this area.
4. To discuss the strengths and limitations of the research.
5. To highlight study implications in terms of TB control policies and future research agenda.

9.1 The performance and operational characteristics of QFT-GIT in Zambia

At the onset of this research project we carried out a number of feasibility studies that looked at performance and operational characteristics of QFT-GIT in Zambia. At that time there were very few feasibility studies looking at factors that may affect QFT-GIT performance in a resource poor setting like Zambia (242). These studies were therefore key in guiding the implementation of the study especially in Zambia where QFT-GIT was not centrally processed during the cohort study. We were able to explain and understand some of the teething problems that we had with the QFT-GIT assay and identify context specific solutions.

Four small studies were used to examine performance and operational characteristics of QFT-GIT in Zambia. The first feasibility study addressed four objectives namely; the sensitivity and test-retest reproducibility of QFT-GIT as well as the effect of CD4+ T-lymphocyte counts and delayed incubation of blood samples on IFN-γ responses.

Sensitivity of QFT-GIT and TST

We found that the sensitivity of QFT-GIT was 85.6% (95% CI: 77.0-91.9) compared to that of TST at the 10 mm cut-off of 51.6% (95% CI: 40.9-62.2). Like TST, we also found that the sensitivity was reduced by severe immunosuppression. In a subgroup analysis of patients with known HIV status, sensitivity was lower in HIV positives, 62.9% (95%CI: 49.7-74.8) compared to HIV negatives, 85.3% (95%CI: 68.9-95.0) (chi2: p=0.03). However, most systematic reviews have shown that QFT-GIT has higher or equal sensitivity to TST in these settings (131). In a recent systematic review evaluating 37 studies that included 5,736 HIV-infected individuals, pooled sensitivity for QFT-GIT was 61%,(95% CI: 41–75%) and neither QFT-GIT nor TSPOT was consistently more sensitive than the TST in head-to-head comparisons (131).

QFT-GIT sensitivity is affected by several biological and operational factors. Sensitivity may be influenced by factors that alter the immune response (247) such as BCG vaccination, HIV, malnutrition, tropical infections and widespread exposure to NTM. Most of these conditions

which are thought to affect QFT-GIT sensitivity are prevalent in Zambia and may have been present in many individuals used in these feasibility studies even though we did not specifically measure them. For example, we found that 46.2% of the TB patients used for sensitivity analysis were underweight and some literature suggested that they may be a relationship between malnutrition and QFT-GIT indeterminate results (248). It is now well recognized that IGRAs perform differently in low vs. high TB burden settings with lower sensitivity in high-incidence countries due to some of these factors (188).

Another significant finding was that with falling CD4 count, there was a trend towards an increase in both indeterminate and negative results overall (test for trend, p<0.001) and in HIV positive patients (test for trend, p=0.03)(222). The majority of HIV positive patients in this study were severely immune-suppressed (CD4/CD8 ratio was 0.45). There is evidence now from many studies that a low CD4+ count (<200 cells/all) is associated with increases in indeterminate results (131, 217, 222, 223).

Test-retest reproducibility

Test-retest reproducibility may not be relevant in routine practice but becomes very important when contemplating serial screening for high risk groups like health workers (181); also useful in cohort studies like this one. We reported a high test-retest reproducibility of QFT-GIT assay of 91.74%. Our study confirmed that QFT-GIT has excellent reproducibility although the percentage was lower than that of the manufacturer (a diagnostic reproducibility of 97.2% (95%CI 94.1%-98.7%) (249). Despite the substantial body of literature on IGRAs over the recent years, few studies have data on test-retest reliability and reproducibility of IGRAs (157, 191, 230, 250), especially under field conditions (251). Some studies have focused on within-person variability of QFT (250, 252).

Effect of delayed incubation on IFN-γ response

Another objective of the feasibility studies was to determine the effect of a 24-hour delay in incubation of blood samples on IFN-γ responses. We showed that a 24-hour delay in incubation decreased IFN-γ responses by 3.34 IU/ml and changed the results for 22.9% patients.(251). Most results changed from either positive or negative to indeterminate. In both the reproducibility and delayed incubation studies, the reason for the indeterminate results for approximately 95% of results was due to low TB Antigen minus Nil and low Mitogen minus Nil responses, suggesting either a poor immune response(251) or degradation of cells in the process.

Indeterminate results such as a high background detected in the negative control tube, or low responses in the positive-control tube, may be explained by technical factors or an impaired immune system (175). A low response to Mitogen (<0.5 IU/mL) indicates an indeterminate result when a blood sample also has a negative response to the TB antigens (249). This pattern may occur with insufficient lymphocytes, reduced lymphocyte activity due to prolonged specimen transport or improper specimen handling, including filling/mixing of blood tubes, or inability of the patient's lymphocytes to generate IFN-γ (249). Elevated levels of IFN-γ in the Nil

sample may occur with the presence of heterophile antibodies, or to intrinsic IFN-γ secretion (249).

One of the duplicate samples (standard incubation 1) was used as a comparison to the set of samples that was delayed (delayed incubation). Either set of duplicate samples could have been used as a comparison to the one that was delayed as the agreement between the duplicate samples was high and the discordance was not significant. The decrease in IFN-γ responses due to delayed incubation has implications for resource limited settings especially in our settings where samples may not be incubated within the recommended time due to operational problems.

The effect of power outages on IFN-γ responses (Study 2)

Our second feasibility study aimed to determine the effect of power outages on IFN-γ responses during incubation. This study was conceived as a result of serious power outages that the country (Zambia) was experiencing which affected the study sites as well. We found that power outages, especially those that occurred during late incubation decreased IFN-γ responses to TB antigens by 0.8 IU/ml. The processing of QFT-GIT is dependent on a stable power supply needed for, particularly, incubation, centrifugation and freezing of samples (if ELISA is performed in batches from a central point). However, incubation remains a critical part of the process as it requires a constant power supply for at least 20-24 hours (manufacturers' recommendation). In certain cases , it is recommended that an uninterruptable power source, for instance, a UPS system or generator be installed in case of power outages in settings where this is a problem. Some equipment may even malfunction owing to frequent power supply interruptions.

Power outages are a potential problem in many developing countries and this may affect processing (especially incubation) and storage of QFT-GIT samples. The decreased IFN-γ response due to power outages during incubation led to minor modification of the QFT-GIT protocol in our sites (251). The incubation time was extended following power outages with the aim of achieving an overall incubation period of 16-24 hours. It remains unknown whether this benefit could have been sustained during long periods of power outages.

Although it could have been possible to counteract the effect of power outages in our sites by using generators, solar energy or better-insulated incubators, this unplanned cost was difficult to implement (251). Since no such measures were in place during the course of the study, management forms were used to capture data on samples that experienced power outages.

Effect of storage temperature on IFN-γ response

Our third feasibility study aimed to determine the effect of increased storage temperature of unused blood collection tubes on IFN-γ responses. This study demonstrated that storage of unused blood collection tubes at 37°C for three months had no effect on IFN-γ responses. In this setting, storage space with the recommended temperature of 4-25°C (air conditioned room or

refrigerator) is limited. Therefore this was reassuring for the study staff especially those who were in the remote areas in Zambia.

General comment on studies above

These studies demonstrated that there are some biological and operational factors that affect the performance of this test in this setting. HIV positivity, low CD4+ T-lymphocytes, delayed incubation of blood samples and power outages all affect test performance. While most of these factors can be controlled, the effect of HIV infection like TST may be a major limiting factor for the role of this test. These feasibility studies emphasize the need for stringent sample collection and processing techniques to ensure the accuracy of QFT-GIT results.

Role of Quality Assurance

A multi-disciplinary Quality Assurance (QA) team was constituted during the course of the QFT cohort study due to the decentralized nature of sample collection, incubation, separation and storage. The aim of the QA team was to ensure that sample processing was done accurately as well as identify and correct gaps and weaknesses. A similar team was not formed in SA because samples are processed centrally and the laboratory already had existing QA systems. The QA visits were done once a year in all the sites although the QA team also conducted other support supervision visits in sites experiencing difficulties.

A QA monitoring tool was designed to help with this process. There were also other routine laboratory checks each time samples arrived at the central laboratories and these included: checking for leakages during transportation, quality of the sample, correct use of barcodes, type of specimen bottles and packaging used.

The financial cost of using QFT-GIT for the diagnosis of TB infection

In the fourth feasibility study, we aimed to determine the financial costing of diagnosing TB infection using QFT-GIT from the provider's perspective. Cost can be a major barrier for the introduction of a test even if other performance factors are favourable. In this study we found that the total cost of performing a QFT-GIT test was estimated at US$ 22.29 in our setting.

In this study, we collected costs using the ingredients approach (243). The ingredients approach to cost estimation entails three distinct phases: identification of ingredients; determination of the value or cost of the ingredients and the overall costs of an intervention; and an analysis of the costs in an appropriate decision-oriented framework. This approach was used for all procedural steps that are required for performing QFT-GIT testing.

In public health interventions, cost analysis should be based on economic costing rather than financial (253). Ideally, a better designed study could have been a cost effective analysis comparing QFT-GIT and TST as tools for diagnosing tuberculous infection. The goal of cost-effectiveness analysis is to identify interventions that provide the greatest health impact with

the lowest cost per unit of output. However, this did not come to fruition during the course of this study because it was not one of the objectives of the study.

There are many guidelines on the use of QFT-GIT for diagnosing TB infection, some of which are based on cost effective studies. The perspective of our study was that of a provider and thus did not take other costs into consideration such as those to the patients. By using the financial approach of costing we did not include indirect costs and thus did not put the opportunity cost into consideration.

Our analysis has some limitations in that the costs did not include the cost of maintenance and servicing of equipment. This is because records on expenditure specific to this study were sometimes difficult to triangulate. The costs of ZAMBART administration were not included in the overhead cost because it was difficult to estimate the percentage of their contribution to the study. However, this would not have a significant effect on the final cost because QFT study expenditures were embedded in the ZAMBART's main expenditures.

9.2 Baseline results

Study population at enrolment

This study is among the first studies in HHCs in SSA to conduct both TST and QFT-GIT tests using a large sample size which illustrates the realistic implementation of QFT-GIT in a setting with a high burden of TB and HIV. It is one of the largest cohort studies evaluating the predictive value of QFT-GIT among HIV positive and negative HHCs in SSA.

The QFT-GIT cohort was nested within ZAMSTAR, which was a big study involving approximately 1.2 million individuals. Although the aim of this section is to discuss baseline results of the QFT-GIT cohort study, a brief discussion of the ZAMSTAR SOCS numbers at enrolment is necessary due to the nested design of the study under discussion. In addition, some of the findings or limitations of this study arise from the design of the main study.

Out of the 16,824 adults enumerated in the ZAMSTAR SOCS study 9,582 (56.8%) agreed to take part in the SOCS study. Reasons for not enrolling into the study show that refusal rate was high (62.6%) as well as absenteeism (29.2%). High refusal rate was probably because ZAMSTAR was the first time most of these communities were exposed to a trial of this magnitude and the concept of research especially the idea of signing for one's consent. Many individuals in these communities had been exposed to routine home based TB/HIV services that didn't require consent. Other reasons for refusal could have been due to lack of community trust, poor explanation of the study by study staff especially in households where individuals had already consented to taking part in one component of the study; both the household intervention and SOCS teams visited the same households. Also some people may have refused because they had to answer three long questionnaires during each visit. While some of these reasons were addressed and improved during the course of the study, for SOCS it was not possible to recruit new individuals in subsequent follow-ups as this was a cohort. It was not possible to

differentiate at this stage the refusals that were specific to the QFT-GIT cohort study as this data was not being collected. Like every study, ZAMSTAR was designed to meet certain objectives with little flexibility making it difficult to collect many additional data specific for this study without making the entire process complex for the study participant.

High rate of absenteeism was due to the fact that most of the individuals in these households were out trying to earn a living through socio-economic activities. Additionally, at the beginning of the study no prior formal appointments were made to visit some of these households; once the index TB patient gave the study team permission to visit the house, household members were visited on the same day although thereafter appointments were made for those who were absent. An individual was described as absent only after three failed visit attempts. Similarly as for refusal, it was not possible to determine which absenteeism was specific for QFT-GIT as this data was not being collected.

We were not able to determine reasons why some 228 HHCs did not have QFT-GIT tests done. However, since QFT-GIT was offered to everyone, it can be assumed that QFT-GIT tests were not done for the same reasons explaining why HHCs were not enrolled into SOCS at the different follow-up times. Such reasons were due to refusal, absenteeism, moved or died. This is why we restricted the analysis to adults enrolled in SOCS and in whom QFT-GIT test was performed.

In this study 88 samples were excluded either because the blood samples were spoilt or insufficient. Usually samples were considered spoilt if blood had hemolyzed in the mitogen tube (if any other tube the sample was run), incubation had been severely affected by disrupted power supply or if not well stored prior to being processed.

The study population at baseline consisted of 1,789 HHCs who were predominantly women (71%) and had a median age of 28 years (IQR: 21-43). This study enrolled few men at enrolment (>70 % of HHCs at enrolment were women) because it was difficult to find men at home as they were out involved in socio-economic activities, some of which included income generating activities. This finding is not unfamiliar for most African settings. Men may also have been absent from home because of social activities such as beer drinking as well. Some of the qualitative work in these communities before the start of the study showed high levels of unemployment in these communities and that men spent a lot of time away from home in taverns and beer halls (ZAMSTAR BBS findings, unpublished). In this study, only 23% of HHCs were employed. Although a majority of HHCs in these communities reported that they did not take alcohol (75.5%) or smoke (82%) this could have been due to reporting or response bias.

As expected in this setting, overall, the prevalence of HIV infection in HHCs was high at 27.9%, a reflection of the burden of disease in the sub-Sahara region. The prevalence of HIV was higher, overall in Zambian communities compared to SA ones probably because Zambia had double the number of communities in SA, hence more HHCs. It is well known that SA has one of the highest prevalence of HIV in the sub-Sahara region (89, 91, 92). In 2009, HIV prevalence in antenatal women was 16.4% in Zambia (93) and 29.4% in South Africa (89, 91, 92).

HIV prevalence was highest in the youngest age group, 15-24 years. Young people, ages 15–24, account for 41% of new HIV infections (among those 15 and over)(254). As expected and widely accepted, HIV prevalence was higher in women compared to men overall and when stratified by country**.** Women represent slightly more than half of all people living with HIV worldwide, and 60% in sub-Saharan Africa. Gender inequalities, differential access to services, and sexual violence increase women's vulnerability to HIV, and women, especially younger women, are biologically more susceptible to HIV. In sub-Saharan Africa, the HIV prevalence rate among young women is more than double that of their male counterparts (254).

Uptake of IPT among self-reported HIV positive HHCs was low at 4.7% (7/148). This is because IPT is not routinely offered by the Ministry of Health in Zambia and in SA its uptake has been problematic even after the policy was revised in 2010 to remove TST as a requirement to start therapy (255). Nevertheless, at the time of this study a pilot project to demonstrate the operational feasibility of providing IPT through the primary health care system was done in the 16 ZAMSTAR sites by ZAMBART project and the MoH in Zambia. However, in this study because of the few numbers, further baseline or survival analysis with IPT data was not possible to do.

Prevalence of Tuberculous infection

Findings in this study suggest a high prevalence of TBI among this population, 63.7% as determined by QFT-GIT (0.35 IU/ml cut off) and 39.7% using TST (10 mm). These findings are consistent with results observed elsewhere (166, 182). In a community based cross sectional study in Ethiopia among 652 healthy pastoralists, the prevalence of TBI was 63.7% using QFT-GIT and 31.2% using TST (10 mm)(182). In another prospective study enrolling 5244 adolescents aged 12-18 years from high schools in Cape Town, SA, 50.9% had a positive QFT-GIT while 42.2% had a TST $\geq$10 mm (166). In a recent systematic review by Fox and colleagues looking at 203 published studies of which 95 studies were from middle and low income countries to determine prevalence of TBI and incidence of TB in HHCs, the prevalence of LTBI (defined as TST $\geq$10 mm or definition applied in paper if not available) was 51.5% (256).

In a cross sectional study done in Zambia by Talati and others to assess the prevalence of and risk factors for a positive test for TBI, among 596 (298 HIV-discordant couples) individuals, the overall prevalence of TBI was 55.5%, 47% and 44.5% using TST, QFT-GIT and TSPOT respectively. In contrast to this study, in the study by Talati and others, a positive TST was defined as ≥5 mm of indurations in HIV-infected persons and ≥10 mm in HIV-seronegative persons and HIV prevalence was very high since half of the participants were HIV positive (257). In another study done in India among 719 health workers, the prevalence of TBI was 40.1% using QFT-GIT and 41.4% using TST (258).

In this study, TBI prevalence was lower by 24 % when defined by TST compared to QFT-GIT (i.e. difference in % positive). Despite our teams being trained and the reading standardized, TBI prevalence as defined by TST (10 mm cut off) was lower than expected probably due to the effect of HIV and the well recognized limitations in its operating characteristics. In Zambia, because of the geographical position of the communities most of the study procedures for both

SOCS and QFT-GIT were decentralized while in SA where possible a central team was maintained. Therefore overall, TST testing in both countries was done by different teams (one team consisted of two nurses and one research assistant) although as much as possible the same injectors and readers were used for a specific community. Use of different teams may have contributed to inter-reader variability. In addition, it is not widely appreciated that a substantial proportion (perhaps up to 50%) of close contacts of microbiologically confirmed index cases, even in many high burden settings, have no immunodiagnostic (positive TST) evidence of LTBI (10).

TBI prevalence in HIV positive HHCs was lower than that in HIV negatives for both QFT-GIT (HIV positives 52.8% Vs. HIV negatives 67.8%) and TST (HIV positives 34.6% Vs. HIV negatives 41.5 %), as shown elsewhere (131, 173). QFT-GIT just like TST is prone to false negatives in HIV positive populations.

In line with recommendations of using a 5 mm cut-off for HIV positives (259), TBI prevalence in this study was 47.03%. In settings with high TB prevalence, use of TST at the 5 mm cut-off even in HIV positives may result in increased false positives; hence others still recommend use of a 10 mm in this group (260). Current evidence suggests that IGRAs perform similarly to the TST at identifying HIV-infected individuals with TBI (131, 173). In the study done in Zambia by Talati and others, HIV positive persons were significantly less likely to have a positive TST, QFT or TSPOT result (differences in % among HIV positive and negatives ranged from 16-26%) (257). In a study done in Western Cape Province in SA, among 43 HIV infected adults and children, prevalence among the different tests was 28% QFT; 61% TSPOT and 41% TST (147). The QFT results are lower than ours due to the small sample size and a different study population (almost half were children). In a study conducted in a SA community a large proportion (58%) of the HIV positive patients had cutaneous anergy to PPD (261).

TBI prevalence was higher in SA communities compared to the Zambian ones, using both QFT-GIT (SA: 71.1% vs. Zambia 58.3%) and TST (SA: 47.2.1% vs. Zambia 34.1%) as in previous findings (95). This is because overall, the burden of infection and disease is higher in the SA compared to Zambia. A large TST survey done in 2005 as a baseline study and for randomization purposes of the ZAMSTAR study found that the ARTI in the Zambia was between 0.8%-2.8% and 2.5% -4.2% in SA, depending on the method used (95). These results confirmed that TB transmission is high in these communities, irrespective of the method used to define it. In the 2012 WHO Global TB report, incidence rate of TB was 444/100,000 for Zambia and 993/100,000 for South Africa thereby confirming findings in this study (85).

Risk factors associated with positive QFT-GIT and TST results

Although neither IGRAs nor TST are gold standards for TBI, an analysis of risk factors associated with test positivity are used as a proxy of the risk factors for *Mtb* infection. In this study, important risk factors associated with QFT-GIT positivity were HIV positivity (aOR: 0.48; p<0.001), residing in an urban area (aOR: 2.37; p<0.03), smear status of index (OR: 1.34; p=0.01) and country (aOR: 1.93; p<0.001) in multivariable analysis adjusted for age, sex, country and

household clustering. Similar results were obtained for TST. QFT-GIT may not be more sensitive than the TST to detect risk factors associated with TBI.

As expected, exploration of QFT-GIT and TST results by age revealed a trend to increased responses with increasing age. Studies (61, 166, 262) have shown that QFT-GIT positivity correlates with increasing age as a result of a higher prevalence of TBI in the older age groups (due to cumulative exposure in settings with a high burden of TB) or suboptimum sensitivity in younger individuals. In two studies done in Columbia and Uganda, exploration of IFN-gamma variations by age revealed a trend to increased responses up to adulthood with CFP, but not with CFP-10 (35, 263). However, children were included in both of these studies.

HIV positivity was less common among those with positive results on QFT-GIT and TST. Some studies have shown that IGRA sensitivity is lower in individuals with HIV and severe immunosuppression (185, 222). Current evidence suggests that IGRAs perform similarly to the TST at identifying HIV infected individuals with TBI (131).

For both QFT-GIT and TST, prevalence of infection was higher in contacts exposed to smear positive index cases compared to smear negative ones, consistent with findings of other studies (264). It is widely accepted that infection is more likely to occur from close contact with a sputum smear positive case (58). The rates of infection in HHCs with a sputum positive index case are 30-50% above community controls (58). In sputum negative culture positive cases, transmission occurs to a lesser extent of 5% above community controls. Sleeping proximity of the contact to the index case was not associated with either QFT-GIT or TST results although this relationship has been well established in other studies that predominantly have HIV negative populations (211, 214). There is growing evidence suggesting a stronger and better defined association between surrogate markers for TB exposure and QFT-GIT results in low TB incidence settings compared to high-TB incidence settings (149).

It is now well accepted that transmission of TB in high incidence settings occurs not only within households but in the community as well (57) and among various social locations (265). A study in Zimbabwe found that the proportion of ELISpot positive contacts was not different from community controls (264) while in another study done in two communities in Zambia, almost 50% of community controls were QFT-GIT positive (63). The mix of intra- and extra- household transmission varies according to the degree of TB endemicity in the general population. In a high TB prevalence setting in Capetown, South Africa, isolates matched on molecular subtyping in less than half (46%) of households with two TB patients and it was estimated that only 19% of *Mtb* transmission occurred within households (57). However, an existing problem is the occurrence of multiple (mixed) infections in high prevalence settings. In such places, transmission routes are less clear. It has been argued that, in settings of high endemicity where a mixture of recent and old infections are commonly found, long term assays are more sensitive than those with shorter culture times (35).

Concordance between QFT-GIT and TST

An assessment of agreement between QFT-GIT and TST tests in this study showed that 28.2% were concordantly negative, 31.6% were concordantly positive, while 40.2% were discordant ($p<0.001$) at TST ≥ 10 mm. There was a low level of agreement between the tests regardless of TST cut-off point (% agreement=59.7%; kappa=0.24) consistent with findings of studies done in high-TB burden settings (183, 211, 264). As IGRAs are designed to be more specific than TST, perfect agreement is not expected (266). However, better agreement has been shown when the comparison is done within specific risk groups like HIV positives (218).

In this study, QFT+/TST- discordance (575/719; 80%) was more frequent than QFT-/TST+ discordance (144/719; 20%) at TST ≥10 mm and this was true of other TST cut-offs and regardless of HIV status. Particularly high number of QFT+/TST- discordant results have been found in studies from poor-resource settings (212). In contrast, studies done in settings with low TB incidence (202, 267) have shown that TST+/QFT- discordance is more common.

In this study, 20% of HHCs were positive by TST but negative by QFT-GIT. The reason for TST+/QFT- discordance has been generally explained by a false positive TST, reflecting former sensitization by either BCG or environmental mycobacteria/NTM or diminished sensitivity of QFT-GIT compared with TST. A study from South Africa found that among those with large TST reaction (>=15 mm), approximately one-third had negative QFT tests (183). In a study from India, 11% of individuals with positive TST (at least 15 mm) were negative by QFT (112).

TST+/QFT- discordance could also indicate a genuine difference between TST and IGRAs in their ability to detect remote and probably cleared infection versus recent, persisting TB infection (52). It has been suggested that TST and IGRAs may not be equivalent in what they measure. T-cells responding to the RD antigens after 24 hours stimulation are predominantly CD4 T-cells of an effector memory phenotype, consistent with having recently encountered antigens *in vivo*. TST reflects the mobilization of a wider spectrum of memory T-cells that are long-lived and may even persist after clearance of live mycobacteria (5).

TST-/QFT+ discordance has been explained by a past history of TB infection, poor TST sensitivity or false positive QFT-GIT (197). However, TST+/QFT- discordance may indicate a diminished sensitivity of QFT-GIT compared with TST in low TB incidence settings rather than high TB incidence settings where it is increasingly being accepted that sensitivities of the two tests are most likely comparable (149). Another reason for TST-/QFT+ response discordance could be related to variations around the respective thresholds, since both tests are continuous measures (52). In a study in Cape Town among 43 HIV positive adults and children (147), in 42% of subjects with discordant results, IGRA values were close to the manufacturers' recommended cut-offs. Low cut-off points may be a possible cause of lower specificity and higher rates of false positives.

In clinical practice, discordance between QFT-GIT and TST follows different guidelines. For instance in the case of an individual with a history of BCG vaccination and a positive TST and

negative IGRA results, if risk for TBI is otherwise low, it is reasonable to assume that the TST result is false positive and to withhold further evaluations (149). In the case of a negative TST and positive IGRA results, if the individual is considered to be at a high risk for TBI the negative TST result alone should not prevent further examination (149). Another consideration when interpreting discordant TST and IGRA results is the observation that TST preceding IGRA testing could boost IGRAs (268). This effect appears more pronounced on the days after the TST and could wane with time. However, this study did not explore reasons for discordance between the two tests as it was outside the scope of this thesis.

9.3 Are IGRAs better than the TST at predicting HHCs at highest risk of progression to active TB?

In 2010, WHO identified the prognostic ability of IGRAs compared to the TST, to accurately identify people living with HIV at a higher risk for progression from latent to active TB as a priority research question in resource limited setting (269). This research gap has not yet been adequately addressed. In this study, I aimed to address this.

The aim of this thesis was to report overall results for Zambia and SA due to the expected small number of TB incident cases (the primary outcome). However, exploratory analyses by country revealed that there were important differences between the countries that could affect or explain the overall results. Therefore in some sections of this thesis, results were presented by country to emphasise these important differences. Some of these differences could be explained by differences in the burden of TB infection and disease and some variation in the implementation of the study between the two countries. Country was also identified as an effect modifier in the association between QFT/TST positivity and TB incidence.

In this study, I report a high incidence rate of TB in HHCs, compared to the general population. Out of the 1,113 HHCs that entered follow-up, 51 (4.6%) developed active TB over 2,433 pyrs, giving an IR of 20.96/1000 pyrs. This represents TB rates that are more than that of the general population in Zambia and SA respectively. TB rates were 993 for SA and 444 for Zambia per 100, 000 population in 2011 according to WHO's global TB report (85). It is well known that close contacts of infectious TB cases such as HHCs are at a higher risk of becoming infected with *Mtb* and development of active TB (256). Studies have shown that individuals with recent infection such as HHCs rapidly progress to disease (64, 66, 270) and that recent infection is ten times more likely to produce a case than a long standing infection (271).

The overall TB incidence rates in this study were also higher than those found elsewhere. In a study in Senegal, among 2,679 HHCs,TB was diagnosed in 52 contacts; IR was 9.27/1000 pyrs (159). Among 2,348 HHCs in Gambia, 26 were diagnosed with definite TB; IR was 6/1000 pyrs (164). Out of 908 children and adolescents HHCs in Turkey, 15 developed TB; IR was 12.5/1000 pyrs (158). In one of the largest prospective studies conducted among 5,244 high school adolescents in Cape Town, SA (166), 67 cases of bacteriologically confirmed TB were detected giving an overall incidence of 4.5/1000 pyrs.

The proportion of HHCs with incident TB reported in this study is consistent with previous findings in contact investigation studies in similar settings (272). HHCs studies among TB patients and large epidemiological surveys have established this effect (273). Morrison and colleagues performed a systematic review to determine the yield of TB in HHCs (273). In this review which included 41 studies the overall yield for all TB (bacteriologically confirmed and clinically diagnosed) was 4.5% (95%CI: 4.3-4.8) of contacts investigated; for cases with bacteriological confirmation the yield was 2.3% (95%CI:2.1-2.5)(273). In this study, the yield of TB was 4.6%.

There was some evidence that QFT-GIT positive (IRR: 1.65; 95%CI: 0.86-3.37; p=0.06) and TST positive (IRR was 1.88 ; 95%CI: 1.04-3.41; p=0.01) HHCs had a two-fold increased risk for developing TB compared to test negative ones, although for QFT-GIT the results were not statistically significant. These results are consistent with results shown elsewhere in similar settings (108).

In this study, IR was highest among HHCs positive by both tests, 32.4 (95%CI: 21.87-47.89) per 1000 pyrs. This IR is much higher than that of longitudinal studies reporting IRs for both TST and IGRA done in Gambia, Turkey, Senegal and India (158, 159, 164, 274). In these studies, the IRs ranged from 3.9 to 22.2 per 1000 pyrs in individuals with positive concordant results; however the study populations were different (children, HHCs, HCWs). In the study in Senegal, HHCs with both TST and ELISPOT positive responses had an IR of TB of 14.74/1000 pyrs (159). In the study in India, HCWs with concordant positive test results had an IR of 3.90 (95%CI:1.62-9.38) per 1000 pyrs although results were not statistically significant (274). In the studies in Turkey and Gambia, the IR among ELISpot-positive and TST-positive were 22.2 (95% CI: 10.6–40.8) and 8.86 (95%CI: 2.4-15.4) per 1000 pyrs respectively.

The IR among discordant pairs in this study were comparable to each other. There was little difference in IRs between the discordant pairs for the three studies done in Gambia, Senegal and India (IRR ranged from 1.1 to 1.3)(159, 164, 274) while the study in Turkey (158) showed an IRR of 1.58 (95%CI: 0.03-30.38)(275). IR was lowest among HHCs with negative concordant results, 14.42 (95%CI: 7.76-26.81), as shown elsewhere (158, 164).

A key finding in this study was the strong evidence supporting the association between QFT-GIT positivity results and development to subsequent TB in both unadjusted and adjusted analyses. Compared with test-negative results, QFT-GIT-positive (aHR: 2.20; 95%CI: 1.14-4.25; p=0.02) and TST-positive (aHR: 2.19; 95%CI: 1.24-3.86; p=0.007) results had similar risks of progression to TB in multivariable analysis adjusted for sex, age and HIV. These findings are consistent with IRR reported in this study for both tests. This is the first time these results are being reported in HHCs in a high TB/HIV burden settings like Zambia and SA although previous studies done in Colombia, Gambia and Senegal have shown similar results (35, 159, 164).

The studies done in Colombia, Gambia and Senegal found that HHCs with positive ELISPOT or TST results at baseline were associated with a two-fold increased risk of developing active TB compared to those who were ELISPOT or TST negative (35, 159, 164). Although all three studies

involved HHCs followed up for 2-3 years, important differences with this study were the use of different assays and the lack of a HIV infected population (2% were HIV positive in the study in Gambia). All three studies used in house ELISPOT or whole blood assays in contrast to the commercial types of IGRAs, like QFT-GIT (35, 159, 164). Despite this, all these studies including a recent systematic review by Rangaka and others (275) confirm similar findings as of this study.

In the systematic review by Rangaka and others (275), 15 studies with a combined sample size of 26,680 participants were evaluated. Compared with test-negative results, IGRA-positive and TST-positive results were much the same with regard to the risk of TB; pooled IRR in the five studies that used both was 2.11 (95% CI: 1.29–3.46) for IGRA vs. 1.60 (0.94–2.72) for TST at the 10 mm cutoff(275). However, heterogeneity was high. In addition, the relative risks differed between studies with possible incorporation or work-up biases (IGRA considered in the diagnosis of TB mainly from high income countries; RR 8.35; 95%CI: 3.19-21.87) and studies without this bias (from low-income or middle in-come countries; RR 2.22; 95%CI: 1.54-3.19)(275).

In the review by Rangaka and others, there was only one study done in SA; the study in high-school adolescents in SA (166, 275). Furthermore, apart from the study done in Kenya among HIV positive pregnant women, no other study had a "reasonable" at risk population of HIV negative and positive individuals (in one study in Gambia, 2% of individuals were HIV positive)(167, 275). Most of the studies done in inpatient or outpatient clinics consisted of HIV positive individuals only (167, 172).

A study in Turkey that looked at children and adolescents (0-16 years) HHCs, 76% whom were given IPT found a 3- to 4-fold increased risk for progression to TB in ELISpot positive HHCs relative to ELISpot-negative ones (158). Among silicosis outpatients (33% were given IPT) in China, Leung and others found that a positive T-Spot.*TB* test significantly predicted the subsequent development of active TB (relative risk: 4.50, 95% CI: 1.03-19.68) and culture/ histology-confirmed TB (relative risk: 7.80, 95%CI: 1.02-59.63)(168). The details of these studies have been well summarized in two systematic reviews (275, 276).

Results by country showed that there was strong evidence that QFT-GIT positive HHCs residing in Zambia had a five-fold increase in IRR compared to QFT-GIT negative ones (IRR: 5.24; 95%CI: 1.59-27.21; p=0.0007). This is the first time this finding is being reported.

For HHCs residing in SA there was weak evidence that QFT-GIT-positive results (IRR: 0.60; 95%CI: 0.25-1.50; p= 0.11) could predict subsequent development of active TB in HHCs. This evidence remained the same in both unadjusted and adjusted analyses. Interestingly, there was some evidence in both unadjusted and adjusted analyses that positive TST results could predict who develops active TB, although numbers were small. For the TST, HR remained similar across all models, as in the overall results and the ones for Zambia. In all models, there was some evidence that TST positive HHCs had a two-fold increased risk of developing TB, although results were not statistically significant due to the small numbers. Hence whilst results may reflect that QFT-GIT was not predictive of who develops TB in SA, TST consistently showed this evidence.

The QFT-GIT results from SA reported in this study are different from a recent study (166) done in the same setting. In the study in Cape Town SA, among 5,244 adolescents (166), IRR was 2.9 (95%CI: 1.55-5.41) in QFT-GIT and 2.7 (95% CI: 1.4–5.0) in TST positives compared to negatives. However, the difference in results with this study could be differences in target population, age groups and inclusion of a HIV positive individuals (166).

Some reasons can be explored to explain the differences in QFT-GIT results between Zambia and SA. Some obvious reasons have been discussed in detail in the introduction and these include differences in the following areas: climatic conditions; urbanization and stage of TB/HIV epidemic (see details in introduction). It is plausible also that because SA has a higher prevalence of infection (also confirmed by this study) and disease the force of infection in SA is very high i.e. that most people had converted by the time TB was being diagnosed. In addition it is also plausible that the QFT-GIT test did not work well due to technical reasons or a reduced sensitivity which was as a result of an advanced HIV epidemic. This study did not have evidence that HHCs residing in SA were more severely immuno-suppressed than those in Zambia but this study showed consistent results in SA in both IRR and HR. It is plausible that the HIV positive HHCs in SA were more severely immuno-suppresed which led to less QFT-GIT positivity and caused interaction.

Predictive value of QFT-GIT versus TST

Overall, the PPV for QFT-GIT was 5.4% while that of TST was 6.6%. These observations mean that the majority of HHCs (more than 95% for QFT-GIT and 93% for TST) would not progress to disease during the observed follow-up time. The overall PPVs reported in this study were not consistent with those reported in a systematic review consisting of studies done mainly from low TB incidence settings (142). In the review by Diel et al, the PPV for QFT-GIT and the T-SPOT.TB both showed a similar PPV for progression;(2.8-14.3% for QFT-GIT; 3.3-10% for TSPOT.TB and 2.3–3.3% for TST)(142). However, limitations of this review were the low number of individuals included in the studies, the short durations of follow-up and absence of studies from high burden settings.

Overall both tests had comparable NPV estimated at 97%. High NPVs were found for five studies performed in low-incidence for both the QFT-G-IT and the T-SPOT.TB (pooled NPV of 97.8% for TSPOT.TB and 99.8% for QFT-G-IT). This indicates that an individual testing negative will most likely not develop TB in the future. Similar conclusions can be drawn for PPV and NPV when results were stratified by country.

Based on a systematic review (275) and international expert opinion, WHO recommended in 2011 that neither IGRAs nor the TST should be used in low- and middle-income countries for the identification of individuals at risk of developing active TB (strong recommendation)(111). At the time of issuing this policy on IGRAs, the quality of evidence for the predictive value of IGRAs was very low. The recommendation by WHO was based on three studies with heterogeneous populations (older males with confirmed silicosis, school-going adolescents, and adult TB contacts including HIV-infected individuals (111). The three studies included one published

study from China (168) and two unpublished (Zambia and South Africa) (Zambia: one presented in this thesis and South Africa now published by Mahomed and others (166)).

Much of the evidence from which the WHO policy was based on showed an association with subsequent incident TB in test-positive individuals compared to test negatives appeared higher for IGRA than for TST; however, this was not statistically significant (IGRA: IRR=3.24; 95% CI 0.62-5.85; p=0.90; TST: IRR=2.28; 95% CI 0.83-3.73). Furthermore, the vast majority of individuals (>95%) with a positive IGRA result did not progress to disease during follow-up.

Therefore as previously reported by others (108, 111) both IGRAs and the TST appear to have modest predictive value and may not help identify those who are at highest risk of progression to disease. Several reasons could be put forward as to why the predictive value of QFT-GIT is likely to be modest. One of the reasons suggested for lack of prognostic value of IGRAs in high burden settings is the endemicity of TB; community transmission may dilute prognostic usefulness (167). In a setting where TB is endemic and households are large and likely to contain co-prevalent TB cases, it is extremely difficult to determine the exact time of TB exposure (where an IGRA assay is likely to perform the best). IFN-γ alone might not be sufficiently predictive of progression (or correlate of protection). A single TST or IGRA result cannot tell us about the underlying phenotype of when infection occurred and how the host immune system responded (159).

Overall incidence rates and risk of progression to active TB in HIV infected HHCs

Even though the evidence so far seems to show that IGRAs have modest predictive ability (108) and may be by themselves only unlikely to be sufficient enough to act as predictive markers for disease, longitudinal data especially in HIV infected populations is still lacking. Currently there are only two published systematic reviews specifically evaluating the predictive value of IGRAs in this area (108, 276).

In this study, overall, the IRR was higher among HIV negative HHCs (IRR: 3.85; 95%CI: 0.90-34.51; p=0.02) compared to HIV positives (IRR; 1.93; 95%CI: 0.88-4.57; p=0.04) for QFT-GIT. Unadjusted analysis showed similar results. Overall, for TST, the IRR among HIV negatives (IRR: 2.21; 95%CI: 0.78-6.72; p=0.05) was similar to that among HIV positives (IRR: 2.32; 95%CI: 1.09-5.00; p=0.009).

Another principal finding was that there was strong evidence of a five-fold increased risk of TB in HIV positive QFT-GIT positive HHCs compared to HIV positive QFT-GIT negative ones (aHR : 4.72; 95%CI: 1.35-16.46; P=0.01) while that of TST remained modest (aHR: 2.37; 95%CI: 0.94-6.02; p=0.07) in the same group.

Overall, among HIV negatives, the PPV and NPV of QFT-GIT was comparable to that of TST (NPV range 98.5%-99.2% and PPV of approximately 3.00% for both tests). These results contribute to the already existing evidence indicating that NPV for QFT-GIT is very high especially for HIV negative populations. In HIV positives, the NPV of both tests remained comparable but lower

than that in HIV negatives (NPV approximately 92.0% for both tests), the PPV for QFT-GIT was lower at 13.8% (95% CI: 8.88-29.22) compared to that of TST at 17.2% (95% CI: 10.33-26.06).

There are at least two systematic reviews examining the role of IGRAs for the diagnosis of LTBI in HIV infected adults (131, 173). Of the 38 studies assessed in the review by Santin and others (173), only three studies conducted in low TB incidence settings (UK, Austria, and Spain) were reported. In the systematic review by Cattamanchi and others (131), these same three studies are used to evaluate the predictive value of IGRAs in HIV infected individuals with an additional one (277).

The systematic review by Cattamanchi and others identified 37 studies that included 5,736 HIV infected individuals (131). In three longitudinal studies, the risk of active TB was higher in HIV-infected individuals with positive versus negative IGRA results. Each study showed that IGRAs had poor positive predictive value but high negative predictive value for active TB. However, the studies had limitations, including small sample sizes with short duration of follow-up (131, 174, 277) and differential evaluation and/or follow-up of persons with positive and negative IGRA results (131, 172, 174, 277). Only one study (172) had an adequate duration of follow-up (1 year or greater) and no study performed adequate outcome assessment (ie, ruled out active TB at baseline and evaluated all participants for active TB during follow-up). In addition, all studies had few (less than 12) incident cases of active TB (131) and the review had limited evidence especially from resource constrained settings.

Incidence rates and risk of progression to active TB in HIV infected HHCs by country

Results were different when the effect of HIV was factored in by country. Generally the adjusted HRs in Zambia were similar to the overall results for both tests while in SA results were different for QFT-GIT. For HHCs residing in Zambia, the IRR was 5.12 (95%CI: 1.45-27.57; p=0.002) in HIV positives while it was 0 among HIV negative for QFT-GIT (no one developed TB among the HIV negative QFT-GIT negative group). This is higher than IRRs reported in some studies that have HIV positive populations (167, 174), apart from the one done in Austria (172). The only comparable study is the one done in China in silicosis patients which is a different population (168). Similar to IRR in Zambia, in multivariable analysis adjusted for age and sex, there was strong evidence of a five-fold increased risk of TB in HIV positives (aHR: 4.77; 95%CI:1.36-16.66; p=0.01) for QFT-GIT. TST results for Zambia for both HIV positives and negatives were similar to the overall ones (table 6.10 and 6.11).

In both Zambia and SA, results suggest that there was no difference between QFT-GIT positive and negatives in HIV negatives HHCs (tables 6.11 and 6.12). These results are unreliable and should be interpreted with caution due to the small number of incident cases in the sub-groups.

In HIV positives in SA, there was weak evidence to suggest that QFT-GIT positivity was protective because the results were not statistically significant in both unadjusted and adjusted analyses (table 6.12).

Other few studies have looked at the risk for progression to TB in HIV infected populations (167, 172-174). Three of these studies (167, 172, 174) showed a higher risk in IGRA-positive compared to IGRA-negative subjects, but one study could not confirm this hypothesis because no cases of TB occurred (173). In this study done in Spain assessing 135 HIV-infected individuals, none of the 103 patients who had a negative or indeterminate QFT-GIT result and negative TST at baseline developed TB after a median follow-up of 20 months. All the 15 patients who were QFT-GIT positive at baseline accepted IPT apart from one (173).

Studies that showed a higher risk in IGRA-positive compared to IGRA-negative subjects in HIV infected populations were done in Austria, United Kingdom and Kenya (167, 172, 174). In the study done in Austria (172) among 822 HIV positive out-patients, active TB occurred among 8.1% (3/37) of QFT-GIT positive; 0% of QFT-GIT negatives (0/738) and 0% (0/47) QFT-GIT indeterminates at baseline during the 19 months of follow-up. The risk ratio for positive vs. negative IGRA for this study was reported as 136.13 (95%CI:7.16-2588.46) (108) but was grossly limited by incorporation or work-up bias. In this study, individuals with a positive QFT-GIT assay result were extensively investigated for active TB (including cultures and PCR) while those with negative results were not; making it likely to miss active TB in the group.

In the UK study with 201 HIV-seropositive individuals, two out of 20 infected patients with positive T-SPOT.TB who did not receive IPT developed active TB during the 12 months follow-up (174). Furthermore, none of the 114 patients who were T-SPOT.TB negative developed TB. In the Kenya study done among 333 HIV positive pregnant mothers, risk ratio for QFT-GIT positivity was 2.69 compared to test-negative mothers (108).

Currently there is no meta-analysis that primarily focuses on the predictive value of IGRAs in HIV infected population. Yet this should be the focus of most predictive value studies so that these people can benefit from IPT. The risk of developing active TB in TST-positive HIV infected individuals is well documented.

Limitations of longitudinal studies evaluating risk of progression to disease

In reviewing these results and other studies looking at the predictability of IGRAs one needs to keep in mind the limitations of these tests and that they detect host immune response, rather than the activity of the pathogen itself (278). Nor can they overcome the diagnostics difficulties posed by remote infection and ongoing transmission after testing (278). In high income low TB incidence countries where re-infection and ongoing transmission is low, IGRAs have been reported to work better (149). Many low TB incidence high-resource countries have incorporated IGRAs in their guidelines with considerable diversity in their approaches (134, 135).

PPV and NPV are dependent on prevalence of disease in the population as well as the sensitivity and specificity of the procedure used. Therefore comparisons should be done with caution across regions with different burdens of disease. Otherwise, positive and negative likelihood ratios are more accurate than NPV and PPV, because likelihood ratios do not depend on prevalence but these were outside the objective of this study. While a positive test may be

helpful in predicting disease, a negative test suggests that risk of progression to disease is low although it does not rule out the possibility entirely (166). High prevalence of disease will increase the PPV but decrease the NPV.

The studies comparing the predictive value of TST and IGRAs for active TB have limitations. There are important differences amongst the studies -different products are compared, different cutoffs are used for TST and different populations have been studied. The designs, measurement differences; quality of study with regard to comparability (adjustments made to effect measures) and outcome (ascertainment of incident TB, losses to follow-up, and reporting of incidence rates vs. cumulative incidence), leading to possible verification bias (108) and lack of adjustment of all confounders may also explain some of the different results obtained (108). This limits the degree to which these studies are comparable. The challenge of most studies done in high and middle income countries is that ethically they were done in individuals who had refused IPT, as it is the standard of care for LTBI.

Risk factors for the development of TB

Note: some of the evidence for this section has already been reviewed (see discussion on risk factors associated with QFT-GIT positivity section).

Most risk factors for infection will apply to disease as well. In this study, there was no difference in the risk of TB by sex. This is inconsistent with what has been known about sex differential in TB from the past although currently there is no consensus.

In most settings, TB incidence and prevalence are higher for males at all ages except in childhood, when they are higher in females (279). The reasons for the higher male prevalence and incidence are poorly understood. Whether, and the extent to which, identifying fewer women with TB globally is due to sex (as a biological determinant) or gender (as a socio-cultural determinant influencing access to TB care) have been issues for discussion and debate (279). While some attribute it to barriers women may face in accessing TB care, risk of exposure to infection, in health seeking behaviour and health systems response and economic consequences, others ascribe it to the natural epidemiology of the disease (279). Some studies have also shown that progression from TB infection to disease is likely to be faster for women compared with men in their reproductive years, and faster for men after 40 years of age (73).

There was some evidence in this study that the age group 26-39 years was associated with TB (HR: 1.95; 95%CI: 0.89-4.27; p<0.001). A prevalence survey conducted in two districts in Lusaka, Zambia found that the prevalence of TB was highest in the 35-44 years age group (aOR 3.31) but there was no difference seen between the prevalence in men and women (68).

In this study, there was no association between socioeconomic status and active TB. Employment was used as a proxy to socioeconomic status. It has been reported that people with low socioeconomic status have a higher risk of being infected and a higher incidence of TB but evidence is not conclusive (280). Part of the limitations of studies looking at the association between socioeconomic factors and TB are due to unclear measurements/definitions of

socioeconomic position at household and/or individual level (74, 280). Some of the recent evidence of the association between socioeconomic status and TB comes from a case-control study nested within a population-based TB and HIV prevalence survey conducted in 2005–2006 in two Zambian communities. Prevalent TB was significantly associated with lower household SEP (aOR = 6.2, 95%CI: 2.0–19.2 and aOR = 3.4, 95%CI: 1.8–7.6 respectively for low and medium household SEP compared to high)(74).

There was some evidence to suggest that a higher level of education was protective against TB (aHR for university/college: 0.74; 95%CI: 0.12-4.53) compared to low education levels (aHR for primary education: 1.66; 95%CI: 0.39-7.13) although results were not statistically significant. A case-control study to evaluate risk factors for TB in South India found that a higher level of education was significantly protective against TB (OR:0.30; 95%CI: 0.11-0.82) (281). Both employment and education can be considered as proxies of a socioeconomic status.

As expected, there was strong evidence that smear positivity of the index (HR: 2.00; 95%CI: 1.04-3.87) was associated with TB. Epidemiological studies conducted during mid-20th century have shown that smear positive cases are more infectious than the others (55, 273).

There was some evidence that sleeping in the same room and bed as the index case was associated with a higher risk of TB (HR:1.88;95%CI:0.64-5.53) although this evidence was not sustained in adjusted analysis. The influence of the proximity to an infectious case was shown in outbreak investigation studies. In one study, passengers seated within two rows of the index TB patient were more likely to have positive TST compared to those in the rest of the section (30.8% versus 3.6%, RR = 8.5, CI = 1.7–41.3)(273).

Although we found weak evidence of an association between incident TB and alcohol (HR: 1.64; 95%CI: 0.86-3.15; p=0.13); incidence rates were higher in those who self-reported to have haven taken alcohol. The association between alcohol use and TB could also be explained by both increased risk of infection related to specific social mixing patterns associated with alcohol use, as well as influence on the immune system of alcohol itself and of alcohol related conditions (282).

In this study, there was strong evidence that occasional smoking, (HR: 4.07; 95%CI: 1.31-12.63; p<0.0001) was associated with TB compared to non-smokers. Studies have shown that smokers are approximately twice as likely to be infected with TB and are more than twice as likely to develop active TB, compared to non-smokers (283). Smoking increases the risks of becoming infected with TB, of developing the active form of disease, and of dying from it (76, 283).

In this study univariable and multivariable analyses for risk factors for incident TB may have been limited by the small number of incident cases. For most risk factors confidence intervals were overlapping even if HRs were suggestive of an effect.

9.4 Are household contacts with high levels of IFN-γ in response to *Mtb* specific antigen in the QFT-GIT assay at higher risk of developing TB?

In the 2010 updated guidelines on the use of IGRAs, CDC recommended that both the qualitative results and the quantitative assay measurements for IGRAs should be reported (132). Reporting IFN-γ measurements does provide useful information in individuals undergoing serial IGRA testing [51]. However, in the absence of interpretive guidelines, this practice could lead to false assumptions and misinterpretation of the results. There are limited data on the significance of changing IFN-gamma levels [52]. It is not clear whether higher IFN- γ responses correlate with greater risk of progression to active TB. The manufacturer for QFT-GIT, Cellestis, also warns that the magnitude of the measured IFN-gamma level cannot be correlated to stage or degree of infection, level of immune responsiveness, or likelihood for progression to active disease (237). In a review published in 2011, Herrera and others (284) recommended that quantitative results should not be used for prognostic or therapeutic monitoring purposes because evidence is lacking or non-supportive.

Therefore the main reason behind addressing the main question in this thesis is because of limited data on the significance of IFN-γ levels (268) in relation to disease progression. It is not clear whether high IFN-γ responses correlate with greater risk of progression to active TB. It has been postulated that individuals with strong and/or rising IFN-γ responses to QFT-GIT may be at greater risk for progressing to active disease (22, 28, 33, 50, 141). It is thought that among recently exposed individuals are those with strong ESAT-6/CFP-10 responses or high levels of IFN-γ in response to *Mtb* specific antigens that offer a vigorous T-cell response as a result of active bacterial replication (39).

Overall, out of the 707 HHCs, 38 (5.4%) HHCs developed active TB over 1558.0 person-years (pyrs) of follow-up, giving an incidence rate of 24.39/1000 pyrs. The median follow-up time was 2.44 years (IQR: 1.59-2.82).

As expected, overall incidence rates were highest in HIV positives, at 63.08 (95% CI: 41.54-95.80) compared to HIV negatives, 13.58 (95%CI: 8.32-22.16). HIV positive individuals have been shown to be at highest risk of progression to disease (131). The principal finding in this study is that there was no difference in incidence rates between HHCs with low and high levels, by country and overall (overall IRR: 1.0 (95% CI: 0.42-2.18)). However, IRRs were different by HIV status. The IRR in HIV negatives was 1.70 (95%CI: 0.51-5.18; p=0.16) while in HIV positives it was 0.79 (95%CI: 0.15-2.70; p=0.38).

The overall findings in this study are similar to those found in another study in Turkey among children (158). Within ELISpot-positive contacts, the size of the baseline IFN-γ response to peptides did not significantly differ between contacts who developed TB and those who remained well (median SFCs per million peripheral blood mononuclear (PBM) cells, 176 (IQR: 88 to 1332) vs. 182 (IQR: 70 to 534); p= 0.27). This was also true for responses to recombinant antigen (median SFCs per million PBM cells, 246 (IQR: 200 to 918) vs. 264 (IQR: 124 to 526); p=0.183)(158).

Another study of 631 HIV infected Ugandan adults found that active TB was not associated with IFN-γ responses to PPD or CFP (285). Unadjusted IRR for PPD was 1.36 while for CFP it was 1.09. When the RR was adjusted for CD4 count, there was still no difference in IFN-γ response to CFP (adjusted IRR: 1.33; 95%CI: 0.60-2.97; p=0.50) while IFN-γ responses to PPD increased to almost two-fold (adjusted IRR; 1.77; 95%CI: 0.85-3.66; p=0.14).

In unadjusted analysis in this study, there was no evidence of an association between high IFN-γ levels and TB incidence overall (unadjusted HR: 1.02; 95%CI: 0.48-2.15; p=0.96). Results were similar when stratified by country. These findings are consistent with evidence from IRRs.

A prospective cohort study of 109 healthy HHCs in Pakistan assessed the patterns of cytokines at 0, 6, 12, and 24 months after exposure to TB (13). In this study, IFN-γ/IL-10 ratio was used which is thought to be a critical determinant of disease severity across the TB spectrum (13). The most significant findings were the exponential increases (approximately 1,000-fold) in both the CFP and the mitogen-induced IFN-γ/IL-10 ratio in healthy HHCs (*n*=26), which peaked at 12 months, compared to the levels in HHCs who developed disease (*n*=7), in whom relatively flat responses were observed during the 24-month period.

The study in Pakistan had the advantage that cytokine testing was done at four time points. However, in this study like many studies that have looked at the prognostic ability of IGRAs a single test showing a high level of IFN-γ response was used which does not provide information on when the response became elevated (whether this was recently or in the past) or whether it has stayed elevated for a long time (whether it has been persistently elevated or not). This has been recognized by others as a limitation of such studies (135).

Although there was no difference between HHCs with low and high IFN-γ levels, other studies have found contrasting findings. Some of the hypotheses behind these studies stem from studying TST longitudinal data. Since it is well documented that individuals with recent TST conversions have a high probability to develop active TB, it is plausible that strong increases in interferon-γ responses after recent exposure might predict progression towards active disease (28, 33, 50). These high levels of IFN-γ production in response to *Mtb*-specific antigens may imply a risk factor for developing active TB (28, 107, 161).

In multivariable analysis adjusted for age and sex, results were similar as to the unadjusted analysis. Although there is substantial evidence indicating age and sex as risk factors for the development of TB in individuals infected with *Mtb*, no specific evidence exists indicating them as important risk factors in the relationship between high IFN-g levels and TB.

Despite the weak evidence from the interaction test (p=0.36), HIV was considered as an effect modifier. HIV was an effect modifier in the relationship between high IFN-γ levels and TB incidence as results were clearly different in HIV negative and positive HHCs. This is because it is likely that power to provide evidence for interaction in this study was reduced due to the small number of incident cases.

Unadjusted HR among HIV negative HHCs was 1.73 (95%CI: 0.63-4.77; p=0.29) and 0.75 (95%0.22-2.55; p=0.65) among HIV positives ones respectively. In a multivariable analysis adjusted for age, sex and country, the HR in remained similar as unadjusted analysis for both HIV negatives and positives.

Several hypotheses can be put forward as to how HIV modifies the relationship between high IFN-γ levels and disease. The answer is based on the well known interaction between TB and HIV and the mechanism is immunological. *Mtb,* interacts with HIV through a number of mechanisms that support disease progression to active TB.

It is well established that HIV impairs the ability to control *Mtb* infection (286). Clinical studies provide compelling evidence that HIV leads to an increased risk of developing TB shortly after HIV infection (286). It is widely accepted that HIV causes a depletion of CD4 T-cells, which is likely to contribute to the susceptibility of co-infected persons to TB, as this T cell subset is important in the control of TB. However, HIV has effects on other cells, including macrophages, and influences cytokine production, which may also prevent a host from containing an initial or latent *Mtb* infection (286).

In a mini-review looking at how HIV-1 exacerbates TB by Flynn and others, a number of hypotheses from indirect evidence have been put forward (286). One area of focus includes changes in *Mtb*-specific T-cell responses. In their review they postulate that HIV increases TB risk through changes in *Mtb* specific T-cell responses in the following hypotheses:
HIV impairs the function of *Mtb* specific T-cells within involved tissue; decreases peripheral *Mtb*-specific T cell responses; reduces *Mtb*-specific T-cell responses in the airways; changes the cytokine profile within granulomas (286). Two hypotheses important for this research are the following:

1. **HIV impairs the function of *Mtb* specific T cells within involved tissue(286).**
   T-cells release cytokines, including IFN-γ TNF, and IL-2, as well as a variety of cytolytic molecules that are important in controlling both *Mtb* and HIV. HIV can exhaust HIV-specific and nonspecific T-cells, which has led to the hypothesis that HIV reduces the number and functionality of *Mtb* specific T-cells in co-infected individuals.

2. **HIV decreases peripheral *Mtb*-specific T cell responses (286).**
   Numerous studies have examined *M*tb T cell responses in individuals infected with M*tb* by stimulating PBMC, BAL fluid, or pleural fluid cells with PPD or CFP, killed *Mtb*, or peptide pools from immunogenic *Mtb*-specific proteins ESAT-6, CFP10. Most of the data support, the idea that HIV impairs the ability of T cells to respond to *Mtb*. The reduction in the observable number of peripheral *Mtb*-specific CD4 T cells may result from their direct infection by HIV in co-infected individuals.

In this study, for all the groups used for sensitivity analyses of the main question, HHCs with the highest IFN-γ levels had increased IRRs ranging from 1.5 to 2 compared to the reference sub-

group. For HIV negatives, HHCs with the highest IFN-γ levels had the highest IRRs in all groups apart from one group. HIV negative HHCs with the highest IFN-γ levels had increased IRRs ranging from 4 to 5-fold compared to the reference sub-group. In comparison, HIV positive HHCs with the highest IFN-γ levels had increased IRRs ranging from 1.6 to 2.6 compared to the reference sub-group.

Two studies in Senegal and Columbia, supported the main hypothesis of this research study, since the magnitude of the ELISPOT response was 5-fold higher in contacts who developed TB compared to those who did not develop TB, and the significant difference in performance of the QFT compared to the TST was only seen in definite TB cases (35, 159). In the study in Columbia, the rate of development of TB was highest in HHCs with high IFN-γ response to CFP-10 at baseline (35)

9.5 Study Limitations and Biases

Selection bias

Selection bias is a systematic error which occurs from the process used to identify the study participants. It leads to preferential selection or participation of subjects according to their exposure status or outcome status. Selection bias in this study may have been due to diagnostic bias, non-response bias and loss to follow up. Loss to follow up was mainly due to mortality, moving out of the study community, withdrawal or being absent.
Selection bias in the study was minimized in the following ways:

1. This study was nested in the ZAMSTAR study which was randomized as described in the methods. Stratification and restriction was used to randomize 24 clusters into intervention arms to avoid enrolment imbalances for its primary outcome.
2. The study population was clearly defined.
3. At least three attempts were made at each of the follow up visits to trace individuals who were absent or had moved. In addition, at enrolment all household HHCs were invited to participate in the study.
4. All individuals recorded as lost to follow up during the first follow up visit were revisited during the second follow up visit to minimize loss to follow ups.
5. Those who completed the study did not have a different disease risk than individuals lost to follow up.

Information Bias

When the information obtained from study participants is systematically inaccurate regarding the disease or exposure under study, information bias may occur. In this study information bias may have been due to errors in measurement introduced by the observer (interviewer bias, biased follow -up), by the study participants (recall bias, prevarication), or by measurements tools such as questionnaires or instruments such as weighing scales.
In this study, information bias was minimized in the following ways:

1. The exposure and the outcome were clearly defined using standard criteria.
2. Standardized measurement instruments were used as described below:
   a) The questionnaire
   1) The questionnaire was field tested/piloted in order to improve and refine it.
   2) The questionnaire used closed precise questions and avoided open-ended questions.
   3) Different questions were used to test the same hypothesis.
   4) Interviewers' techniques were standardized through training so that questions could be asked in the same way using the exact translation.

   (b) QFT and TST tests

   The exposure was defined using internationally recommended validated commercial tests for tuberculous infection.

3. Data on outcome was collected without prior knowledge of exposure status of a participant i.e. sputa from HHCs were collected without prior knowledge of enrolment QFT-GIT results. Although the research assistants and nurses were not formally blinded to exposure status of the HHCs at enrolment, the results for QFT-GIT test were only returned to the HHCs (and hence known by the research assistants) after 1-2 months of being recruited into the study. This is because QFT-GIT was processed in batches from the central laboratories. This minimized interviewer bias.
4. Written protocols were used to standardize procedures on data collection.
5. Information provided by the participant through the questionnaire on outcome was validated against other records (clinic TB register records and study laboratory results).
6. Multiple sources of information were used e.g. questionnaire, direct measurements.

Response Bias

Response bias occurs when study participants are more likely to suppress information that they feel would incriminate them as the cause of their own illness. This could have occurred when HHCs when questioned about risk behavior like smoking and drinking alcohol.

Prevalent-cohort Bias (287)

This study may have been subjected to some degree of prevalent-cohort bias. This has been described as bias arising from recruiting participants assumed to be disease free at enrolment when they are actually not(287). This may be due to the difficulty in identifying when transmission occurred, the period of latency and other risk factors. This creates a cohort that is rife with incidence-prevalence bias. In this study the true disease status of HHCs at enrolment was not known and whether the disease process had already begun. Due to this difficulty and to avoid overestimation the true number of incident cases, HHCs who developed active disease within two months of being in the study were excluded from analysis. These were assumed to be co-prevalent cases. Exclusion from analysis of individuals assumed to have co-prevalent TB has been done in studies with high prevalence of TB and HIV(159). However, there seems to be no consensus on how these time points (2 or 3 months) are selected or whether they are dependent on individual studies.

The inability to screen individuals for TB at enrolment was because the ZAMSTAR study design allowed little flexibility.

Confounding

Unlike selection and information bias, confounding is one type of bias that can be, to some extent, adjusted in the analysis.

Confounding occurs when the causal effect of the factor we are interested in is "mixed up" with the causal effects of other factors. Confounding is concerned with "alternative" explanations for the effect seen between the exposure of interest and the outcome. Randomisation, matching and restriction can be tried at the time of designing a study to reduce the risk of confounding. Confounding can be controlled at the analysis stage by stratification and multivariable (adjusted) analysis.

In the ZAMSTAR trial, control of confounding at the design stage was done through randomization and restriction. Randomisation was an attempt to evenly distribute potential confounders in study groups. It reduced potential for confounding by generating groups that are fairly comparable with respect to known and unknown confounding variables. However, it did not guarantee control of confounding and is useful only for intervention studies. **Restriction** eliminated variation in the confounder but caused loss of statistical power.

All relevant variables other than the exposure were taken into account to explain the results. Important imbalances on risk factors associated with active TB that were not taken into consideration in the ZAMSTAR randomization could arise. For this reason, clustering at household level was adjusted for and possible confounders were identified.

The possible confounders were

1. age
2. sex
3. socio-economic status
4. alcohol consumption
5. smoking
6. educational level

Non measurable confounders

Another source of bias could have been non-measurable confounders.

Individuals lost to follow up

A limitation of this study was the number of individuals lost to follow up. 25.5% of HHCs were lost to follow-up between the different time points. While we did not find any significant differences in the basic characteristics of those followed versus those lost to follow-up, the

study was vulnerable to unknown sources of bias. Furthermore, because no verbal autopsies were conducted many of those who died could have been as a result of TB.

Bias from missing results

We had missing data on individuals that had refused to have skin tests or did not return for the reading as well as on a number of risk factors. A study of this magnitude done across diverse communities and countries made data collection over the study period a challenge. Although some missing data (see data analysis) were excluded from analysis, bias was not expected to result from this elimination since the data was not systematically missing. Missing data generally leads to misclassification if the data are estimated, imputed or otherwise placed in the analysis in the absence of certainty about the exposure and disease category.

Our results may have been severely compromised by missing data on some risk factors. This was difficult for survival analysis; as the number of TB cases reduced depending on the definition, some risk factors had to be grouped. Therefore although the baseline chapter presents more groups for individual risk factors, these were later grouped due to the few outcomes.

Diagnostic or Work-up (verification) bias

Differential evaluations and/or follow up of individuals with positive and negative IGRA results can underestimate active TB in those with negative results. Incorporation or work-up bias was unlikely to have influenced results since those who were QFT-GIT or TST positive at baseline were not investigated for TB.

Other Study Limitations

a) HHCs at enrolment had mixed infections (acute, latent, and previously infected) and in an environment with ongoing, intensive nosocomial exposure, persistence of infection and/or re-infection might occur. All these influenced the outcome differently.
b) Incident cases may have been underestimated due to HIV positive individuals on IPT and ART. This effect was minimal since few HHCs were on ART and IPT (4.7% of HHCs were on IPT).
c) The true number of secondary cases in this study may have been underestimated. Some of those that had died or were lost to follow up may have had TB.
d) Both TST and QFT-GIT assay cannot distinguish between infection and disease and there is no standard for the diagnosis of TBI. Infection prevalence estimates were affected by test limitations discussed in the introduction and literature review. Due to the limitations of the TST, testing done across sites and countries could have affected results. However, SOPS were standardized across countries and training included exchange visits between Zambia and SA so that the trainers were using the same methods throughout.
e) Underlying ZAMSTAR Interventions (Enhanced case finding and household TB/HIV intervention) may have increased TB cases found and introduced bias.

f) TB diagnosis using register: It is possible that we did not capture everyone with TB within the community since some TB patients may access treatment from other places (clinics not within the ZAMSTAR community, private clinics, traditional healers) or are not recorded in the register. Therefore patient treatment cards were also used in such instances.
g) There were also difficulties to recruit HHCs into the study in cases where the index has died. Staff were trained in how to improve information giving in such instances.

Conclusions

The principal finding in this study is that there was no difference in incidence rates between HHCs with low and high levels (overall IRR: 1.0 (95% CI: 0.42-2.18)).

Another principal finding was that there was strong evidence of a five-fold increased risk of TB in HIV positive QFT-GIT positive HHCs compared to HIV positive QFT-GIT negative ones (aHR : 4.72; 95%CI: 1.35-16.46; P=0.01). For all the groups used in the sensitivity analysis of the primary question, HHCs with the highest IFN-γ levels had increased IRRs ranging from 1.5 to 2 compared to the reference sub-group. The feasibility studies emphasized the need for stringent sample collection and processing techniques to ensure the accuracy of QFT-GIT results.

10.0 The Role of IGRAs as diagnostic tests in high TB/HIV endemic resource settings and the evolving future research

In this chapter I will highlight the role of IGRAs as diagnostic tests in high TB/HIV endemic resource settings and some evolving IGRA and TST research in the context of this thesis.

10.1 IGRAs as diagnostic tests in high TB/HIV endemic resource constrained settings.

The utility of IGRAs in high-burden settings remains unclear. In contrast, in low TB burden settings with high BCG vaccination programs, IGRAs are used as screening tests for TB infection. Many countries in such settings have published guidelines on IGRAs although some are not evidence based.

There is evidence that IGRAs performance varies across high TB burden vs. low TB burden settings (149).

In high-burden settings, many factors may influence the results of IGRAs. These may be influenced by factors that modulate the immune response (7) such as HIV co-infection, BCG vaccination, poverty related malnutrition, tropical infections and widespread exposure to non-tuberculous mycobacteria.

IGRA performance depends on intact cellular immune responses; however, malnutrition and infection with helminths are known to alter such responses. The effects of malnutrition and helminth infection on cell mediated immunity include reduced IFN-γ production and altered balance between Th1-type and Th2-type responses, respectively. In the severely malnourished host, the absolute number and function of T lymphocytes are compromised, which corresponds to reduced IFN-γ production in human and experimental animal studies (288). A recent study in Bangladesh showed that malnutrition and helminth infections were associated with indeterminate QFT-GIT results in children (288).

Although the specific antigens encoded within the RD-1 of Mtb are absent in many NTM, their presence in other species like in *M. leprae* and *M. kansasii* can pose challenges if prevalent. Theoretically, the presence in the host of some NTM could cause a false-positive IGRA result. The presence of environmental mycobacteria in Zambia communities was evident in a recent TST survey (95). A study in Malawi (152) found a high rate of IFN-γ responses to PPD of atypical mycobacteria, especially to those of MAIS complex (*M.avium, M.intracellulare, M. scrofulaceum and M. marinum*)which was strongly associated with positive TST response to human PPD. Only *M. kansasii* can cause disease similar to tuberculosis and it does so only infrequently (113).

To be useful in settings with high prevalence of TB and HIV, IGRAs should not only detect recent infection but should be of better sensitivity for HIV positive populations than the TST. This is because HIV is the strongest risk factor for developing TB in those with latent or new *Mtb* infection (111). HIV-positive individuals have a 5-10% annual risk (119, 289) of active disease while HIV negatives have a lifetime risk of 5-10%. There is now sufficient evidence to show IGRA

sensitivity varies across populations and tends to be lower in high-endemic countries and in HIV-infected individuals (131).

Studies from low-burden countries indicate that the IGRAs correlate better, along a gradient of exposure, than the TST. Nevertheless, in high-burden settings, the TST performs reasonably well and correlates as well, or better, with proxy measures of exposure (149). Studies from Uganda, India and the Gambia showed that the TST correlated well with proximity to an index case and performed better than the IGRA along an *Mtb* gradient of exposure.

According to WHO, novel technologies successfully introduced into developed countries require adaptation to match the needs of developing countries (71), which includes feasibility and cost effectiveness considerations. In developing countries, potential drawbacks may include the cost, the need for highly skilled staff and a good laboratory infrastructure (with capacity to run ELISA or ELISPOT), the high HIV prevalence as well as logistical issues (e.g. power shortages) peculiar to these settings. In addition, strict assay techniques have to be followed for accurate results. For example, QFT-GIT indeterminate results can occur due to improper assay techniques (e.g. time delays that lead to loss of viable T cells in the specimens)(112). Our own experience of using QFT-GIT on a large scale identified some of these operational factors that affected the robustness of this test in Zambia (251).

Given the current grey areas in the use of IGRAs especially in poor resource settings and their sub-optimal sensitivity, the decision to use either IGRAs or TST should be based on country guidelines and resource and logistical considerations (131).

10.2 Evolving IGRAs research

Technical modifications of IFN-γ-based tests are being explored (290). The attempts to improve IFN-γ-based tests include:

1 Incorporation of additional antigens of high specificity.
2 Use of alternative *Mtb* specific antigens/peptides.
3 Use of different marker from IFN-γ, for example IP-10, IL-2, MCP-2.
4 Alternative readouts to measure IFN-γ release, which are different from ELISA or ELISPOT.
5 Simultaneous measurement of chemokines and interleukins (130, 290, 291).
6 Applicability of IGRAs with extrasanguinous samples i.e. biological sample different from blood (BAL, pleural fluid, CNS).

Novel test formats that include other antigens, additional markers, the comparative use of various patient specimens and advanced techniques, have been suggested as a replacement for today's commercially available IGRAs for a better diagnosis of true TBI. Novel concepts have been investigated that include the use of different epitopes of RD antigens, readout different from IFN-γ such as chemokines or cytokines, new antigens different from the RD genomic region, such as those defined as Rv1733c, Rv2029c, additional cytokines or characteristic phenotypic markers (5).

Diagnostic sensitivity of IGRA can also be enhanced by incorporation of a novel RD1-encoded antigen, Rv3879c, without compromising diagnostic specificity (5). In a large prospective study of patients with suspected TB, incorporation of a novel RD-1-encoded antigen, RV3879c, alongside ESAT-6and CFP-10, significantly improved diagnostic sensitivity over the standard ELISpot test (5). Similarly, the addition of Rv2645 in QFT-GIT significantly improved sensitivity of this assay in diagnosing active TB over QFT-G without compromising specificity (290).Alternative readouts to measure IFN-γ release by *Mtb*-specific T-cells have also been explored, for example, using flow cytometry (290).

Other biomarkers, alone or in combination, could enhance the diagnostic performance of IGRAs. Downstream chemokines induced by IFN-γ, such as IP-10 or CXCL10, monocyte chemoattractant protein (MCP)-2 may serve as a more amplified readout than IFN- γ itself, thereby yielding higher sensitivity (290).It has been shown that IFN-γ-inducible IP-10 and IL-2 could be alternative or adjunct biomarkers to IFN-γ (292). A study conducted in Nigeria among 59 children showed that IP-10 was expressed in high amounts in response to *Mtb* specific antigens and performed with excellent concordance with the QFT-IT test and the agreement between the IP-10 and QFT-IT was stronger than with the TST (292).The combined readout of IFN-g/IP-10 has been suggested as a potentially more sensitive marker of *Mtb* infection than current IGRAs (290).

IGRAs were developed and licensed for use on blood. However, it is known that *Mtb*-specific T-cells are recruited at the site of infection, where their frequency is increased compared to peripheral blood. As a result, there is increased research activity on the applicability of IGRAs with extrasanguinous samples (e.g. pleural fluids, materials from bronchoalveolar lavage, ascitis, or liquor cerebrospinalis)(175).

Other biomarkers for disease progression

There is need for further research to enhance our understanding of the biology of *Mtb* and interactions with the human host. Such knowledge gaps impede the development of biomarkers that can distinguish between latent and active TB, especially in HIV-infected adults and children.

Research regarding predictive biomarkers for disease progression in susceptible individuals for early intervention is still ongoing (Table 10.1). Despite these diagnostic advancements, there are currently no reliable surrogate markers for disease progression. Some studies have suggested that relative mRNA concentrations of interferon γ, interleukin 4,and interleukin 4δ2 (a splice variant of interleukin 4) might be better predictors than interferon γ alone, since ratios of interferon γ or interleukins 4 and 4δ2 fall as healthy contacts develop tuberculosis, and increase as patients with tuberculosis are cured (104). The ratio of interleukin 4 to 4δ2 is also increased in longstanding latent tuberculosis infection, presumably suggesting low risk of reactivation (22).

Table 10.1: Biomarkers associated with disease progression (11)

Cytokines & chemokines	Diagnosis	Correlate of risk or of protection	References
IFN-γ	Latent or active TB	Vaccine efficacy or disease progression	70-73
CXCL10,IL-10	Active TB	Increased after BCG	74-77
IL4↑	Active TB	Progression	78,79
IL-4δ2/ IL-4 ratio	Extent of disease	Not determined	54,82
IFN-γ/IL-4 ratio	Latent or active TB	Not determined	54
IL-17 and TNF	Latent or active TB	Increased after BCG	81

***Correlates of risk of tuberculosis are markers that are associated with low risk of disease development or the absence of markers associated with high risk of disease, whereas correlates of protection against tuberculosis reliably predict the level of protective efficacy induced by a vaccine on the basis of differences in the immunological measurements of vaccinated and unvaccinated groups. ‡**

Evolving TST research

An attempt to improve the skin test was recently made using a recombinant dimer of ESAT-6 (rdESAT-6), a protein encoded by the region of difference 1 (RD1) of the *Mtb* chromosome (293). This region, and thus the proteins and their epitopes, is not present on BCG and on most NTMs, making it a perfect candidate for a more specific skin test. Inoculation of rdESAT-6 in the skin of subjects with TBI or previous TB was proven to induce local responses similarly to TST, but no skin reaction was observed in BCG-vaccinated individuals (294).

Recently, results of a phase I trial of a skin test that uses rdESAT-6 instead of tuberculin have demonstrated safety and tolerability of such a test (293). In combination CFP-10 antigen to increase diagnostic sensitivity, such a skin test could overcome some of the obstacles currently related to the use of the TST. If clinical trials show superiority to the TST this test could be made widely available for the diagnosis of TBI in resource-limited settings where the use of IGRA is prohibited by their costs and demands for an established laboratory infrastructure.

Vaccines

Current TB vaccine development efforts aim at preventing primary *Mtb* infection through preinfection vaccination, or at interrupting the transition from TBI to active disease through post-BCG booster vaccination approaches. The number of promising tuberculous vaccine candidates has increased in the last decade. Currently there are eight vaccine candidates in human phase I and phase IIa trials (10). The identification of those in need of vaccine-induced protection and preventive chemotherapy is of major importance not only for the individual but also for disease control.

Future research needs

Future research needs in line with identifying predictive biomarkers for TB infection include the following:

- Larger prospective studies assessing the positive and negative predictive values of IGRAs for the diagnosis of LTBI especially in children and other high-risk groups such as immunocompromised populations.
- Prospective studies on the efficacy of preventive therapy based on IGRA results.
- Research to develop IGRAs that incorporate new *Mtb*-specific antigens and alternative cytokines that would enhance sensitivity, allowing TBI to be distinguished from active TB.
- Future studies will also need to better illuminate the kinetics of Mtb–specific immune responses in relation to actual tuberculosis risk (107).
- Studies to evaluate the feasibility and cost of IGRAs in the diagnosis of TBI and active TB in different settings and for different purposes (e.g. contact screening, serial testing of healthcare workers).

Randomized controlled trials

In the absence of a gold standard for diagnosing LTBI, the best way to assess the predictive value of the test for subsequent development of TB, in theory, could be to compare IGRAs with TST in a controlled trial of treatment for test-positive individuals, thereby circumventing the ethical hurdle of withholding interventions from individuals at risk of TB (173, 278). However, a very large sample size would be needed and with suboptimum acceptance, adherence and effectiveness of available treatment regimes, the number of TB cases averted might be few for the efficient assessment of simple diagnostic devices (278). Randomized controlled trials alone are unlikely to improve existing technology but more basic research is needed to identify a suitable biomarker for progression to active disease (278).

TB Biomarker Signatures (Gene expression profiling).

Comprehensive analysis of gene expression patterns associated with different *Mtb* infectious status have important implications for developing novel diagnostic biomarkers to differentiate active disease from LTBI. Gene expression profiles provide valuable clues for better understanding of progression from latent infection to active disease and pave the way for defining predictive correlates of protection in TB (295, 296).

Many studies have been done to clarify the cytokine and chemokine responses to *Mtb*-specific antigens that are involved in the progression from latent infection to active disease (19,20,21 from Chanyi). However, these studies revealed only limited insight into human resistance or susceptibility to *Mtb* infection. More recently, genome-wide transcriptome analyses have been widely used to explore the complex interaction between human and bacteria (295, 297, 298).

Using blood transcriptional profiling, several signature gene sets have been identified in adult cohorts from South Africa, The Gambia and The United Kingdom (295, 297). Although peripheral blood transcriptional signatures discriminating between TB, LTBI and HC subjects have been identified in adult studies, concerns about the specificity of these signature sets have been raised (295, 297, 299). More recently, single candidate biomarkers were combined into multicomponent signatures to increase power and specificity. Such signatures appear to be more powerful biomarkers than individual genes or proteins (295, 298, 300).

Berry and others (297) provided one of the first complete descriptions of the human blood transcriptional signature of TB in a study done in more than 400 participants with active TB, LTBI and healthy controls from London and Cape Town, South Africa. This study suggested that the signature of active TB, observed in 10–20% of patients with latent TB, may identify individuals who will develop active disease but longitudinal studies are needed to confirm this (297).

In another study, Chanyi and others performed genome-wide transcription analysis of PPD-stimulated PBMCs from subjects with TB, LTBI and HC, and identified unique transcript profiles in individuals with tuberculous infection and active disease (296). In this study, they found that the combination of CXCL10, ATP10A and TLR6 could be used as novel biomarkers to differentiate TB from LTBI. Transcriptional profiling is becoming more and more frequent as an additional immunological approach in clinical trials of new TB vaccines, where gene expression signatures are compared in samples from various time points (301).

An ideal test for diagnosing TB infection

An ideal test for LTBI diagnosis should meet the following criteria: High sensitivity in all populations at risk; high specificity regardless of BCG vaccination and infection with NTM; reliability and stability over time; objective criteria for positive result, affordability and easy administration and ability to distinguish recently infected individuals with increased risk of progression to active TB. Such a test, if widely implemented and accompanied by successful treatment, could revolutionize TB control (109).

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Appendices

Appendix 1.: Ethics approval for the ZAMSTAR study

THE UNIVERSITY OF ZAMBIA

RESEARCH ETHICS COMMITTEE

Telephone: 256067
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Dean's Office
P.O. Box 50110
Lusaka, Zambia

Assurance No. FWA00000338
IRB00001131 of IOR G0000774

15 December, 2004
Ref: 007-10-04

Dr Helen Ayles, BSc, MB.BS, MRCP, DTM&H, MSc, PhD
Zambart Project
Department of Medicine
University Teaching Hospital
LUSAKA

Dear Dr Ayles,

RE: SUBMITTED RESEARCH PROPOSAL

The following research proposal was presented to the Research Ethics Committee Meeting on 8 November, 2004 where changes were recommended. We would like to acknowledge receipt of the corrected version with clarifications. The proposal has now been approved. Congratulations!

Title of proposal: **"ZAMSTAR: Zambia and South Africa Tuberculosis and AIDS Reduction Study"**

Conditions:

- This approval is based strictly on your submitted proposal. Should there be need for you to modify or change the study design or methodology, you will need to seek clearance from the Research Ethics Committee.
- If you have need for further clarification please consult this office. Please note that it is mandatory that you submit a detailed progress report of your study to this committee every six months and a final copy of your report at the end of the study.
- Any serious adverse events must be reported at once to the Committee.
- Please note that when your approval expires you may need to request for renewal. The request should be accompanied by a Progress Report (Progress Report Forms can be obtained from the Secretariat).

[redacted]

Prof. J. T. Karashani, MB, ChB, PhD
CHAIRMAN
RESEARCH ETHICS COMMITTEE
Date of approval: 15 December, 2004 Date of Expiry: 14 December, 2005

Appendix 2. : Ethics approval for the Quantiferon-TB Gold Study

THE UNIVERSITY OF ZAMBIA

RESEARCH ETHICS COMMITTEE

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Assurance No. FWA00000338
IRB00001131 of IOR G0000774

13 February, 2007
Ref.: 007-10-04

Dr Helen Ayles
ZAMBART Project
Department of Medicine
University Teaching Hospital
P.O. Box 50110
LUSAKA

Dear Dr Ayles,

RE: **ZAMSTAR: ZAMBIA AND SOUTH AFRICA TUBERCULOSIS AND AIDS REDUCTION STUDY APPROVED 15 DECEMBER, 2004**

Reference is made to your letter dated 22 January, 2007 concerning the above-mentioned study.

We note from your letter that you are seeking approval of the amended research documents as follows:-

1. Information sheets;
2. Consent forms;
3. In the recruitment of secondary outcome cohorts (SOCS), questionnaires, information sheets and consent forms, you wish to include an additional test for adults only. In these adults you are currently taking 6 mls of venous blood for HIV testing but you wish to take an additional 3 mls for testing of latest TB infection using Quantiferon Gold in-tube assay and to perform a tuberculin skin test according to standard protocol.

We also take note that the submitted documents are all in English and that the translated versions will be submitted later.

This Committee has no objection to the amendments as requested in your letter.

Yours sincerely

[redacted]

Prof. J. T. Karashani, MB, ChB, PhD
CHAIRMAN

Appendix 3. : SOCS enumeration form at baseline

VISIT:	

BARCODE:

ADDRESS

HOH QUESTIONNAIRE DONE:	
TOTAL NUMBER OF PAGES:	

GPS-X	S	-			º			,				‘	
GPS-Y	E	0			º			,				‘	

Address:	
Compound:	

Number of Study Cards Issued for this Address::		

SIGNED OFF:	DATE								CODE			SIGN
	D	D	M	M	Y	Y	Y	Y				

CODES for Enumeration Form

Consent:	**N**=no consent	**Y**=consent	**M**=Moved Out	**D**=Died	**A**=absent/not found
Shared … with Index/Other TB	**B=**same Bed	**R=**same Room	**H=**same House	**O**=Other house	**U=** Unknown/Don’t remember

No.	Adult / Child Barcode	First name	Surname	Sex	Age	Consent On	Shared with Index / Other	Consent	Q –done Y	Q –done N	Blood Y	Blood N	QFT Y	QFT N	TST Y	TST N

SIGN OFF : FIELD

SIGN-OFF : DATA CENTRE

RESEARCH ASSISTANT (RA):

DATE SENT TO DC: D D M M Y Y Y Y

DC1:

Note: For visit 3, this form was amended to include sputum

Appendix 4.: SOCS Information Sheet

You are invited to take part in a study called Zamstar (Zambia South Africa TB and AIDS Reduction Study) looking at ways to reduce TB and HIV in your community…….. (Fill in community name). Three hundred-and-fifty (350) adults and three hundred (300) to four hundred (400) children <5 years of age from 150-200 households from your community will take part. Information about the study is supplied in this document. Project staff will be on hand to explain the contents and answer all your questions. Please make sure that you understand everything described in this document. If you decide to participate, you will be asked to give written consent before you take part.

Who is doing the study?

This study is being performed by ZAMBART Project from the University of Zambia and London School of Hygiene and Tropical medicine

Dr/Mr./Mrs……is responsible for the day to day running of the study.

The study has been approved by the Medical Ethics Committees of the University of Zambia and the London School of Hygiene and Tropical Medicine.

In your area we are working with the health centre committee (Community advisory board).

What is the purpose of the study?

In spite of a good TB tracing and treatment programme in your community, the number of TB cases remains high. The purpose of this study is to determine the most effective way of reducing TB and HIV in the community. ZAMSTAR as a study is not meant to provide health care that you would ordinarily get from your local health centre, but to assist with this care.

What is TB?

Tuberculosis (TB) is an infectious disease caused by bacteria (germs), which are spread through droplets (coughing). TB infection affects mainly the lungs, but can also affect other parts of the body. Many people with TB are not diagnosed quickly, and go on infecting other people without knowing it. It is however possible to diagnose TB quickly through examining sputum. TB can be cured once diagnosed.

TB is sometimes associated with HIV, the virus that can cause AIDS. We would like to find out whether this is the case here and so will be asking you to provide a blood sample that we will test for HIV. We will be testing these samples in groups labeled only with a barcode or number and so we will not be able to provide you with the results immediately.

If you would like to find out your HIV status, please tell the nurse who is taking the blood and he/she will ensure that a small sample is tested for HIV here at the clinic laboratory. We would encourage you to find out your HIV status by visiting the voluntary counseling and testing centre at …….. (Fill in name of local VCT). To ensure that you get the results of your blood test you will need to take your study card and number along with you.

Why diagnose TB infection?

A person who gets TB first becomes infected and then may develop the disease after some years. People infected with tuberculosis are well.

We would like to find out how many people are infected with tuberculosis in order to consider medical treatment to protect them from becoming unwell with tuberculosis disease and to provide them with all the information required on what to do if they start to become unwell.

Until recently the tuberculin skin test (TST) was the only way to diagnose infection with TB. Nowadays, there is a new test – the QuantiFERON®-TB Gold In-Tube (QFT-Gold IT). This test is considered to be more reliable than TST. Differently from TST, this test is performed on blood.

It is important for you to know the following:

1. If you give blood for testing for tuberculosis infection, we will use your blood only for TB testing and no further test for any other disease will be performed on it.
2. Blood samples will be labeled with barcodes and results will be reported to you only.
3. If your result is positive, this DOES NOT mean you have TB, but only that you have been infected with TB.
4. If your result is positive, we will come back to you and we will give you more information about what to do next.

What is required of you?

1. You will be asked to complete a questionnaire about your health at the local health centre.
2. You will be asked to provide a blood sample for examination for HIV (For adults aged above ≥ 15 years). Just more than a teaspoon of blood (6mls) will be taken from you on 3 occasions; now, 18 months from now and 36 months from now (total volume of blood = less than a tablespoon [18mls]).
3. You will be asked to provide a blood sample for examination for Tuberculosis infection (For adults aged above ≥ 15 years). As for HIV testing, this will require not more than a teaspoon of blood (3mls, approximately 1 ml for each blood tube). Blood samples will be taken from you on 3 occasions; now, 18 months from now and 36 months from now (total volume of blood = less than a tablespoon [9mls]).
4. Each time we take blood we will use a separate sterile needle.
5. We will test all children under 5 years and all adults for tuberculosis infection by doing a skin test. This test involves giving an injection into the skin with a small needle and then reading the reaction to the injection 3 days later. Each individual will be injected with a separate sterile needle. We will not take any blood from the children.

Are there any risks for people who take part in this survey?

The main risk from participating in the study is that we will take 3 blood samples from you. This may cause some transient discomfort and you may develop a bruise at the site of the blood draw.

The tuberculin skin test is done by injection into the skin using a small needle. We will not be taking any blood from the children in the study. We will look at the injection site after 3 days to see if there is any reaction on the skin. The main risk from this procedure is that there may be some discomfort at the time of injecting and the skin may become red and swollen. If this happens we will give you some cream to apply to the area which will make the swelling go down quickly.

Benefits

The main benefit is for your community where we are finding out whether we can reduce TB and HIV by providing certain interventions.

For the children in the study, any child who has a positive skin test will be referred to the clinic to be examined to rule out tuberculosis. Children who do not have tuberculosis but have evidence of exposure to tuberculosis will be referred for preventive treatment for TB.

You may wish to find out your HIV status, and by participating in this study that will be possible. You will receive standard counseling and testing and your blood will be tested using a rapid HIV test using the same procedures as are in current usage at this clinic. You must sign an additional consent form for this to be done and for you to receive the result. If you are HIV positive we will refer you for further treatment, care and support, including preventive treatment for TB if you are also found to have TB infection.

Participation on this study is completely on a voluntary basis. You are free to withdraw from the study at any stage, without any consequences for you or your family. No financial reward will be given to any persons taking part in this study.

Alternatives to the study

Access to health services (TB and VCT) for you or your family will not be influenced if you choose not to participate on the study.

Confidentiality of information and privacy of the participant

All personal information obtained from individuals or households during this study will remain strictly confidential. The answers will be transferred to a computer, but your name will not be included, and you will be identified by a coded number only. No information about individuals will be released to any other parties but the research team, without your further consent. Completed questionnaires will be stored in a safe place. No information regarding personal details which could identify individuals or individual households will be disclosed.

The data collected from this study will be published in scientific journals, but without names being available to anyone.

Thank you for reading this information sheet. If you have any questions, please feel free to ask them now. The interviewer will be pleased to answer them. If you wish to take part, please read and sign the consent form. If you wish children less than 5 years in your household to take part, please read and sign a separate consent form on their behalf. Please keep this information sheet in a safe place.

Appendix 5.: SOCS Informed consent form

Adult Barcode:

1. I confirm that I have read the information sheet, and that the information and procedures involved in my taking part in the survey have been explained to me.
2. I confirm that I have had the opportunity to ask questions about the study and that I am satisfied with the answers and explanations that have been provided.
3. I have been given time and opportunity to read the information carefully, to discuss it with others and to decide whether or not to take part in this survey.
4. I understand that I can withdraw any time from the study.
5. I understand that I will provide a blood sample for HIV testing. I further understand my HIV results will be strictly confidential. I understand that I can receive my HIV results on my request following counseling.
6. I understand that I will provide a blood sample and undergo a skin test for TB infection testing. I further understand my TB results will be strictly confidential. I understand that if these results are positive, I will receive information about what to do in case of symptoms occurrence.
7. I understand that study staff will visit me after 18 and 36 months to conduct similar tests.
8. I agree to take part in the study.

Participant's information:

Signature (or fingerprint): _____

Surname: _____ Name: _____ (please print)

Date _____

The person who conducts the informed consent discussion must sign and date this form.

Signature: _____date _____

Surname:_____ Name: _____(please print)

Signature of witness, if applicable.

Witnessed by: (print name): _____

Signature of Witness: _____ date _____

Appendix 6. : Individuals adult Questionnaire (visit 1)

ADDRESS BARCODE	ADULT BARCODE

SECTION A – GENERAL INFORMATION

		Diagnosis	M18 (Visit 2)	M36 (Visit 3)
Q01_VIS	**VISIT**	1	2	3
Q02_COD	**INTERVIEW'S CODE**			

		D	D	M	M	Y	Y	Y	Y
Q03_DAT	**DATE OF INTERVIEW**								

		M	F
Q04_SEX	**SEX**	1	2

		D	D	M	M	Y	Y	Y	Y
Q05_DOB	**DATE OF BIRTH (01/01/1800 IF UNKNOWN)**								
Q05_1DOB	**If not known, what was your age in years on your last birthday? (999 IF UNKNOWN)**								

Q06_ETH **To which ethnic group do you belong?**

Black Zambian	**1**		**Black SA**	**2**	
Black Other	**3**		**White**	**4**	
Coloured	**5**		**Asian**	**6**	
Other	**7**	**Specify:**			

Q07_REL **What is your religion?**

Catholic	**0**		**Protestant**	**1**	
Muslim	**2**		**Hindu**	**3**	
SDA	**4**		**JW**	**5**	
Pentecostal/Evangelical	**6**		**No Religion**	**7**	
Other	**8**	**Specify:**			

		No	Yes	Unknown
Q08_AS	**Have you ever attended a school?**	0	1	9
	(IF YES CONTINUE.IF NO OR UNKNOWN GO TO Q9)			
Q08_1_HG	**What is the highest grade attended?**			

		No	Yes	Unknown
Q08_2_AC	**Have you attended College or Higher Institution?**	0	1	9

		No	Yes	Unknown
Q09_IH	**In the past 6 months have you made any contribution in cash or kind to the household?**	0	1	9

Q10_NAH **In the last month, how many nights have you spent away from home? (WRITE NUMBER IN BOX)** [ ][ ]

I would now like to ask you some questions about your current drinking and smoking habits

		No	Yes	Unknown
Q11_DA	**During the last 4 weeks have you taken any drinks containing alcohol?**	0	1	9
Q12_DD	**During the last year have you ever drunk so much that you were unable to remember what you were doing?**	0	1	9

Q13_SH **How would you classify your smoking habits?**

Daily Smoker	**1**	**Occasional Smoker**	**2**
Ex-smoker	**3**	**Never Smoked**	**4**

(IF SMOKER OR EX-SMOKER CONTINUE, IF NEVER-SMOKER SKIP TO Q14)

Q13_1 SD	**How long have you/did you smoke for? (years)**			
Q13_2 SA	**How many cigarettes per day do/did you smoke?**			

		No	Yes	Unknown
Q14_SM	**Do you ever smoke marijuana/dagga etc?**	0	1	9
Q15_RD	**Do you take any other recreational drugs?**	0	1	9

I would now like to ask you some questions about your sexual relationships

Q16_MS **What is your marital status now?**

Single	**1**	**Married/living as married**	**2**
Widowed	**3**	**Divorced/separated**	**4**

		No	Yes	Unknown
Q17_SX	**In the last 6 months have you had sex? (IF YES CONTINUE. IF NO OR UNKNOWN GO TO NEXT SECTION B, Q18)**	0	1	9

		No	Yes	Unknown
Q17_1_OP	**In the last 6 months at any stage, have you had more than one sexual relationship at the same time? (IF YES CONTINUE. IF NO OR UNKOWN GO TO Q17_2)**	0	1	9

Q17_1_1_CM **Did you use condoms with these partners?**

Never	**1**	**Always**	**2**
Sometimes	**3**	**Unknown/ no answer**	**9**

		No	Yes	Unknown
Q17_2_POP	**In the last 6 months do you think that your main partner has had other partners apart from you?**	**0**	**1**	**9**

SECTION B – TB AND HIV QUESTIONS – ASK ALL RESPONDENTS

I would now like to ask you some questions about your health, specifically about TB and HIV

		No	Yes	Unknown
Q18_TBT	**Are you currently on TB Treatment?**	**0**	**1**	**9**
	(IF YES CONTINUE. IF NO OR UNKNOWN GO TO Q19)			
Q18_1_TBT	**What is your TB registration number?**			

		No	Yes	Unknown
Q19_HIV	**Have you ever been tested for HIV?**	**0**	**1**	**9**
	(IF YES CONTINUE. IF NO OR UNKNOWN GO TO SECTION C, Q22)			

		Negative	Positive	Unknown
Q19_1_HIV	**Would you mind telling me what the result was?**	**0**	**1**	**9**
	(IF POSITIVE CONTINUE. IF NEGATIVE OR UNKNOWN GO TO SECTION C, Q22)			

		No	Yes	Unknown
Q20_ARV	**Have you ever taken ARV treatment?**	**0**	**1**	**9**
	(IF YES CONTINUE. IF NO OR UNKNOWN GO TO Q21)			

		No	Yes	Unknown
Q20_1_CARV	**Are you currently taking ARV treatment?**	**0**	**1**	**9**
	(IF YES CONTINUE. IF NO OR UNKNOWN GO TO Q21)			

		D	D	M	M	Y	Y	Y	Y
Q20_1_2_SARV	**When did you start ARV treatment?**								

Q20_1_3_WARV	**Which health center do you receive treatment from?**
	(NAME OF CLINIC/HEALTH CENTER)

		No	Yes	Unknown
Q21_IPT	**Have you ever been on preventive therapy for TB (IPT)?**	**0**	**1**	**9**

SECTION C – STIGMA QUESTIONS – FOR ALL RESPONDENTS

Tuberculosis Stigma Indicators: For all Respondents. Read Out the Statements Slowly, One at a Time and Record Response.

I am going to read to you some statements that have been heard in relation to TB and HIV. I would like you to tell me whether you agree or disagree with these statements.

		Agree	Disagree
Q22_TSCT	**You can catch tuberculosis if you touch a patient who is diagnosed as having TB**		
	You can catch tuberculosis by sharing eating utensils with someone who has TB		
	You can catch tuberculosis from having sex with someone who has TB		
	TB Patients should be isolated from others in the community		

Q23_TSBJ	**It is women who spread TB in our communities**		
	It is men who spread TB in our communities		
	It is outsiders who spread TB in our communities		
	TB is a punishment for bad behaviour		
	TB patients are careless		
	TB patients are disgusting		
	TB patients are promiscuous		

		Yes	No
Q24_TES	**Do you personally know anyone with TB who has experienced any of the following: *(YES/NO ANSWERS)***		
	Been excluded from a social gathering		
	Abandoned by spouse/partner		
	Isolated by their household		
	Lost housing/denied housing for rent		
	Lost respect or standing in the community		
	Been teased, insulted or sworn at		
	Been gossiped about		
	Whose children or family have been isolated/shunned		
	Been treated worse than patients with other diseases by health staff		
Q_25_TD	**Do you personally know of anyone who has tried to hide their TB diagnosis?**		

		Agree	Disagree
Q_26_THIV	**Do you agree or disagree with the following statements**		
	TB is curable in those living with HIV		
	All TB patients have HIV/AIDS		

N.B. ASK TB PATIENTS ONLY. SKIP TO Q32 IF NOT A TB PATIENT.

		Yes	No
Q27_TPES	**Since you fell sick with TB have you experienced any of the following:**		
	Been excluded from a social gathering		
	Abandoned by spouse/partner		
	Isolated by your household		
	Lost housing/denied housing for rent		
	Lost respect or standing in the community		
	Been teased, insulted or sworn at		
	Been gossiped about		
	Your children or family have been isolated/shunned		
	Been treated worse than patients with other diseases by health staff		

(IF YES TO ANY OF THE ABOVE CONTINUE, IF NO TO ALL OF ABOVE SKIP TO Q29)

		Yes	No
Q28_TIS	**Do you think it was reasonable for you to be treated in this way?**		

Q29_TIS2	**Since you were diagnosed with TB have you felt:**		
	Less of yourself because of your TB		
	Guilty about having TB		
	Unclean or dirty because of your TB		
	Careless to have got TB		
	Afraid of being seen at the TB corner / clinic		

		Yes	No
Q30_TBD	**Have you tried to hide your TB diagnosis from anyone?**		
Q31_OTD	**Have you told anyone outside of your household about your TB diagnosis**		

HIV Stigma Indicators: For all Respondents. Read Out the Statements Slowly, One at a Time and Record Response.

I am now going to read to you some statements that have been heard in relation to HIV. I would like you to tell me whether you agree or disagree with these statements.

		Agree	Disagree
Q32_HSCT	**You can become infected with HIV if you are exposed to the saliva of someone who is HIV positive**		
	Children can become infected with HIV if they play with children who are HIV positive		
	You can become infected with HIV by caring for/looking after someone who has HIV		
Q33_HSBJ	**It is women who spread HIV in our communities**		
	It is men who spread HIV in our communities		

	It is outsiders who spread HIV in our communities		
	HIV is a punishment for bad behaviour		
	HIV is a punishment from God		
	People with HIV are promiscuous		

Q34_HES	**Do you personally know anyone who is HIV positive who has experienced any of the following:**	**Yes**	**No**
	Been excluded from a social gathering		
	Abandoned by spouse/partner		
	Isolated by their household		
	Lost housing/denied housing for rent		
	Lost respect or standing in the community		
	Been teased, insulted or sworn at		
	Been gossiped about		
	Whose children or family have been isolated/shunned		
	Been treated worse than patients with other diseases by health staff		

Q35_HD	**Do you personally know anyone who has disclosed their HIV status directly to you or publicly in the last 12 months?**		

IF THE RESPONDENT HAS NOT BEEN TESTED FOR HIV, OR IS HIV NEGATIVE OR UNWILLING TO DISCLOSE THEIR HIV STATUS THE INTERVIEW WILL END HERE. THANK THEM FOR THEIR TIME AND ASSISTANCE
IF THE RESPONDENT HAS TOLD YOU THAT THEY ARE HIV POSITIVE CONTINUE WITH THESE QUESTIONS FOR PEOPLE LIVING WITH HIV ONLY.

		Yes	**No**
Q36_HPD	**Have you disclosed your HIV status to anyone (except to me for the purpose of this study)**		
	(IF YES CONTINUE, IF NO SKIP TO Q36)		
Q36_1	**Have you disclosed your HIV status to your main sexual partner?**		
Q36_2	**Have you disclosed your HIV status to other household members?**		
Q36_3	**Have you disclosed your HIV status to anyone outside of the household?**		

Q37_HES	**In the last year have you experienced any of the following due to your HIV status:**	**Yes**	**No**
	Been excluded from a social gathering		
	Abandoned by spouse/partner		
	Isolated by their household		
	Lost housing/denied housing for rent		
	Lost respect or standing in the community		
	Been teased, insulted or sworn at		

	Been gossiped about		
	Whose children or family have been isolated/shunned		
	Been treated worse than other patients by health staff		

(IF YES TO ANY OF THE ABOVE CONTINUE, IF NO TO ALL OF ABOVE SKIP TO Q39)

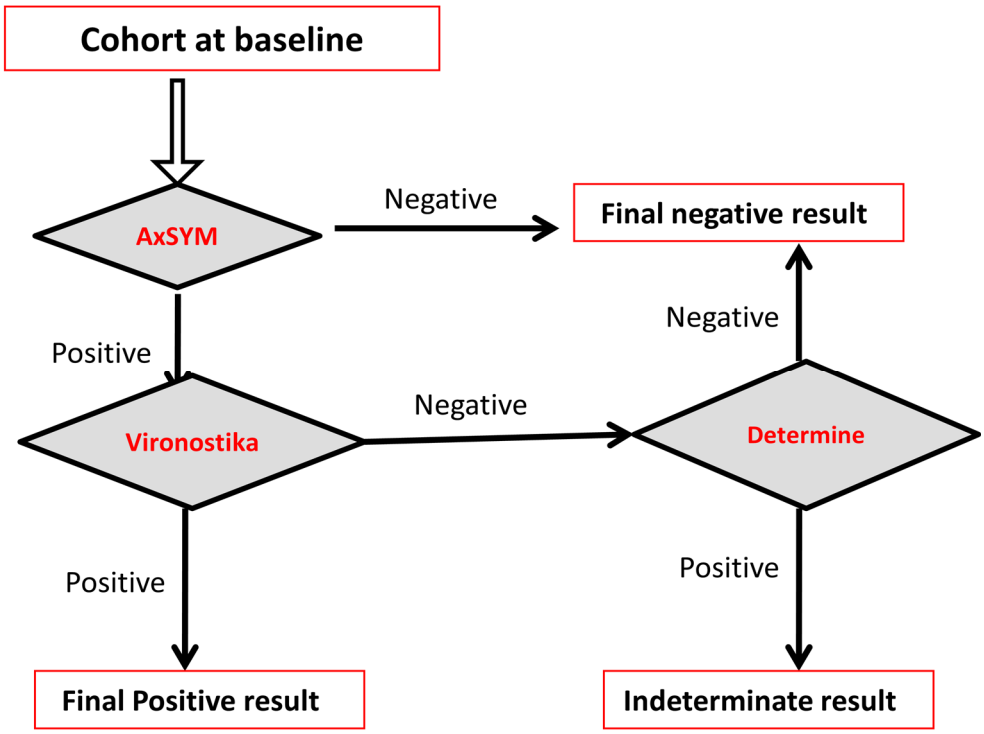
		Yes	**No**
Q38_HIS	**Do you think it was reasonable for you to be treated in this way?**		
Q39_HIS2	**In the last year have you ever avoided or withdrawn from any of the following due to your HIV status**	**Yes**	**No**
	Applying for school, further training or a scholarship		
	Promotion or job opportunity		
	Travel		
	Seeking health care		
	Relationship with friends or family		
	Having a sexual relationship, getting married or having a child		
Q40_HIS2	**In the last year, because of your HIV status, have you felt:**	**Yes**	**No**
	Less of yourself because of your HIV		
	Guilty about having HIV		
	Unclean or dirty because of your HIV		
	That you deserved to get HIV		

THANK THE RESPONDENT FOR THEIR PARTICIPATION

	Interviewer's Code	**Date**								**Signature**
		d	**d**	**m**	**m**	**y**	**y**	**y**	**y**	
Interviewer										
Field Manager										
1st data entry										
2nd data entry										

Appendix 7.: HIV Testing Algorithm

1. The first line testing is AxSYM HIV test although there are slight differences in the generation of assays used between the two countries. Samples processed in South Africa are first tested on a 4th generation assay, AxSYM HIV Ag/Ab Combo Assay (Abbott). Samples processed in Zambia are first tested on a 3rd generation assay, AxSYM HIV 1/2gO (Abbott). Interpretation of results for AxSYM HIV Ag/Ab Combo Assay are as defined by the manufacturer: Specimens with S/CO (sample rate/cutoff rate) < 0.90 are considered negative; ≥ 1.00 as reactive (positive); 0.90 - < 1.00 as gray zone.
2. For both countries, the second line testing is done on the VironostikaUni-form11 Ag/Ab assay (Manual ELISA test). Interpretation of results for Vironostika Uniform tests are as described by the manufacturers (manual ELISA test). A cut-off value = Mean negative control (NCx) + 0.100 is used. Samples are positive if sample absorbance is ≥ cut-off value. Samples are negative if sample absorbance is ≤ cut-off value.
3. Discrepant samples i.e. those positive on the first line testing, and negative on second line testing are tested with the Determine HIV ½ Ag/Ab Combo rapid test.
4. Interpretation of Determine rapid HIV test is as described by the manufacturers.



Appendix 8.: SOP for QuantiFERON® – TB Gold IT

Summary and Explanation of the Test.

- The QuantiFERON® – TB Gold IT test is a test for Cell Mediated Immune (CMI) responses to peptide antigens that simulate mycobacterial proteins. These proteins, ESAT-6, CFP-10 and TB7.7 (p4), are absent from all BCG strains and from most non-tuberculosis mycobacteria with the exception of *M. kansasii*, *M. szulgai*, and *M. marinum*. Individuals infected with M. tuberculosis complex organism usually have lymphocytes in their blood that recognise these and other mycobacterial antigens. This recognition involves the generation and secretion of the cytokine, IFN-γ. The detection and subsequent quantification of IFN-γ forms the basis of the test.

- Numerous studies have demonstrated that peptides antigens used in this test stimulate IFN-γ responses in T-cells from individuals infected with *M. tuberculosis*, but generally not from uninfected or BCG vaccinated people without disease or risk for LTBI. However, medical treatments or conditions that impair immune functionality can potentially reduce IFN-γ responses.

- QuantiFERON® – TB Gold IT test is both a test for LTBI and may be useful in diagnosing *M. tuberculosis* complex in sick people. A positive result supports the diagnosis of tuberculosis disease: however infection by other mycobacteria (e.g. *M. kansasii*) could also lead to a positive result. Other medical and diagnostic evaluations (i.e. risk assessment, radiography) are necessary to confirm or exclude tuberculosis disease.

- The QuantiFERON® – TB Gold IT test is performed in two stages:

  i. Blood is collected into each of the QuantiFERON® – TB Gold IT tubes which include:

  - Nil control (Grey cap)
  - TB Antigen (Red Cap)
  - Mitogen control (Purple Cap)

  ii. Following a 20 to 24 hour incubation period, the tubes are centrifuged, the plasma is removed, and the amount of IFN-γ measured by ELISA.

Sample Collection and Incubation

Samples will be collected at either household or clinic level as part of the SOCS procedure.

1. Before sample collection make sure:

- The phlebotomy equipment is ready
- Each blood collection tube is correctly labeled with the individual's barcode ID
- Each record form is labeled with the individual's barcode ID

2. For each subject collect **1 ml** of blood by venepuncture directly into **each** of the QuantiFERON-TB Gold blood collection tubes (three tubes per patient).

- As 1 ml tubes draw blood relatively slowly, keep the tube on the needle for 2-3 seconds once the tube appears to have completed filling, to ensure that the correct volume is drawn.

The black mark on the side of the tubes indicates the 1ml fill volume. QuantiFERON TB Gold blood collection tubes have been validated for volumes ranging from 0.8 to 1.2 ml. If the level of blood in any tube is not close to the indicator line, it is recommended to obtain another blood sample. If this happens, remember to put the individual's ID bar code on the tube.

3. Mix the contents of the tube with the blood thoroughly by turning the tube end-over 8 to 10 times or shaking the tube for 5 seconds ensuring that the entire inner surface of the tube has been coated with blood.

4. Keep the samples at room temperature in an **UPRIGHT** position and transfer to a 37^0C incubator within 30 minutes of collection, either a portable incubator in the vehicle, or a small incubator in the clinic lab.

5. Report the date and time of blood collection on the record form.

Important notes:

- **Do not refrigerate or Freeze the blood sample before incubation!**
- **Before incubation of the samples, make sure they have been properly mixed as described above.**

6. Incubate the samples for 20-24 hours maximum:

- All samples MUST be incubated for the same time period (make sure you record the time of start of the incubation period and respect the agreed 20-24 hours of incubation).
- Samples must NOT exceed 24 hours of incubation.

7. Report the time of incubation's start on the record form.

Laboratory Procedures

South Africa and Lusaka Sites in Zambia:

- Portable incubators will be used:
  - Make sure that you plug in the portable incubator at the start of the day to allow it to equilibrate its temperature.
  - Ensure that all switches are in the correct position and that the temperature is 37°C (+/-1 °C) when putting blood tubes in.
  - Record any problems on the sample form e.g. power supply failures, failure to maintain correct temp etc.
- Samples must be taken to the research laboratory using the portable incubators the same day of collection and then transferred to laboratory incubator until the end of the incubation period.
- The laboratory staff member receiving the specimens must sign on the record form as appropriate.
- Once the samples are delivered to the laboratory, make sure the portable incubators are brought back and are available for the field work.

Zambia except for Lusaka sites:

- Blood samples will be incubated in small incubators in the ZAMSTAR clinics. They will remain in the incubators for the full incubation period.
- If blood is to be taken in the household in a site where this will take more than 30 minutes to get back to the clinic, a portable incubator should be used in the field (in the car) and then the blood transferred into the incubator at the clinic as soon as possible.
- Samples will be processed in the laboratories in the clinics.

In the central laboratory in South Africa and Zambia and in the peripheral laboratories in Zambia (all sites EXCEPT for Lusaka) the following procedures will be conducted:

STAGE 1: Bar-coding and harvesting of plasma.

1. At the end of the incubation period the samples must be removed from the incubator and the date and time recorded on the sample form.

2. Samples should be centrifuged as soon as possible after the end of the 20-24 hours of incubation.

3. If samples cannot be processed immediately after this period of time (e.g. due to unforeseen circumstances such as power failure or weekends), the blood collection tubes may be held in the fridge between 2°C and 27°C for up to 3 days prior to centrifugation.

4. Centrifuge the blood collection tubes for 10 min at 1500 to 2200 RCF (g). The gel plug will separate the cells from the plasma, if this does not occur, the tubes should be re-centrifuged at higher speed.

5. Report the date and time of plasma extraction on the record form.

6. After centrifugation, arrange centrifuged tubes in the blood stand making sure each triplet of tubes refer to the same patient. Check that the barcodes on the 3 tubes are all the same and that the record form matches the barcode.

7. For each triplet of blood collection tubes do the following:

A. RED CAP Blood collection tube

- Take one of the red-topped (or pink) 1.5 ml eppendorf provided, labeled with a unique random number.
- Harvest 150µl plasma from red cap blood collection tube into the 1.5 ml red topped (pink) eppendorf tube.
- Report the barcode number of the eppendorf on the record form.

B. GREY CAP Blood collection tube

- Take one of the grey topped (or grey) 1.5 ml eppendorf provided, labeled with a unique random number.
- Harvest 150µl plasma from the grey cap blood collection tube into the 1.5 ml grey topped (grey) eppendorf tube.
- Report the barcode number of the eppendorf on the record form.

C. PURPLE CAP Blood collection tube

- Take one of the purple topped (purple) 1.5 ml eppendorf provided, labeled with a unique random number.
- Harvest 150µl plasma from the purple cap blood collection tube into the 1.5 ml purple topped (purple) eppendorf tube.
- Report the barcode number of the eppendorf on the record form.

8. Place the 3 eppendorfs (1 red topped (pink), 1 grey topped (grey) and 1 purple topped (purple)) in a plastic bag and seal.

9. Store the plasma at +2-8°C (in the fridge) for 4 to 8 weeks.

10. Store the record form in the proper folder or in the envelope in the specimen bag.

Zambia sites except Lusaka:

All specimens will be transported to Lusaka on the agreed day of the month in a cooler box. The specimens with completed laboratory forms (and matching SOCS envelopes) will be carried by the data person to Lusaka and handed over and signed for by the laboratory staff.

STAGE 2: Freezing Samples

To be performed at the central laboratories in both Zambia and South Africa.

Note: In Lusaka Sites and in South Africa transport of samples will not be needed as samples are already stored in the Central lab. At the end of the 4-8 weeks, take the samples stored at 2-8°C and follow the procedures as described below.

- After 4-8 weeks at +2-8°C, or whenever the samples from Zambian sites (except Lusaka) arrive at the central laboratory, plasma will be stored at -70°C until ELISA is performed.
- For each specimen bag, do the following:

1. Complete the information on the record form detailing the date of delivery at the central lab and the receiving technician (if applicable).
2. Scan the barcode from each quantiferon recording form and the corresponding eppendorfs (with the unique random number).
3. Allocate the eppendorfs to specified storage boxes/racks/freezers.
4. Take the eppendorfs out of the plastic bags and store them in the specified freezer space at -70°C.
5. Record the date of freezing on the record form.
6. Enter the data on the record forms in the database.

STAGE 3. Human IFN-γ ELISA

This will be done in batches when enough samples are ready to be tested. It is best for samples to be tested in batches of 28 subjects (i.e. 28 sets of the 3 tubes)

1. Bring all plasma samples and reagents, **except for Conjugate 100X Concentrate,** to room temperature (22°C ± 5°C) before use. Allow at least for 60 minutes for equilibration.

2. Remove strips that are not required from the frame, reseal in the foil pouch, and return to the refrigerator for storage until required.
   Allow at least one strip for the QuantiFERON® – TB Gold standards and sufficient strips for the number of subjects being tested. After use retain frame, and lid for use with the remaining strips.

3. **Reconstitute the freeze dried Kit Standard** with the volume of deionised or distilled water indicated on the label of the Standard vial. Mix gently to minimise frothing and ensure complete solubilisation. Reconstitution of the Standard to the stated volume will produce a solution with a concentration of 8.0 IU/ml.

Note: the reconstitution volume of the Kit Standard will differ between batches.

Use the reconstituted Kit Standard to produce a 1 in 4 dilution series of IFN-γ Green Diluent (GD).

S1 (Standard 1) contains 4IU/ml, S2 (Standard 2) contains 1IU/ml, S3 (Standard 3) contains 0.25IU/ml, and Standard 4 contains 0 IU/ml (GD alone). The standard should be assayed at least in duplicate.

Recommended procedure for duplicate standards
a. Label 4 tubes "S1", "S2", "S3", "S4"
b. Add 150 µl of GD to S1, S2, S3, S4
c. Add 150 µl of the Kit Standard to S1 and mix thoroughly.
d. Transfer 50 µl from S1 to S2 and mix thoroughly.
e. Transfer 50 µl from S2 to S3 and mix thoroughly.
f. GD alone serves as zero standard.

Recommended procedures for triplicate standards
a. Label 4 tubes "S1", "S2", "S3", "S4"
b. Add 150 µl of GD to S1, S2, S3, S4
c. Add 210 µl of GD to S2, S3, S4
d. Add 150 µl of the Kit Standard to S1 and mix thoroughly.
e. Transfer 70 µl from S1 to S2 and mix thoroughly.
f. Transfer 70 µl from S2 to S3 and mix thoroughly.
g. GD alone serves as zero standard.

Note: prepare fresh dilutions of the Kit Standard for each ELISA session.

4. **Reconstitute freeze dried Conjugate 100X Concentrate** with 0.3 mL of deionised or distilled water. Mix gently to minimise frothing and ensure complete solubilisation of the Conjugate.

   Prepare working Strength conjugate by diluting the required amount of reconstituted Conjugate 100X Concentrate in Green Diluent as set out in **Table 8.1 – Conjugate Preparation**.

Table 8.1: Conjugate preparation

Number of strips	Volume of Conjugate 100X Concentrate	Volume of Green Diluent
2	10 µL	1.0 mL
3	15 µL	1.5 mL
4	20 µL	2.0 mL
5	25 µL	2.5 mL
6	30 µL	3.0 mL
7	35 µL	3.5 mL
8	40 µL	4.0 mL
9	45 µL	4.5 mL
10	50 µL	5.0 mL
11	55 µL	5.5 mL
12	60 µL	6.0 mL

- Mix thoroughly, but gently to avoid frothing.
- Return any unused Conjugate 100X Concentrate to 2°C to 8°C immediately after use.
- Use only Green Diluent.

5. Prior to assay, mix the plasma to ensure that IFN-γ is evenly distributed throughout the sample.

6. Add **50 µL** of freshly prepared Working Strength conjugate to the required ELISA wells using a multi-channeled pipette.

7. Add **50 µL** of the test plasma samples to appropriate wells using a multichannel pipette (see figure J. 1).

8. Finally, add **50 µL** each of the Standard 1 to 4.

9. Mix the conjugate and plasma samples/standards thoroughly using a microplate shaker for 1 minute.

10. Cover each plate with a lid and incubate at room temperature (22°C ± 5°C) for 120 ± 5 minutes.

- plates should not be exposed to direct sunlight during incubation

Figure J.1: Recommended sample layout for the Nil, TB Antigen and Mitogen tubes (28 tests per plate)

	1	2	3	4	5	6	7	8	9	10	11	12
A	1N	1A	1M	S1	S1	S1	13N	13A	13M	21N	21A	21M
B	2N	2A	2M	S2	S2	S2	14N	14A	14M	22N	22A	22M
C	3N	3A	3M	S3	S3	S3	15N	15A	15M	23N	23A	23M
D	4N	4A	4M	S4	S4	S4	16N	16A	16M	24N	24A	24M
E	5N	5A	5M	9N	9A	9M	17N	17A	17M	25N	25A	25M
F	6N	6A	6M	10N	10A	10M	18N	18A	18M	26N	26A	26M
G	7N	7A	7M	11N	11A	11M	19N	19A	19M	27N	27A	27M
H	8N	8A	8M	12N	12A	12M	20N	20A	20M	28N	28A	28M

- S1 (Standard 1), S2 (Standard 2), S3 (Standard3)
- 1N (Sample 1. Nil Control plasma)
- 1A (Sample 1. TB Antigen plasma)
- 1M (Sample 1. Mitogen Control plasma)

11. During the incubation, dilute one part Wash Buffer 20X Concentrate with 19 parts deionised or distilled water and mix thoroughly. Sufficient Wash Buffer 20X Concentrate has been provided to prepare 2L of working Strength wash buffer.

Wash wells with **400 µL** of Working Strength wash buffer for at least 6 cycles. **An automated plate washer is recommended.**

- Thorough washing is very important to the performance of the assay. Ensure each well is completely filled with wash buffer to the top of the well for each wash cycle. A soak period of at least 5 seconds between each cycle is recommended.
- Standard laboratory disinfectant should be added to the effluent reservoir, and established procedures followed for the decontamination of potentially infectious material.

12. Tap plates face down on absorbent towel to remove residual wash buffer. Add **100 µL** of Enzyme Substrate Solutions to each well and mix thoroughly using a microplate shaker.

13. Cover each plate with a lid and incubate at room temperature (22°C ± 5°C) for 30 minutes.

- Plates should never be exposed to direct sunlight during incubation.

14. Following the 30 minutes incubation, add **50 µL** of Enzyme Stopping Solutions to each well and mix.

- Enzyme stopping solution should be added to wells in the same order and approximately the same speed as the substrate in step 13.

15. Measure the Optical Density (OD) of each well within 5 minutes of stopping the reaction using a microplate reader fitted with a 450 nm filter and with a 650 reference filter. OD values are used to calculate results.

Quality Control of the Test

For the ELISA to be valid:

- The mean OD value for Standard 1 must be ≥ 0.600.
- The %CV for Standard 1 and Standard 2 replicate OD values must be ≤ 15%.
- Replicate OD values for Standard 3 and Standard 4 must not vary by more than 0.040 OD units from their mean.
- The correlation coefficient (r) calculated from the mean absorbance values of the standard must be ≥ 0.98.

  The QuantiFERON® – TB Gold IT Analysis Software calculates and reports these quality control parameters.

  Note: If the above criteria are not met, the run is invalid and must be repeated.

- The mean OD value for the Zero Standard (Green Diluent) should be ≤ 0.150. If the mean OD value is > 0.150 the plate washing procedure should be investigated.

Interpretation of the Results

Nil (IU/mL)	TB Antigen minus Nil (IU/mL)	Mitogen minus Nil (IU/mL)[1]	QuantiFERON® – TB Gold IT Result	Report/Interpretation
≤ 8.0	< 0.35	≥ 0.5	Negative	*M. tuberculosis* infection NOT likely
	≥ 0.35 and <25% of Nil value	≥ 0.5		
	≥ 0.35 and ≥ 25% of Nil value	Any	Positive[2]	*M. tuberculosis* infection likely
	< 0.35	< 0.5	Indeterminate[3]	Results are indeterminate for TB-Antigen responsiveness
	≥ 0.35 and <25% of Nil value	< 0.5		
> 8.0[4]	Any	Any		

Key:

1. Responses to the Mitogen positive control (and occasionally TB Antigen) can be commonly outside the range of the microplate reader. This has no impact on test results.

2. Where *M. tuberculosis* infection is not suspected, initially positive results can be confirmed by retesting the original plasma samples in duplicate in the QuantiFERON® – TB Gold ELISA. If repeat testing of one, or both replicates, is positive, then the individual should be considered test positive.

3. Indeterminate results should be uncommon and may be due to the immune status of the individual being tested, but many also be related to a number of technical factors:

- Longer than 16 hours from blood draw to incubation at 37°C.
- Storage of blood outside the recommended temperature range (22°C±5°C).
- Insufficient mixing of blood collection tubes.
- Incomplete washing of the ELISA plate.

4. In clinical studies, less than 0.25% of subjects had IFN-γ levels of > 8.0 IU/mL for the Nil Control.

Note: the magnitude of the measured IFN-γ level cannot be correlated to stage or degree of infection, level of immune responsiveness, or likelihood of progression to active disease.

Appendix 9.: QuantiFERON® – TB Gold IT Recording Form

ADULT BARCODE	

QGIT01_ **Visit No**

QGIT02a_ **Nurse Code**

QGIT03_**Date of sample collection** — **D D M M Y Y Y Y**

QGIT03a_**Time of sample collection** — **h h m m**

QGIT04_**Date of incubation's start** — **D D M M Y Y Y Y**

QGIT05_**Time of incubation's start** — **h h m m**

QGIT05a_**Temp of incubation's start (^{0}C)**

QGIT06_**Date of Incubation's end** — **D D M M Y Y Y Y**

QGIT07_ **Time of incubation's end** — **h h m m**

QGIT07a_**Temp of incubation's end (^{0}C)**

QGIT08_**Date of plasma extraction** — **D D M M Y Y Y Y**

QGIT09_ **Eppendorf barcodes**

GREY CAP TUBE

RED CAP TUBE

PURPLE CAP TUBE

QGIT10_**Date of storage at –20 Celsius** — **D D M M Y Y Y Y**

QGIT11_**Date of delivery at central lab** — **D D M M Y Y Y Y**

QGIT12_**Date of storage at – 80^{0}C** — **D D M M Y Y Y Y**

QGIT13a_ **Signature** _____

Appendix 10. : Standardized QuantiFERON –TB Gold IT responses to participants

Quantiferon results: response to participants

QGIT is a new blood test, still under research and not yet fully understood by researchers throughout the world.

If a participant has TB symptoms, regardless of the QGIT results they should be referred to the clinic for further screening. Below are suggested guidelines of how these results can be explained to participants.

1. Positive Quantiferon test results. This means that the participant has been exposed to TB or is infected with TB. This does not mean that they have TB disease, as many people in the community are exposed to TB and get infected without getting the disease. The participant does not need to be referred to the clinic for further investigations due to a positive result unless they are unwell.

2. Negative Quantiferon test result. This means that the participant has not been exposed to TB or is not infected with TB.

3. Indeterminate Quantiferon test result: The test is neither positive nor negative. This means that the test has failed to work. The TB infection status of the participant could not be determined although it may be able to do so at a later stage when the test is repeated (Note: Indeterminate results will not be routinely repeated). Failure of the test to work can be a result of many factors related to the participant (low immune response), process (blood collection and sample management) or laboratory technical errors.

4. Discordant QGIT and TST results: Discordant results are when the two tests do not agree (QGIT +ve and TST –ve or QGIT -ve / TST +ve). The two tests are different and measuring different aspects of the immune system and may not necessarily agree. When results are discordant, the TST (which has been used for a long time and better understood) result should take preference over the QGIT result.

5. No test result available due to errors: In some cases the participant had given blood for QGIT, but no test could be done in the ZAMBART laboratory. Possible technical and logistical errors are for example: (1) the volume of the blood sample was insufficient (2) the tube broke during transport (3) the tube was not well closed and blood leaked out (4) the blood had clotted.

Appendix 11. : QuantiFERON® –TB Gold IT Monitoring Tool

Name of site: _____ Date: _____ Quarter under review: _____

Functional area	Question	Indicator	Specific indicator	Source Of data	Method	Field Evaluation
General work place and sop observation	Is all the necessary equipment for sample processing in stock?	Blood collection bottles (red, grey, purple) butterflies/vacutainer, Needles and holder, mobile and laboratory incubator centrifuge		Evaluator	Observation	See table 1
	Do staff know the QGIT SOP (select one staff responsible for centrifugation and incubation randomly)?	Staff accurately narrates how samples are processed according to SOP		Nurse or Microscopist	Interview	See table 2 (A and B)
	Do all the three bottles for one person (grey , red, purple) have the same barcode?	Barcodes on the three bottles (red, grey, purple)	Check specimens collected on evaluation day and check the barcode label	Evaluator	Observation	Yes Table 4
	Is blood properly mixed before incubation as per SOP?	Observe them mix samples collected on evaluation day		Evaluator	Observation	Yes No
	After blood collection how long does it take before samples are put in the incubator?	Time	Take note of the time between collection and incubation	Nurse or Microscopist	Interview	Time:
	Are the management forms properly and accurately filled in?	Management form	Pick ten forms randomly and check if their accurately and properly filled	Microscopist or nurse	Interview	See table 3

Functional area	Question	Indicator	Specific indicator	Source Of data	Method	Field Evaluation
Laboratory procedure after incubation	After sample incubation how long does it take before samples are centrifuged?	Time		Microscopist or nurse	Interview	Time _____
	Are the samples properly centrifuged before separation?	Blood cells should be at the bottom, gel in the middle and serum on top	Check samples centrifuged on evaluation day	Evaluator	Observation	Yes No
	Is centrifuged serum transferred to their corresponding colour eppendorfs (grey to grey, red to red, purple to purple)?		Observe them transfer the serum into eppendorfs	Evaluator	Observation	Yes No
	After centrifugation how long does it take before the plasma is extracted?			Microscopist or nurse	Interview	
	Are the eppendorfs kept in the freezer after plasma extraction?	Serum eppendorfs in the freezer		Evaluator	Observation	Yes No
	What is the approximate temperature of the freezer used?	Temperature	Check whether they use a deep freezer (-20) or an ordinary freezer (4) degrees	Evaluator	Observation	Temperature ----------
	How often do power cuts occur? (during which time night or day)		Interview the person in charge of sample incubation	Microscopist or nurse	Interview	Hours per week ----------

The Following section evaluates equipment used in the processing, knowledge of QuantiFERON SOP and completeness of recorded forms.

Equipment	Field Evaluation	Number in Stock	Comment
Collection bottles (grey, red, purple)	Yes / No		
Vacutainer needles and holder/butterflies	Yes / No		
Mobile incubator (where applicable)	Yes / No		
Working Laboratory incubator(where applicable)	Yes / No		
Working Centrifuge	Yes / No		

SOP Content for Sample Incubation (for responsible person)	Field Evaluation	Comment
Know the maximum time before sample incubation	Yes / No	
Know what temperature samples should be incubated	Yes / No	
Know how long samples should be incubated	Yes / No	
SOP Content for Sample Centrifugation (for responsible person)	**Field Evaluation**	**Comment**
Know what is meant by fully centrifuged	Yes / No	
Know the temperature at which serum is stored	Yes / No	
Check for completeness of management Form	**Field Evaluation**	**Comment**
Nurse name	Yes / No	
Nurse code	Yes / No	
Date of sample collection	Yes / No	
Time sample collection	Yes / No	
Date of start incubation	Yes / No	
Time of incubation start	Yes / No	
Date of incubation end	Yes / No	
Time of incubation end	Yes / No	
Date of serum separation	Yes / No	
Barcodes label properly and accurately filled	Yes / No	
Is the same barcode on all three specimen	**Field evaluation**	**Comment**
Study participant 1	Yes / No	
Study participant 2	Yes / No	
Study participant 3	Yes / No	

Accuracy = $\frac{\text{Number of forms checked}}{\text{Number of forms correctly filled}}$ x 100

Accuracy = $\frac{\text{Number of bottle sets checked}}{\text{Number of bottle sets with same barcodes}}$ x 100

Comments on Sample collection, storage and processing

_____
_____
_____
_____
_____
_____

In general comment on what happens to the samples in case of power outage

_____
_____
_____
_____
_____
_____

Appendix 12. : TST recording form

ADDRESS BARCODE	**ADULT BARCODE**

PPD batch used		**Dose**	0.1ml (2TU)

		Baseline	**M18 (Visit 2)**	**M36 (Visit 3)**
Q01_VIS	**Visit**	**1**	**2**	**3**

Q03_DAT **Today's date**

D	**D**	**M**	**M**	**Y**	**Y**	**Y**	**Y**

Q09_ASSINJ **SOCS nurse code injecting PPD**

Q10_ASSREAD **SOCS nurse code reading TST**

Q11_TSTDONE **Date TST done**

D	**D**	**M**	**M**	**Y**	**Y**	**Y**	**Y**

Q12_TSTREAD **Date TST read**

D	**D**	**M**	**M**	**Y**	**Y**	**Y**	**Y**

Q13_TSTIND **Induration (mm)**

	Code	**Date**								**Signature**
		d	**d**	**m**	**m**	**y**	**y**	**y**	**y**	
Field Manager										
1st data entry										
2nd data entry										

Appendix 13. : Sputa transportation form for SOCS Visit 3

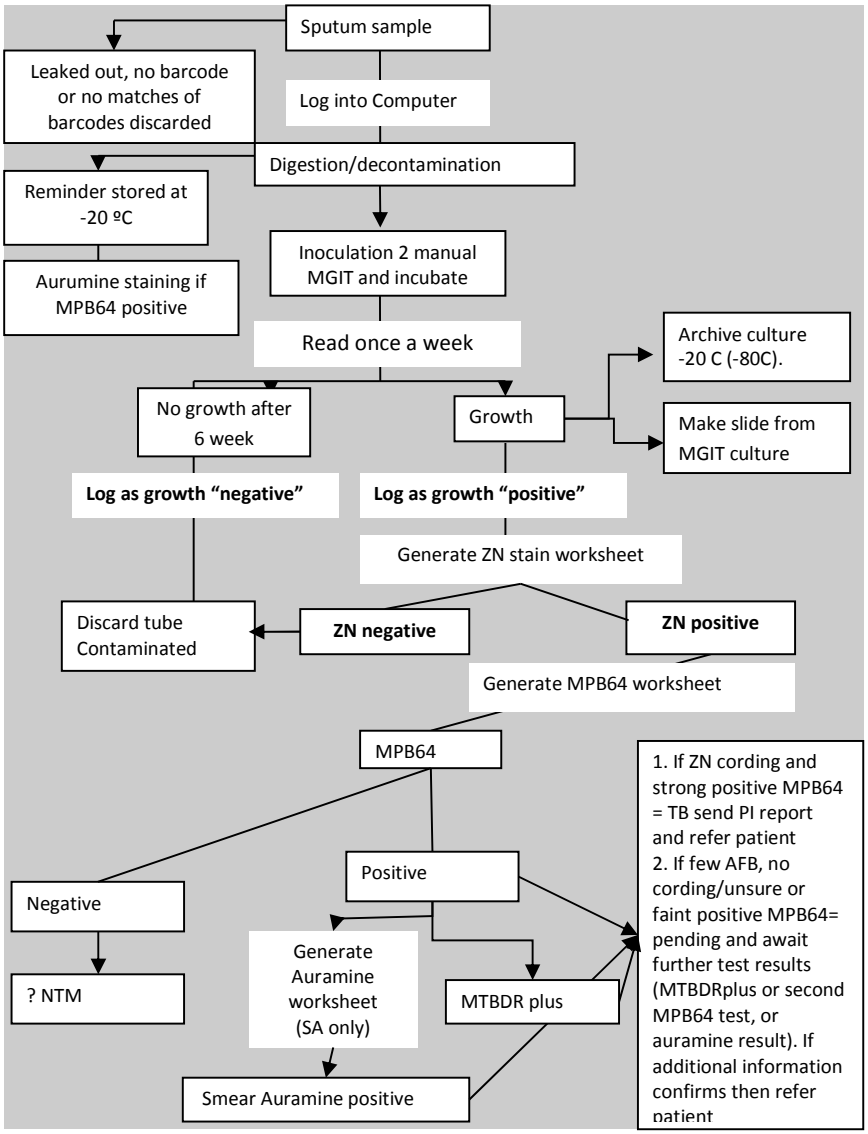
ZAMSTAR	Transportation Form	Date Samples collected:	Page

Site				Date sent to Zambart House/Post courier				Total No. of samples sent:			
Individual Barcode	Sent	*Received*	*Received at CTL*	Individual Barcode	Sent	*Received*	*Received at CTL*	Individual Barcode	Sent	*Received*	*Received at CTL*
	☐	☐	☐		☐	☐	☐		☐	☐	☐
	☐	☐	☐		☐	☐	☐		☐	☐	☐
	☐	☐	☐		☐	☐	☐		☐	☐	☐
	☐	☐	☐		☐	☐	☐		☐	☐	☐
	☐	☐	☐		☐	☐	☐		☐	☐	☐

Delivered By ITL/Post Courier:_____ **Received By:**_____

Date Received at CTL:_____

Appendix 14. : Algorithm for sputum processing and decision on whom to follow-up



Appendix 15. : Culture positive follow up form for Visit 3

Q01_SURNAME		**Q02_FIRST NAME**	
Q03_SEX		**Q04_AGE**	
Q05_ADDRESS			
Q06_LATITUDE		**Q07_LONGITUDE**	

Section B – Availability of Individual

Q08_IND_AVAILABLE FOR FOLLOW-UP	Yes	No			
Q09_REASON_NOT_ AVAILABLE	Died	Moved	Absent	Refused	Unknown

Section C – Clinical Review Symptoms (More Options are Possible)

Q10_SYMPTOMS	Cough	Chest Pains	Cough Up Blood	Fever
	Weight Loss	Night Sweats	Difficulty Breathing	

Section D – FU SPUTUM Smear Results – Fill Follow-Up Smear Results from Local Clinic Lab

Q11_SPOT	Negative	Scanty (1-9)	1+	2+	3+	Not Done
Q12_MORNING	Negative	Scanty (1-9)	1+	2+	3+	Not Done

Section E – Chest X-Ray

Q13_XRAY	Abnormal TB	Abnormal Not TB	Normal	Not Done

Section F – FU CULTURE Results – Complete when Follow_Up Results are Available from CTL

Q14_CULTURE	TB Isolated				TB Not Isolated			
Q15_DST	Q15_INH		Q15_SM		Q15_RIF		Q15_EMB	
	S	R	S	R	S	R	S	R

Section H – Decision

Q16_DECISION	TB	No TB
Q17_CLINICAL_DECISION	Yes	No
Q18_TREATMENT_STARTED	Yes	No
Q19_DATE_TREATMENT_STARTED		
Q20_TB TREATMENT_NUMBER		
Q21_TB TREATMENT_OUTCOME		
Q22_REMARK		
SIGNATURE	**DATE SIGNED OFF**	D D M M Y Y Y Y

Appendix 16. : Adult Questionnairre for Visit 3

SECTION A – GENERAL INFORMATION

		M36 (Visit 3)
Q01_VIS	**Visit**	3
Q02_COD	**Interviewer's code**	

		D	D	M	M	Y	Y	Y	Y
Q03_DAT	**Date of Interview**								

I would now like to ask you some questions about your current drinking habits

		No	Yes	Unknown
Q11_DA	**During the last 4 weeks have you taken any drinks containing alcohol?** Pamasabatainaiyapita, munamwapo moba uliwonse? *Bushe pa milunguine (4) iyapita ,mwalinwapo ubwalwa?*	**0**	**1**	**9**
Q12_DD	**During the last year have you ever drunk so much that you were unable to remember what you were doing?** Mu chaka chathaichi munamwapo moba ndikulezelakwambirikufikilakutisimunakumbukilechi menemunalikuchita? *Muliumwakawapwilemwaliikolwapoicakutimwalaban efyomwalecita?*	**0**	**1**	**9**

I would now like to ask you some questions about your sexual relationships

Q16_MS **What is your marital status now?**
Kodi ndinuokwatilakapenaosakwatilapaliinonthawi?
Bushe:

Single Osakwatila *Tamwaupa/tamwaupwa?*	1	**Married/living as married** Okwatila / kukhalamongaOkwatila *Mwalyupa/mwalyupwa/mwikapamonga baupana*	2
Widowed Ofedwamwamunakapenamufelekazi *Abena mwenu balifwa?*	3	**Divorced/separated** Chikwatichinathaolokupatukanachabe *Mwalilekana/tamwikalapamo?*	4

		No	Yes	Unknown
Q17_SX	**In the last 6 months have you had sex?** Pa mweziisanundiumodziyapitapomunagonapondimkazi/mwamuna *Mu myeshimutanda (6) iyapita, mwalilalapo no mwanakashi/umwaume?*	0	1	9

(IF YES CONTINUE. IF NO OR UNKNOWN GO TO NEXT SECTION B, Q18)

		No	Yes	Unknown
Q17_1_OP	**In the last 6 months at any stage, have you had more than one sexual relationship at the same time?** Kodi pa mweziisanundiumodzi (6) yapitapoiyi, munagonanapokapenakukumanandimwamunakapenamkaziwinakuchoselakoamenemumakumananayenthawizonse**?** *Mu myeshimutanda (6) iyapitamwalilalapo no mwanakashi/umwaumeumbiukucilipabomula lanabosana?*	0	1	9

(IF YES CONTINUE. IF NO OR UNKNOWN GO TO Q17_2_POP)

Q17_1_1_CM	**Did you use condoms with these partners?** Kodimunasebenzesamupilawakondomupali abo munagonananao? *Bushemwalibomfesheumupilanaba bantu mwalelenabo?*			
Never Simunasebenzese *Awe*	1	**Always** Nthawi zones *Lyonse*	2	
Sometimes Nthawizina *Limolimo*	3	**Unknown/ no answer** Siniziwa *Nshishibe*	9	

		No	Yes	Unknown
Q17_2_POP	**In the last 6 months do you think that your main partner has had other partners apart from you?** Pa mweziisanundiumodziyapita, muganizakutiabwenzianuanagonapondimunthuwina? *Mu myeshimutanda (6) iyapita, bushemuletontonkanyaukutiabatemwikabenu abo mulalanabosanabalikwatabambi abo balelalanaboukucilapaliimwe?*	0	1	9

SECTION B – TB AND HIV QUESTIONS – ASK ALL RESPONDENTS

I would now like to ask you some questions about your health, specifically about TB and HIV
Ndifunakufunsamafunso a khanizaumoyowanumakamaka pa TB na HIV
Ndefwayanombaukumwipushakoamepusho pa bumiwenumakamakapali TB na HIV

		No	Yes
Q18_TBT	**Since 2006, have you been on TB treatment?** Kucokera mu choka cha 2006, kodimunakhalakopamankhwala a chifuwa cha TB? *Ukufumamumwakawa 2006, bushemwalibapopamutiwa TB?*	**0**	**1**

(IF YES CONTINUE. IF NO GO TO Q19)

Q18_3_TBT	**How many times have you been on TB treatment since 2006?** Kodi munakhalapo kangati pa mankhwala a chifuwa cha TB kucokera mu chaka cha 2006? *Miku inga iyo mwalipo pamuti wa TB ukufuma mumwaka wa 2006*?	**1**	**2**	**3**

		No	**Yes**
Q18_4_TBT	**Can I see your TB card(s)?** Nipempakuona TB (ma TB) card yano? *Ndelombaukumona TB (nangulaama TB) card yenu?*	**0**	**1**

Q18_5_TBT **If yes, using TB card (s). Research assistant to check and complete the following**

	TB Regnum	**Date TB treatment started**								**Clinic where TB treatment was given**	**Type of TB on card[1]**				**Type Of Patient[2]**		
		D	**D**	**M**	**M**	**Y**	**Y**	**Y**	**Y**								
1											**1**	**2**	**3**	**9**	**1**	**2**	**9**
2											**1**	**2**	**3**	**9**	**1**	**2**	**9**
3											**1**	**2**	**3**	**9**	**1**	**2**	**9**

[1]Type of TB on card	**1 = Pulmonary Smear positive, 2 = Pulmonary Smear negative, 3 = Extra pulmonary, 9 = Unknown**
[2]Type of Patient	**1 = New, 2 = Retreatment, 9 = Unknown**

Q18_6_TBT **If no TB card (s) available, Research assistant to ask the following:**
Which month and year(s) did you start TB treatment?
Kodindimweziutindichaka(Zaka) pamenemunayambakulandirathandizo la mankhwalaachifuwa cha TB?
Ni mumweshinshikabilimwakanshimwatendekeukubapamutiwa TB?
Where did you receive your TB treatment?
Kodindikutikomwemunalandilirathandizo la mankhwala a chifuwa cha TB?
Nikwisamwalesendelaumutiwenuwa TB?
Was it sputum positive?
Kodizinkholodwa (Zikolala) zanuzinalimatenda a TB?
Busheubulwelebwa TB babusangilemufikolala?

	Month started (eg February)	**Year started**	**Which TB treatment Centre did you receive your treatment from?**	**Was it Sputum Positive?**		
				No	**Yes**	**Unknown**
1				**0**	**1**	**9**
2				**0**	**1**	**9**
3				**0**	**1**	**9**

		No	Yes	Unknown
Q19_HIV	**Have you ever been tested for HIV?** Kodimunapimitsakomagazi? *Bushemwalipimwapopalwakashishi ka HIV?*	**0**	**1**	**9**

(IF YES CONTINUE. IF NO OR UNKNOWN GO TO SECTION C, Q22_TSCT)

		Negative	Positive	Unknown
Q19_1_HIV	**Would you mind telling me what the result was?** Kodi mungakondwele kuniuza zimene anapezamo? *Bushekutimwanjebakofyatumbwikemo?*	**0**	**1**	**9**

(IF POSITIVE, CONTINUE. IF NEGATIVE OR UNKNOWN GO TO END)

		No	Yes	Unknown
Q20_ARV	**Have you ever taken ARV treatment?** KodimunamwapomankhwalayamaARVs? *BushemwalitalaamunwapoamaARVs?*	**0**	**1**	**9**

(IF YES CONTINUE. IF NO OR UNKNOWN GO TO Q21_IPT)

		No	Yes	Unknown
Q20_1_CARV	**Are you currently taking ARV treatment?** Kodimukumwamankhwalaya ARVs palipano? *Bushemulenwaama ARVs paliinonshita?*	**0**	**1**	**9**

(IF YES CONTINUE. IF NO OR UNKNOWN GO TO Q21_IPT)

		D	D	M	M	Y	Y	Y	Y
Q20_1_2_SARV	**When did you start ARV treatment? (01/01/1800 if unknown)** Kodimunayambalitikumwamankhwalaya ARVs? *Nilisamwayambileukunwaama ARVs?*								

		No	Yes	Unknown
Q21_IPT	**Have you ever been on TB Preventive Therapy (IPT)?** Kodimunamwapomankhwalayochingiliza TB (IPT)? *Bushemwalitalaamunwapoumutiwakucingililwakubulwelebwa TB (IPT)?*	**0**	**1**	**9**

Thank the Respondent for their Participation

	Interviewer's code	Date								Signature
		d	d	m	m	y	y	y	y	
Interviewer										
Field Manager										
1st data entry										
2nd data entry										

Appendix 17. :Information sheet for QuantiFERON-TB Gold sensitivity and reproducibility studies.

An Investigation into the Sensitivity and Reproducibility of the QuantiFERON-TB Gold In-Tube Assay in Zambian Adults with Smear-Positive Tuberculosis.

Information about this Research Study

This study is being conducted by Dr Edward Raby, a student from the London School of Hygiene and Tropical Medicine as part of his Masters' degree. He is working with a Zambian doctor, Dr Joseph Banda.

We would like to invite you to take part in a research study. Before you make a decision, you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish. Ask us if there is anything that is not clear or if you would like more information.

What is the purpose of the study?

A new blood test for tuberculosis (TB) has recently been developed and is used in routine practice in the United States of America and the United Kingdom. However, the blood test has not yet been widely used or tested in Zambia. In order to better understand how useful this test will be here, we need to see how well it works in people who have TB.

Why have I been invited?

The clinic staff have identified you as having tuberculosis.

Do I have to take part?

It is up to you to decide. We will describe the study and go through this information sheet, which we will then give to you. We will then ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

What will happen to me if I take part?

There are three parts to the study: (1) a blood test, (2) a skin test and (3) a short questionnaire about your health. This will take no more than thirty minutes today and five minutes next week to get the result of the skin test. If you have not begun your TB treatment yet, we will also ask you to give a second blood sample in 2 weeks time.

What will happen to the blood?

We will take the blood to the laboratory in Lusaka and use the blood sample to study the new TB blood test. With the same blood sample we will also do a special test called a CD4 count that tests the way your body fights infection.

Zambian medical guidelines recommend an HIV test in all people who have TB. If you have not had this done, and would like one, you will be referred appropriately. An HIV test is not a compulsory part of this study and you can take part without having one.

What are the possible disadvantages and risks of taking part?

You may get a bruise from the blood test. The skin test involves a small injection under the skin. This may itch over the next week. You will have to spend about 30 minutes longer in the clinic today.

What are the possible benefits of taking part?

As we already know you have TB, there will be no benefit to you of having the new TB blood test. It will not affect your treatment. However by having the test you are helping us understand how well the test works which may benefit other people in the future who have TB that is difficult to diagnose.

If you have HIV and your CD4 count is very low you would be likely to benefit from treatment for HIV and this will be offered to you. If your CD4 count is not very low, then you would be unlikely to benefit from HIV treatment at the moment.

What will happen if I don't want to carry on with the study?

It is up to you whether or not you would like to take part in the study. If you agree to take part, you can change your mind at any time and leave the study. After leaving the study you will still receive all the usual care in the clinic.

Will my taking part in this study be kept confidential?

This consent form is the only thing that will have your name on. This form will be kept in a locked cabinet that only Dr Raby and Dr Banda will have access to. All other papers and samples will be identified only by a study number and will not have your name on. They will not be used for any other purpose than the present study. If the results of the study are published or presented they will not identify you by name.

What if there is a problem?

If you have questions about this research study you may contact Dr. Edward Raby by email at edward.raby@lshtm.ac.uk.

Alternatively, contact Dr. Joseph Banda at ZAMBART offices based at the School of Medicine at Ridgeway Campus Telephone number 0211 254710 or mobile +26 0955905569 at any time. This study has been reviewed and approved by research ethics committees at both the University of Zambia and the London School of Hygiene and Tropical Medicine.

Appendix 18. : Consent form for QuantiFERON-TB Gold sensitivity and reproducibility studies.

An Investigation into the Sensitivity and Reproducibility of the QuantiFERON-TB Gold In-Tube Assay in Zambian Adults with Smear-Positive Tuberculosis.

Consent Statement

I have read the provided information about this study, or it has been read and explained to me. I have been informed of the potential risks involved in participating in this study as the coordinator or doctor has carefully explained these to me. I have been given a chance to ask questions about any queries that I may have and I feel that all of my questions have been answered. I have been informed that participating in this study is my choice and that even if I choose to be in this study today, I may withdraw from it at any time in the future. I have been informed that if I participate now, or quit/withdraw from the study at a later time, this will not affect my care at this clinic in any way. I have been informed that all information about me, my illness and my family will be kept private at all times. My signature below indicates that I agree to be in this study.

Name of the Participant (Please Print) _____

Signature of the Participant _____ Date _____

I have read this consent to the patient.

Signature of Person who read consent _____ Date _____

I have read this document to the patient in a language that they understand.

Signature of Interpreter _____ Date _____

Using language that is understandable and appropriate, I have explained this research project to the person named above.

Signature of Investigator_____ Date _____

Appendix 19. :Questionnaire for QuantiFERON-TB sensitivity and reproducibility studies

An Investigation into the Sensitivity and Reproducibility of the QuantiFERON-TB Gold In-Tube Assay in Zambian Adults with Smear-Positive TB.

Study Number	
Date & Time of Enrolment (DD/MM/HR:MIN)	
Age (Years)	
Sex (M/F)	
Height (cm)	
Weight (kg)	
Mid Upper Arm Circumference(cm)	
Date of TB Diagnosis (DD/MM/YYYY)	
Grade of smear positivity (1+, 2+ or 3+)	
Date Started Treatment (DD/MM/YYYY)	
Previous TB (Year)	
BCG Scar (YES/NO)	
HIV Test (Pos/Neg/Unknown)	
Last HIV Test (DD/MM/YYYY)	
ART (Yes/No)	
ART Date Started (DD/MM/YYYY)	
Accepted HIV Test Today? (Yes/No)	
Diabetes (Yes/No)	
Other medical condition	

OPEN ACCESS Freely available online

PLoS one

Risk Factors Associated with Positive QuantiFERON-TB Gold In-Tube and Tuberculin Skin Tests Results in Zambia and South Africa

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Abstract

Introduction: The utility of T-cell based interferon-gamma release assays for the diagnosis of latent tuberculosis infection remains unclear in settings with a high burden of tuberculosis.

Objectives: To determine risk factors associated with positive QuantiFERON-TB Gold In-Tube (QFT-GIT) and tuberculin skin test (TST) results and the level of agreement between the tests; to explore the hypotheses that positivity in QFT-GIT is more related to recent infection and less affected by HIV than the TST.

Methods: Adult household contacts of tuberculosis patients were invited to participate in a cross-sectional study across 24 communities in Zambia and South Africa. HIV, QFT-GIT and TST tests were done. A questionnaire was used to assess risk factors.

Results: A total of 2,220 contacts were seen. 1,803 individuals had interpretable results for both tests, 1,147 (63.6%) were QFT-GIT positive while 725 (40.2%) were TST positive. Agreement between the tests was low (kappa = 0.24). QFT-GIT and TST results were associated with increasing age (adjusted OR [aOR] for each 10 year increase for QFT-GIT 1.15; 95% CI: 1.06–1.25, and for TST aOR: 1.10; 95% CI 1.01–1.20). HIV positivity was less common among those with positive results on QFT-GIT (aOR: 0.51; 95% CI: 0.39–0.67) and TST (aOR: 0.61; 95% CI: 0.46–0.82). Smear positivity of the index case was associated with QFT-GIT (aOR: 1.25; 95% CI: 0.90–1.74) and TST (aOR: 1.39; 95% CI: 0.98–1.98) results. We found little evidence in our data to support our hypotheses.

Conclusion: QFT-GIT may not be more sensitive than the TST to detect risk factors associated with tuberculous infection. We found little evidence to support the hypotheses that positivity in QFT-GIT is more related to recent infection and less affected by HIV than the TST.

Citation: Shanaube K, Hargreaves J, Fielding K, Schaap A, Lawrence K-A, et al. (2011) Risk Factors Associated with Positive QuantiFERON-TB Gold In-Tube and Tuberculin Skin Tests Results in Zambia and South Africa. PLoS ONE 6(4): e18206. doi:10.1371/journal.pone.0018206

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Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Tuberculosis continues to be a major public health problem in sub-Sahara Africa. The incidence of tuberculosis [1] is being accelerated by high rates of HIV co-infection [2,3]. Targeted testing and treatment of latent tuberculosis infection (LTBI) especially among HIV positive individuals is an important strategy to reduce the incidence of tuberculosis [4].

Currently, LTBI detection relies on the tuberculin skin test (TST) in most countries with high incidence of tuberculosis although this is not routinely performed and is perceived as a barrier to accessing TB preventive therapy [5]. However, TST has many reported limitations. These include low specificity in populations with high levels of BCG vaccination or significant exposure to non-tuberculosis mycobacteria (NTM), and reduced sensitivity in immunocompromised individuals such as those with HIV infection [6,7].

T-cell based interferon-gamma release assays (IGRAs) such as the QuantiFERON-TB Gold In-Tube (QFT-GIT) can now also be used to detect LTBI [8]. IGRAs are in-vitro blood tests based on interferon-γ release after T-cell stimulation by antigens more specific to *Mycobacterium tuberculosis (Mtb)* than the purified protein

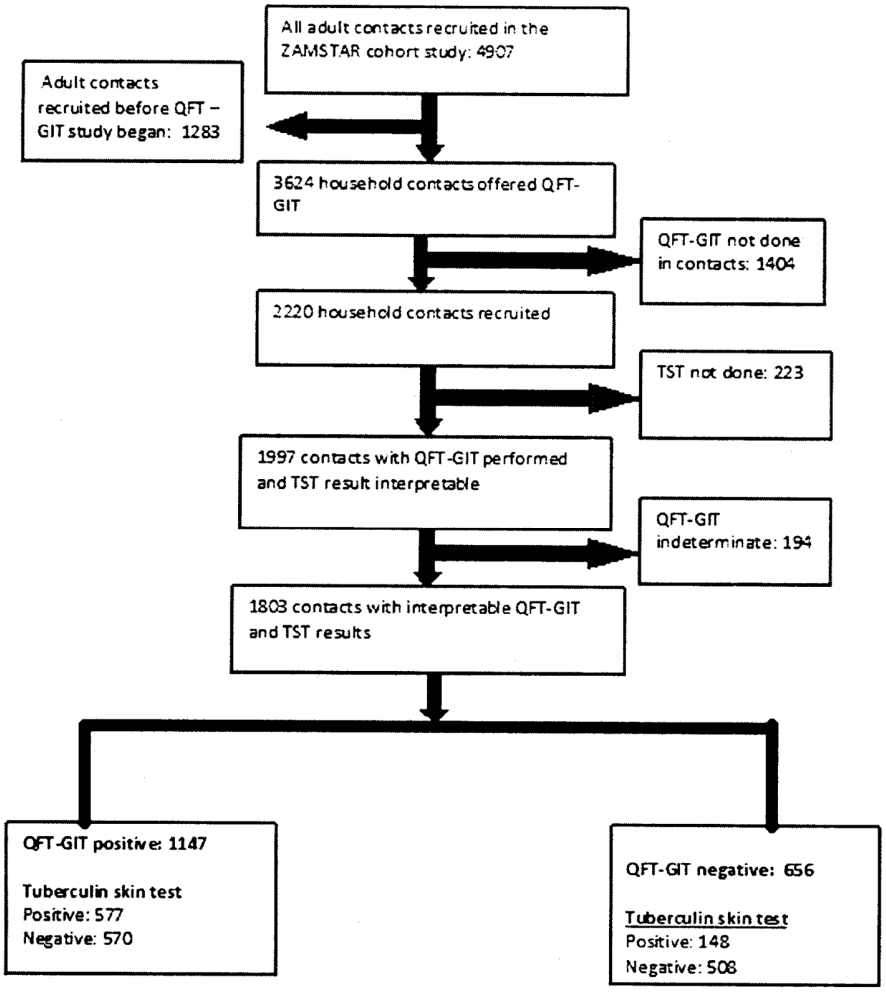

Figure 1. Flow diagram of study participants. QFT-GIT (QuantiFERON-TB Gold In Tube) not done was due to refusal (18.4%), being absent (18.3%), insufficient blood samples (0.4%) or missing data (1.6%). TST (Tuberculin skin test) not done was due to refusal (5.6%), not returning for reading (3.6%) or missing data (0.8%). Individuals with QFT-GIT/TST not done and those with indeterminate QFT-GIT results were excluded from analysis.
doi:10.1371/journal.pone.0018206.g001

derivative used in TST. IGRAs are therefore designed to have high specificity that is unaffected by BCG vaccination and cross-reactivity with most NTM [8]. There is also some evidence of greater sensitivity among HIV positive individuals [9,10] compared to the TST. Current literature suggests that IGRAs detect responses of effector T-cells that have recently encountered antigens *in vivo*, while TST reflects the mobilization of a wider spectrum of memory T-cells that are long-lived [11].

The use of IGRAs in developed countries is rapidly expanding but their performance in settings with a high prevalence of tuberculosis and HIV still requires further research [12,13]. There is growing evidence that IGRAs performance varies in different settings [14]. In high-TB burden settings, the results of IGRAs may be influenced by factors that affect the immune response [15] such as HIV co-infection, BCG vaccination, malnutrition, tropical infections and widespread exposure to NTM. Recent studies done in low and middle income settings [16] showed a large reduction in the proportion of positive test results for both QFT-GIT and TSPOT in HIV infected individuals. Another recent study in Bangladesh showed that malnutrition and helminth infections were associated with indeterminate QFT-GIT results in children [17].

Significant challenges exist in directly assessing whether IGRAs are superior to TST in diagnosing LTBI as there remains no gold

Table 1. Characteristics of the study population.

	Study participants n (column %)
Total	**2220**
Sex	
Male	666 (30.1)
Female	1545 (69.9)
Missing	9
Age group (years)	
15–24	835 (38.3)
25–34	556 (25.5)
35–44	302 (13.9)
45–54	246 (11.3)
55–64	134 (6.1)
>65	106 (4.9)
Missing	41
Age in years: Median 28 (IQR:21–42); mean 33	
Highest level of education	
Not attended school	142 (6.5)
Primary school	689 (31.9)
Secondary school	1126 (52.1)
College or University	203 (9.4)
Missing	60
Smoking habits	
Daily smoker	220 (10.0)
Occasional smoker	73 (3.3)
Ex-smoker	104 (4.7)
Never smoked	1801 (81.9)
Missing	22
[1]Alcohol consumption	
No	1648 (75.2)
Yes	544 (24.8)
Missing	28
Household size (adults)	
1–3	331 (15.0)
4–6	848 (38.4)
7–9	554 (25.1)
≥10	477 (21.6)
Missing	10
HIV status	
Negative	1271 (62.4)
Positive	765 (37.6)
Missing	184
Smear status of index	
Smear negative	741 (49.2)
Smear positive	766 (50.8)
Missing	713

[1]Defined as alcohol consumption four weeks prior to the interview.

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standard against which to compare either test. In the absence of a practical gold standard for *Mtb* infection, exposure to an infectious TB index case has been used as a surrogate measure of infection [18,19,20,21]. Studies from low-TB burden countries indicate that the IGRAs correlate better, along a gradient of exposure, than the TST [14]. Nevertheless, in high-TB burden settings, the TST performs reasonably well and correlates as well, or better, with proxy measures of exposure [14]. There are limited data on the comparison of QFT-GIT and TST in relation to *Mtb* exposure as a surrogate measure of infection and the influence of age [19].

In this study, we describe the prevalence of tuberculous infection among household contacts of recently diagnosed tuberculosis patients as measured by QFT-GIT and TST in 24 communities with a high prevalence of TB and HIV in Zambia and South Africa. We also determine risk factors associated with positive QFT-GIT and TST results and the level of agreement between the tests in each community. We use data from two recent TST surveys [22] to explore the correlation between community TB transmission and infection prevalence in contacts as measured by QFT-GIT and TST. A TST survey, if conducted correctly and technically interpretable, allows an estimation of the extent of *Mtb* transmission that has occurred in the community [23].

Finally, we formally assess the extent to which our results are compatible with expected findings on the basis of a number of prior hypotheses about the characteristics of each test. Previous studies have given rise to prevailing views about the expected performance of both TST and IGRAs [7,11,14,16]. We therefore explore whether our data support the hypotheses that positivity in QFT-GIT is more related to recent infection and less affected by HIV than the TST.

Methods

Ethics statement

Ethics approval for the study was obtained from the research ethics committees of the University of Zambia, the London School of Hygiene and Tropical Medicine and Stellenbosch University. All individuals involved in the study gave written informed consent.

Study setting

This cross-sectional study was nested within a large community randomized trial of interventions to reduce tuberculosis transmission, the Zambia South Africa TB and AIDS Reduction Study, (ZAMSTAR) in 24 selected communities in Zambia and South Africa [24]. We defined a "community" as the population (minimum size of 25,000) accessing one tuberculosis diagnostic centre and this was the unit of randomization for the ZAMSTAR trial. The communities selected were in five provinces of Zambia (16 communities) and in Western Cape Province of South Africa (8 communities) and included both urban and rural communities. The design of the ZAMSTAR study is described elsewhere [24,25].

Baseline measurement of tuberculous infection in all ZAMSTAR communities was estimated by means of TST surveys among primary school children [22]. These community-wide surveys served three objectives: to characterize ZAMSTAR communities, with regards to TB infection, in relative terms; to inform the randomization of the communities into intervention arms; and to provide data for one of ZAMSTAR's secondary outcomes.

Zambia and South Africa have among the highest tuberculosis incidence [26] and HIV seroprevalence rates [27] in Africa and globally. The estimated HIV prevalence in new tuberculosis cases is 70% [26].

Participants

From April 2007 to July 2008 we recruited newly notified adult tuberculosis cases from the 24 ZAMSTAR communities, subse-

Table 2. TB infection prevalence estimates and Cohen's kappa coefficients per community.

Community code	Geography	Urban/rural	[1]HIV prevalence	Number with both TST & QFT results	QFT-GIT positive (%)	TST positive (10 mm) (%)	Kappa
SA1	Province	Urban	Moderate	109	78	42	0.17
SA 3	Metropole	Urban	High	98	77	77	0.49
SA 6	Province	Rural	Moderate	70	77	27	0.15
Z 4	Lusaka	Urban	High	69	72	41	0.25
SA 5	Metropole	Urban	Moderate	52	71	62	0.28
Z 5	Copperbelt	Urban	High	45	71	13	−0.09
SA 2	Province	Rural	Moderate	87	70	28	0.16
SA 4	Metropole	Urban	High	140	68	61	0.18
Z1	Lusaka	Urban	High	93	67	49	0.36
Z 15	Luapula	Rural	Moderate	24	67	33	0.25
SA 7	Metropole	Urban	High	74	66	76	0.38
Z 11	Luapula	Rural	Moderate	40	65	73	0.02
SA 8	Metropole	Urban	High	124	65	24	−0.01
Z 7	Lusaka	Urban	High	92	61	38	0.19
Z 6	Lusaka	Urban	High	84	61	61	0.48
Z 8	Southern	Urban	High	121	60	42	0.41
Z 3	Copperbelt	Urban	High	48	60	17	0.16
Z 10	Central	Urban	High	68	59	34	0.08
Z 12	Copperbelt	Urban	High	97	59	15	0.19
Z 13	Central	Urban	High	103	50	23	0.16
Z 2	Copperbelt	Urban	High	90	48	28	0.32
Z 16	Southern	Rural	Moderate	18	44	17	0.16
Z 9	Southern	Urban	High	29	38	7	0.22
Z 14	Southern	Rural	Moderate	28	29	11	0.25

Communities arranged from highest to lowest TB infection prevalence estimates as defined by Quantiferon-TB Gold In-Tube (QFT-GIT) test. SA: South African community; Z: Zambian community; TST (Tuberculin skin test). Geography, urban/rural and HIV prevalence as described elsewhere [22,25].

[1]A panel of eight experts critically examined data from ante-natal clinic surveillance, prevention of mother-to-child transmission programmes, voluntary counseling and testing clinics and provincial demographic and health survey data and made an informed decision whether to categorize HIV prevalence as 'high' or 'moderate' for each community [25].

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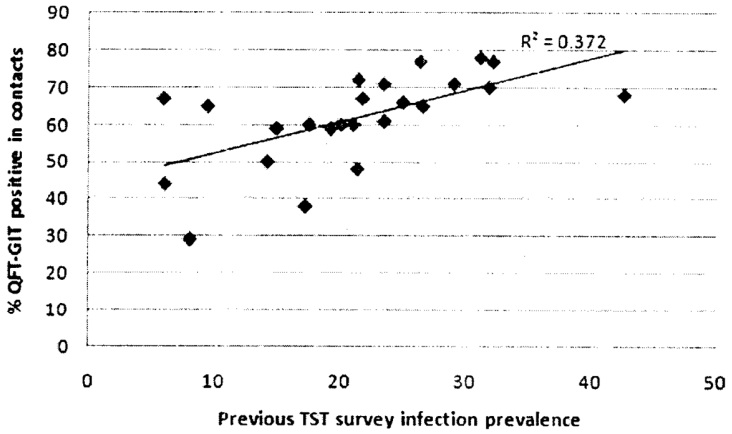

Figure 2. Scatter plot of positive QFT-GIT results in contacts and infection prevalence results from previous TST surveys. Previous TST surveys among children were conducted within the same communities as those of contacts. Infection prevalence in children was defined as TST ≥10 mm.

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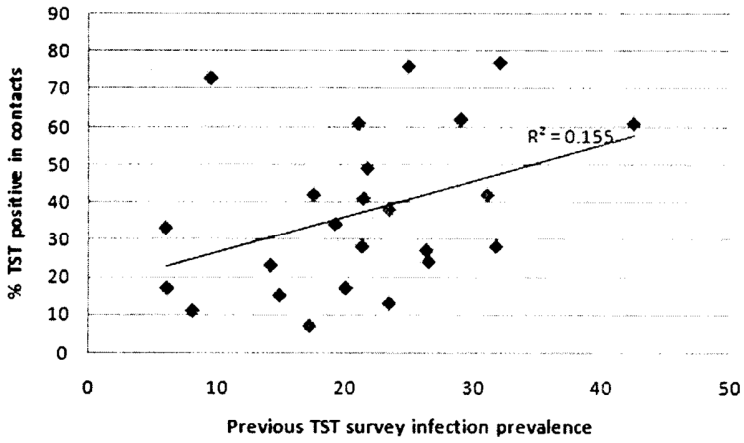

Figure 3. Scatter plot of positive TST results in contacts and infection prevalence results from previous TST surveys. Previous TST surveys among children were conducted within the same communities as those of contacts. Infection prevalence in contacts and children was defined as TST ≥10 mm.
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quently referred to as index cases, to the study. All tuberculosis cases (pulmonary smear positive, smear negative or extrapulmonary) were eligible if recruited within a month after being notified in the tuberculosis register and started on treatment at a government clinic. We obtained written informed consent from those accepting to take part in the study. In addition, we sought permission from these index cases to visit their households where we invited household members to participate. We made at least three attempts to visit household members who were absent during the first visit to the household.

We defined household contacts as individuals at least 15 years old, who generally slept in the home, ate with the index case and who identified a common household head. We asked all household contacts for individual signed consent before participating in the study. This study focuses on this population of household contacts of newly diagnosed tuberculosis cases.

Measures

Consenting household contacts had blood drawn for HIV antibodies and QFT-GIT testing. Tuberculin skin tests were also performed. A standardized questionnaire was administered to all contacts by trained interviewers, who collected information on risk factors associated with tuberculous infection. Sputum microscopy for index cases was performed as part of the clinic routine services and the results were recorded in the TB registers.

HIV testing was done using the Abbot Murex HIV Ag/Ab combination ELISA (Murex Biotech, Dartford, United Kingdom). All individuals were encouraged to attend counseling and HIV testing at the local health centre. In South Africa, HIV positive individuals were advised to go for TB preventive therapy in accordance with National Tuberculosis Control Program guidelines [28] while in Zambia this is not yet government policy. However, in Zambia, preventive therapy was offered to eligible contacts through collaboration with another study operating in the ZAMSTAR sites.

QFT-GIT procedure. QFT-GIT test was performed according to the manufacturer's instructions [29]. For four Zambian and all the South African communities, QFT-GIT processing was done centrally at our research laboratories in Lusaka and Stellenbosch University Medical School respectively. However, for twelve Zambian remote communities, QFT-GIT processing was decentralized. In these communities, blood samples were collected, incubated, separated and stored locally. Tubes were incubated for 16–24 hours at 37°C and plasma was harvested and frozen at −20°C. Frozen samples from these sites were transported monthly to the central laboratory in Lusaka where the ELISA was performed manually in batches.

TST procedure. The skin testing was conducted using 2 TU (Tuberculin Units) of PPD RT23 with Tween, supplied by the Statens Serum Institut (Copenhagen, Denmark). All tests were administered and read by nurses who were trained according to the standard IUATLD protocol [23]. A dose of 0.1 ml was injected intradermally on the left forearm. Skin reactions were read using calipers 72 hours later. A positive TST was defined as an induration of ≥10 mm. Blood for QFT-GIT was drawn before TST was administered usually on the same day.

Statistical analysis

Data were double entered into a "Microsoft SQL Server" database and checked for errors. Analysis was performed using STATA (version 11.0).

The characteristics of the study population were described using frequencies and percentages for categorical variables and the median and interquartile range for quantitative variables. *Prevalence of infection* was defined as the number of QFT-GIT or TST positive results divided by the total number of individuals with interpretable (positive and negative) results. Individuals having missing TST or indeterminate QFT-GIT results were excluded from the analysis. These did not differ significantly from those that had interpretable results (results not shown). Furthermore, household contacts on TB treatment were excluded from analysis.

The level of agreement between test results was assessed for each community using Cohen's kappa coefficient. By convention, kappa values of less than 0.4 generally indicate poor agreement. Correlation between continuous interferon-γ values (IU/ml) and TST induration (mm) was assessed using Spearman's correlation coefficient for each community.

Table 3. Univariable and multivariable odds ratios showing risk factors associated with positive QuantiFERON-TB Gold In Tube assay results.

	QFT positive		
	n (row %)	Crude OR (95% CI)	Adjusted OR (95% CI)[1]
Total	1147/1803 (63.6%)		
Sex			
Male	333(63.5)	1	1
Female	809 (63.7)	1.08 (0.76–1.25)	0.93 (0.72–1.20)
Missing	5		
Age group (years)			
15–24	417 (60.8)	1	1
25–34	276 (61.3)	1.00 (0.74–1.34)	1.00 (0.74–1.34)
35–44	159 (66.5)	1.35 (0.93–1.96)	1.34 (0.92–1.94)
45–54	139 (68.5)	1.49 (1.0–2.22)	1.47 (0.98–2.20)
55–64	87 (75.6)	2.46 (1.43– 4.23)	2.56 (1.47–4.48)
>65	53 (64.6)	1.42 (0.79– 2.53)	1.46 (0.79–2.70)
Missing	16		
Highest level of education			
Not attended school	75 (64.7)	1	1
Primary school	378 (67.1)	1.11 (0.68—1.81)	1.4 0 (0.82–2.38)
Secondary school	551 (60.9)	0.79 (0.49–1.28)	0.99 (0.57–1.71)
College or University	111 (67.3)	1.12 (0.62–2.02)	1.40 (0.73–2.69)
Missing	32		
Smoking habits			
Never smoked	913 (62.7)	1	1
Ex-smoker	42 (53.9)	0.73 (0.43–1.24)	0.60 (0.34–1.07)
Occasional smoker	43 (70.5)	1.40 (0.73–2.66)	1.13 (0.57–2.25)
Daily smoker	137 (72.9)	1.69 (1.14–2.50)	1.14(0.73–1.77)
Missing	12		
Alcohol consumption			
No	844 (62.9)	1	1
Yes	285 (65.4)	1.11 (0.85–1.45)	1.04 (0.78–1.38)
Missing	18		
Household size (adults)			
1–3	170 (61.8)	1	1
4–6	415 (62.7)	1.03 (0.73–1.46)	1.30 (0.90–1.87)
7–9	302 (64.4)	1.14 (0.78– 1.67)	1.46 (0.97–2.19)
≥10	258 (65.3)	1.16 (0.77–1.75)	1.68(1.09–2.61)
Missing	2		
HIV status			
Negative	728 (69.0)	1	1
Positive	335 (54.6)	0.48 (0.37–0.61)	0.51 (0.39–0.67)
Missing	84		
Smear status of index			
Smear negative	373 (60.8)	1	1
Smear positive	426 (67.6)	1.48 (1.09– 2.01)	1.25 (0.90–1.74)
Missing	348		
Sleeping proximity to index			
Different house	72(57.6)	1	1
Same house	355 (63.6)	1.31 (0.78–2.18)	1.07 (0.61–1.86)
Same room	36 (56.3)	0.92 (0.42–2.02)	1.19 (0.52–2.72)

Table 3. Cont.

	QFT positive		
	n (row %)	Crude OR (95% CI)	Adjusted OR (95% CI)[1]
Same bed	142 (62.3)	1.26 (0.71–2.23)	1.16 (0.62–2.14)
Unknown	221 (63.1)	1.26 (0.73–2.16)	1.10(0.61–1.99)
Missing	321		

[1]Odds ratios-adjusted for sex, age and community using random effects logistic regression.
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The distribution of positive reactions to each test in relation to established individual and household level risk factors for LTBI was described. The strength of relationship between risk factors and QFT-GIT/TST positivity was assessed using random effects logistic regression. The random effects approach specified the household of residence as the clustering variable. All models were adjusted for age, sex and community of residence. We present adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for each risk factor.

Finally, we formally assessed four hypotheses related to the expected performance of the tests. We explored whether our data were compatible with expected findings on the basis of a number of prior hypotheses about the characteristics of each test. Ideally, we would have had a gold standard measure of LTBI against which to compare both tests. However, no such test currently exists. Furthermore, the natural history of LTBI remains a source of debate [11] and as in other studies of LTBI we had no direct measure of exposure to *Mtb* either recently or in the past. However, we did have data on three proxies related to prior exposure to *Mtb* (age) or recent exposure to *Mtb* (proximity to and infectivity of the index case).

Thus, we tested: (i) whether HIV infection was associated with a negative TST result, among those with a positive QFT-GIT, since we expect HIV infection to cause more false negative results with TST than QFT-GIT. We restricted the analysis to individuals with a positive QFT-GIT result and used random effects logistic regression models as described previously.

We then explored: (ii) whether age was more strongly associated with a positive TST result than with a positive QFT-GIT result since we expected TST to be more likely to detect evidence of lifetime infection with *Mtb* while QFT-GIT was more likely to detect recent infections; (iii) whether living with a smear positive index case was more strongly associated with a positive QFT-GIT result than a positive TST result, since we expected that QFT-GIT would be more strongly associated with recent infections than TST; and (iv), for the same reason as (iii), whether sleeping in the same room as the index case was more strongly associated with QFT-GIT than TST positivity.

For these final three hypotheses we conducted matched-pairs analysis using conditional logistic regression in an approach similar to that used by Ewer and others (21), where the outcome is the result of the TST or QFT-GIT test. We used Wald tests to assess the strength of evidence for an interaction between the test used and the factor of interest (age, living with a smear positive index case, sleeping in same room as index case). In these models we specified the individual identifier to indicate the paired data, and calculated confidence intervals using robust standard errors taking account of household level clustering.

Results

Figure 1 shows the flow chart of the study participants. A total of 2220 contacts were recruited across the 24 communities. As shown in table 1, the study population was predominantly women (69.9%), most had attended secondary education (52.1%) and had no history of smoking (81.9%) or alcohol consumption (75.2%). 765/2036 (37.6%) of household contacts were HIV positive, and 766/1507 (50.8%) lived with a smear positive TB index case.

A total of 1803 individuals had interpretable results available for both QFT-GIT and TST (figure 1). Of these, 1147 (63.6%) tested positive with QFT-GIT while 725 (40.2%) tested positive with TST (figure 1). In all but two communities infection prevalence as measured by QFT-GIT was higher than that of TST (table 2). Overall, infection prevalence as measured by TST was higher for South African communities (arithmetic mean 50%, range 24–77%) than for the Zambian communities (arithmetic mean 31%, range 7–73%). Results were similar for QFT-GIT (results not shown).

There were 577 (32%) individuals with concordant positive results and 508 (28.2%) had concordant negative results. QFT+/TST− discordant results were more common than QFT−/TST+ results (31.6% vs 8.2%). There was a low level of agreement overall in the 24 communities (% agreement = 60.2%; kappa = 0.24 (arithmetic mean 0.23, range −0.09–0.49)), and in each of the communities (range of kappas: 0–0.49) (table 2). Using different cut-off values for the TST did not improve overall test agreement in the 24 communities (kappa: 0.26, 0.24 and 0.14 for TST cutoffs of 5, 10 and 15 mm respectively). When results were stratified by HIV status, agreement appeared slightly better in HIV positive (% agreement = 65.1%; kappa 0.322) compared to HIV negative (% agreement = 57.4%; kappa 0.19) contacts. There was a positive correlation (correlation coefficient square, $r^2 = 0.372$) between positive QFT-GIT results in contacts and infection prevalence results in children (figures 2), however, this was weaker for the TST (correlation coefficient square, $r^2 = 0.155$) (figure 3).

Tables 3 and 4 show risk factors associated with positive QFT-GIT and TST results respectively. Both QFT-GIT and TST results were associated with increasing age (adjusted OR [aOR] for each 10 year increase for QFT-GIT 1.15; 95% CI: 1.06–1.25; $p<0.001$ for linear trend, and for TST aOR: 1.10; 95% CI 1.01–1.20; $p = 0.025$ for linear trend). HIV positivity was less common among those with positive results on QFT-GIT (aOR: 0.51; 95% CI: 0.39–0.67; $p<0.001$) and TST (aOR: 0.61; 95% CI: 0.46–0.82; $p = 0.001$).

There was some evidence of an association between smear positivity of the index and QFT-GIT (aOR: 1.25; 95% CI: 0.90–1.74) and TST (aOR: 1.39; 95% CI: 0.98–1.98) results.

Both QFT-GIT (aOR for household size: 1.04; 95% CI: 1.00–1.09, $p = 0.65$ for linear trend) and TST (aOR: 0.97; 95% CI: 0.93–1.01, $p = 0.005$ for linear trend) results were not associated with increasing household size.

Finally, we tested the four specific hypotheses described in the methods. As shown in table 5, we found little evidence to support the hypotheses that positivity in QFT-GIT is more related to recent infection and less affected by HIV than the TST.

Table 4. Univariable and multivariable odds ratios showing risk factors associated with positive tuberculin skin test results.

	TST ≥10 mm		
	n (row %)	Crude OR (95% CI)	[1]Adjusted OR (95% CI)
Total	725/1803 (40.2%)		
Sex			
Male	203 (38.7)	1	1
Female	520 (40.9)	1.22 (0.92–1.62)	1.18 (0.90 – 1.56)
Missing	2		
Age group (years)			
15–24	263 (38.4)	1	1
25–34	177 (39.3)	1.10 (0.79–1.54)	1.05 (0.77–1.45)
35–44	102 (42.7)	1.41 (0.93–2.13)	1.49 (1.00–2.21)
45–54	88 (43.3)	1.34(0.87–2.07)	1.34 (0.89–2.04)
55–64	58 (50.4)	2.01(1.17–3.47)	2.03 (1.19–3.45)
>65	28 (34.1)	1.05(0.55–2.00)	1.12 (0.58–2.17)
Missing	9		
Highest level of education			
Not attended school	39 (33.6)	1	1
Primary school	236 (41.9)	1.47 (0.85–2.55)	1.53 (0.88–2.66)
Secondary school	365 (40.3)	1.22 (0.72–2.10)	1.29 (0.72–2.31)
College or University	74 (44.8)	1.49 (0.77–2.90)	1.13 (0.57–2.25)
Missing	11		
Smoking habits			
Never smoked	573 (39.3)	1	1
Ex-smoker	32 (41.0)	1.19 (0.64–2.20)	1.09 (0.59–2.02)
Occasional smoker	31 (50.8)	1.51 (0.75–3.02)	1.26 (0.63—2.54)
Daily smoker	85 (45.2)	1.33 (0.87–2.02)	1.10 (0.70–1.73)
Missing	4		
Alcohol consumption			
No	544 (40.6)	1	1
Yes	174 (39.9)	0.93 (0.68–1.27)	0.94 (0.69–1.28)
Missing	7		
Household size (adults)			
1–3	127 (46.2)	1	1
4–6	261 (39.4)	0.68 (0.44–1.03)	0.77 (0.52–1.13)
7–9	192 (40.9)	0.72 (0.46–1.14)	0.91 (0.59–1.40)
≥10	144 (36.5)	0.57 (0.35–0.94)	0.71 (0.44–1.13)
Missing	1		
HIV status			
Negative	465 (44.1)	1	1
Positive	207 (33.8)	0.57 (0.43–0.76)	0.61 (0.46 – 0.82)
Missing	53		
Smear status of index			
Smear negative	230 (37.5)	1	1
Smear positive	290 (46.0)	1.65 (1.15–2.36)	1.39 (0.98 – 1.98)
Missing	205		
Sleeping proximity to index			
Different house	43 (34.4)	1	1
Same house	202 (36.2)	1.08 (0.62–1.89)	0.76 (0.44–1.30)
Same room	20 (31.2)	0.80 (0.34–1.92)	0.94 (0.41–2.15)
Same bed	91 (39.9)	1.37(0.74–2.55)	0.80 (0.44–1.46)

Table 4. Cont.

	TST ≥10 mm		
	n (row %)	Crude OR (95% CI)	[1]Adjusted OR (95% CI)
Unknown	128 (36.6)	1.11 (0.61–2.00)	0.74 (0.41–1.33)
Missing	241		

[1]Odds ratios-adjusted for sex, age and community using random effects logistic regression.

doi:10.1371/journal.pone.0018206.t004

Discussion

We conducted a large scale evaluation of the prevalence of LTBI as detected by TST and QFT-GIT among household contacts of tuberculosis patients in 24 high HIV and TB prevalence communities in Zambia and South Africa. Our findings suggest a high prevalence of LTBI among this population. QFT-GIT estimates were higher than those of TST in all but two communities. LTBI prevalence was higher in South African communities compared to the Zambian ones, as in previous findings [22].

LTBI was more common among older individuals and those who were HIV negative, similar to previous studies in this setting [9,10,30]. HIV positivity was less common among those with positive results on QFT-GIT and TST. We found little evidence to support the hypothesis that HIV infection was associated with TST negativity among QFT-GIT positive individuals as would have been expected if HIV causes more false negatives with TST than QFT-GIT. Both TST and QFT-GIT are prone to false negatives results among different population groups [9,30]. In a study done in Zambia, low CD4+ counts in HIV positive TB patients were associated with increases in both indeterminate and false-negative QFT-GIT results [9]. Current evidence suggests that IGRAs perform similarly to the TST at identifying HIV-infected individuals with LTBI [16].

For both QFT-GIT and TST, prevalence of infection was higher in contacts exposed to smear positive index cases compared to smear negative ones, consistent with findings of other studies [31]. Sleeping proximity of the contact to the index case was not associated with either QFT-GIT or TST results. In contrast, a study done in Cape town found an association between *Mtb* contact scores and increasing exposure [19], simliar to findings in the Gambia [21]. Both of these studies had smaller sample sizes compared to our study and were done among HIV negative [19] or few HIV postive contacts [21]. There is growing evidence suggesting a stronger and better defined association between surrogate markers for TB exposure and QFT-GIT results in low TB incidence settings compared to high-TB incidence settings [14,32,33,34] although this is still inconclusive.

Our results suggest that tuberculous infection in adults may often be unrelated to household transmission. It is well recognized that transmission of tuberculosis in high incidence settings occurs not only within households but in the community as well [35,36] among various social locations [37]. A study in Zimbabwe found that the proportion of ELISpot positive contacts was not different from community controls [31]. In another study done in two communities in Zambia, almost 50% of community controls were QFT-GIT positive [38]. In our study, positive QFT-GIT results in contacts correlated well with infection prevalence results from previous TST surveys, providing further evidence that community transmission seems to play a bigger role in positivity than household exposure. However, in a large study in Colombia, IFN-γ responses to CFP-10 were consistently higher in household contacts of all ages compared to subjects in the source population [34]. Nevertheless, a seven day whole blood culture in-house assay was used, which primarily detects central memory responses. It has been argued that, in settings of high endemicity where a mixture of recent and old infections are commonly found, long term assays are more sensitive than those with shorter culture times [34].

We found little evidence in our matched pair analysis to support the idea that age was more strongly associated with a positive TST result than with a positive QFT-GIT result since we anticipated that TST was more likely to detect evidence of lifetime infection with *Mtb* while QFT-GIT was more likely to detect recent infections. Our results using conditional logistic regression showed that age was associated with positive QFT-GIT and TST results and there was a trend to increased responses with increasing age for both tests suggesting cumulative exposure to *Mtb*. In the study done in Colombia [34], exploration of IFN-γ variations by age revealed a trend to increased responses up to adulthood with CFP, but not with CFP-10, similar to observations in Uganda [39]. However, children were included in both of these studies.

We show a low level of agreement between the tests in all communities, consistent with findings of studies done in high-TB burden settings [19,31,40]. As IGRAs are designed to be more specific than TST, perfect agreement is not expected [41]. However, better agreement has been shown when the comparison is done within specific risk groups like HIV positives [42]. Although kappa statistics have been widely used as a measure of agreement between IGRAs and TST, alternative statistical approaches have recently been proposed such as latent class analysis [43] but have yet to gain wider acceptance. Similar to other studies reported from poor-resource settings [44], there were particularly high number of QFT-GIT+/TST- discordant results, in contrast to studies done in settings with low TB incidence [45,46] where TST+/QFT-GIT- discordance is more common.

Our study had both strengths and limitations. Ours is among the first studies to conduct both TST and QFT-GIT tests using a large sample size which illustrates the realistic implementation of QFT-GIT in a setting with a high burden of TB and HIV. However, as for all studies of this nature we had no gold standard measure of LTBI against which to compare our tests. As such, we were unable to comment directly on the accuracy of either test, but rather to compare the findings of each test in relation to prior beliefs about their properties. It is plausible that our failure to prove our hypotheses may have been due to test limitations typical in such high-TB burden settings. In addition, individuals in these settings may have mixed infections due to multiple *Mtb* exposure.

Our results may have been severely compromised by missing data on some risk factors. Despite efforts to standardize TST training and reading across the two countries, use of different teams may have contributed to inter-reader variability. Although most contacts reported that they had never smoked or taken alcohol, we believe this was due to reporting bias. We had no data

Table 5. Hypotheses of expected performance of QFT-GIT and TST in our setting and the results obtained.

Prevailing Understanding	Hypothesis	Result
TST is more likely to give false negative results in HIV positives than QFT-GIT.	1. HIV is a risk factor for TST negativity conditional on a QFT-GIT positive result.	Adjusted odds ratio for HIV on TST positivity among QFT-GIT positives = 0.94 (95% CI:0.62-1.40) Wald-test p = 0.75
QFT-GIT positivity is related to recent acquisition of *Mtb* infection whilst TST detects old infections.	2. Age trend is stronger for TST than QFT-GIT because age is as proxy for likelihood of lifetime exposure to *Mtb*.	Wald-test for age'*'diagnostic test' interaction parameter in conditional logistic regression; p = 0.94
	3. Stronger association between residence with a smear positive TB case and QFT-GIT positivity than for TST positivity, because smear status is a marker of infectivity and thus of likelihood of recent exposure to *Mtb*.	Wald-test for 'smear status of index case'*'diagnostic test' interaction parameter in conditional logistic regression; p = 0.45
	4. Stronger association between sleeping in same room as index case and QFT-GIT positivity than for TST positivity, because sleeping in the same room is a marker of likelihood of recent exposure to MTB.	Wald-test for 'sleeping in same room as index'*'diagnostic test' interaction parameter in conditional logistic regression; p = 0.76

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on likely exposure to NTM which may provide an alternative reason for false positive results, especially with TST.

Probably the most important characteristic of tests of LTBI is the extent to which they predict subsequent clinical tuberculosis. The data we present here are cross-sectional in nature; however, they come from a larger longitudinal study whose participants have been followed up for later development of active TB.

Conclusion

QFT-GIT may not be more sensitive than the TST to detect risk factors associated with tuberculous infection. Given the lack of strong associations with either TST or QFT-GIT with risk factors generally accepted to be related to household infectivity, these results suggest that tuberculous infection in adults in these communities may often be unrelated to household transmission. We found little evidence to support the hypotheses that positivity in QFT-GIT is more related to recent infection and less affected by HIV than the TST.

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Author Contributions

Conceived and designed the experiments: HA NB CS PG-F. Performed the experiments: KS AM AS K-AL. Analyzed the data: KS JH KF PG-F. Contributed reagents/materials/analysis tools: AS K-AL CS. Wrote the paper: KS JH KF HA PG-F NB BH. Critical review of the paper: PG-F HA JH KF NB.

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Intra-assay reliability and robustness of QuantiFERON®-TB Gold In-Tube test in Zambia

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SUMMARY

BACKGROUND: Interferon-gamma (IFN-γ) release assays (IGRAs), such as the QuantiFERON®-TB Gold In-Tube test (QFT-GIT), are becoming a preferred method for diagnosis of tuberculosis (TB) infection in many industrialised countries. However, data on the effectiveness of IGRAs in high TB-HIV (human immunodeficiency virus) endemic and resource-limited settings, such as Zambia, are limited.

OBJECTIVE: To determine the intra-assay reliability and robustness of QFT-GIT in a field setting in Zambia.

DESIGN: During July–October 2007, 109 adult smear-positive TB patients were recruited to determine QFT-GIT reliability and the effect of a 24-h delay in incubation. Two simulated laboratory experiments were also performed using 9–14 volunteers, to explore the effect of power outages during incubation and storage temperature of collection tubes on IFN-γ responses.

RESULTS: QFT-GIT intra-assay concordance was 91.7% ($\kappa = 0.8$). Discordance was observed for nine patients, of whom six were HIV-positive. There was evidence of an association between HIV status and discordant results (OR 1.98, 95%CI 1.06–3.67, $P = 0.03$). A 24-h delay in incubation changed results for 25 of the 109 (22.9%) patients. Power outages that altered incubation time reduced IFN-γ responses.

CONCLUSION: Although QFT-GIT seems reliable in this setting, we have identified operational factors that affect its robustness. These factors may influence the effectiveness of this test in similar resource-limited settings.

KEY WORDS: QuantiFERON-TB Gold In-Tube; reliability; HIV; tuberculosis; Zambia

INTERFERON-GAMMA release assays (IGRAs), such as QuantiFERON®-TB Gold In-Tube (QFT-GIT), are replacing the tuberculin skin test (TST) as the preferred method for the diagnosis of latent TB infection (LTBI) in many industrialised countries.[1–3] In these settings, IGRAs are believed to have many advantages compared to the TST, especially an increase in specificity.[4,5] However, in high tuberculosis (TB) endemic and resource-limited settings such as Zambia, data on the effectiveness of IGRAs are limited and their future role in LTBI diagnosis is still uncertain.

In resource-limited settings, many factors may influence the results of IGRAs. In a study conducted in Zambia,[6] low CD4+ counts in human immunodeficiency virus (HIV) positive TB patients were associated with increases in both indeterminate and false-negative results. QFT-GIT results may also be influenced by factors that modulate the immune response,[7] such as poverty-related malnutrition, tropical infections and widespread exposure to non-tuberculous mycobacteria. Furthermore, stringent control of multiple variables, from blood collection, processing and storage in the field to performance of the test in the laboratory, may be difficult to control in resource-limited settings. Other potential drawbacks include the cost of the test, the need for highly skilled staff and a laboratory equipped to run enzyme-linked immunosorbent assays (ELISAs). A recent study in Cape Town, South Africa, evaluating the short-term reproducibility of QFT-GIT, emphasised the importance of rigorous laboratory techniques.[8]

We report on four studies that determine QFT-GIT reliability and robustness in a field setting in Zambia. The first study determines the intra-assay reliability of QFT-GIT. The remaining three studies examine the effect on the interferon-gamma (IFN-γ) response of three factors affecting QFT-GIT robustness, namely a 24-h delay in the incubation of blood samples, power outages during incubation of blood samples and increased storage temperature of unused blood collection tubes. This is the first time that all of these factors have been examined in our resource-limited setting.

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METHODS

Study setting and background

The studies described in this paper were conducted in Zambia, which has one of the highest TB incidence rates in Africa (506 per 100 000 population),[9] as well as one of the highest HIV prevalence rates, at 14.3% among 15–49-year-olds.[10] The estimated prevalence of HIV in new TB cases is 70%.[9]

These studies stem from challenges reported from an ongoing QFT-GIT cohort study nested within a large community randomised trial, Zambia/South Africa TB and AIDS Reduction (ZAMSTAR),[11] being conducted across 16 sites in Zambia and 8 sites in Western Cape, South Africa.[11] These difficulties stem in part from the decentralised nature of sample collection, incubation, separation and storage in Zambia. Blood samples of the cohort are collected and processed at remote sites. Frozen samples from these sites are transported every month to a central laboratory, where ELISA is performed manually in batches. The present study therefore addresses challenges experienced only in Zambia.

The ethics committees of the London School of Hygiene & Tropical Medicine and the University of Zambia approved the protocol for QFT-GIT reliability and delayed incubation studies. The other studies were laboratory based.

Intra-assay reliability of QFT-GIT and delayed incubation studies

A total of 109 smear-positive TB patients aged >18 years and seen within 1 month of commencing treatment were recruited to determine QFT-GIT intra-assay reliability. These patients attended four government clinics in Lusaka from July to October 2007. Blood was collected in three sets of collection tubes, two of which were handled as duplicates (standard incubation 1 and 2). All duplicate samples were tested by the same operator, on the same ELISA plate and in the same laboratory.

Intra-assay reliability was defined as the variation in measurements taken by a single operator when samples were tested in duplicate on the same assay plate and under the same conditions.

For the third set of tubes, the incubation period was delayed by 24 h (24-h delayed incubation). This was done by leaving the blood collection tubes on the bench at room temperature (20–25°C) before incubation. QFT-GIT testing was then performed according to the manufacturer's instructions.[12]

Effect of power outage on IFN-γ response

To explore the effect of power outages during incubation of blood samples on IFN-γ response, simulated laboratory experiments were performed. The patterns of power outages during incubation reported from the QFT-GIT cohort study sites were summarised into four categories: no power outage (standard incubation), early power outage, late power outage and early power outage with extension. Four sets of blood collection tubes were obtained from each of nine volunteers and incubated according to the four categories. The first set was incubated for 24 h with no power outage (standard incubation). The second set was incubated for 2 h; power was turned off for 5 h, then switched back on for 17 h (early power outage). The third set was incubated for 16 h, power was switched off for 5 h, then turned back on for an additional 3 h (late power outage). The fourth set was incubated for 2 h, power was switched off for 5 h, turned back on for 17 h, and incubation extended by 5 h to compensate for the time lost during the power outage (early power outage with extension).

The total incubation time was 24 h each for standard incubation and early power outage with extension, and 19 h each (excluding the 5 h of power outage) for early and late power outage experiments.

Effect of storage temperature on IFN-γ response

A simulated laboratory experiment was performed to determine the effect of increased storage temperature of unused blood collection tubes on IFN-γ response. Blood was collected from each of the 14 volunteers in two sets of blood collection tubes. One set had been stored at 37°C for 3 months before blood was drawn and the other had been stored in an air-conditioned storage area where the temperature was maintained at <25°C. The manufacturer's recommended storage temperature is 4–25°C.

QFT-GIT procedure

The QFT-GIT test was performed according to the following manufacturer's instructions,[12] unless otherwise stated: 1 ml of blood was drawn into each of the three blood collection tubes coated with saline, mitogen and peptides of early secreted antigenic target-6 (ESAT-6), culture filtrate protein 10 (CFP-10) and TB7.7. Tubes were incubated for 16–24 h at 37°C and plasma was harvested and frozen at −20°C. ELISA was performed using standard kits. The raw data were entered into the QFT-GIT analysis software. This reported results as positive (if nil ≤ 8.0 international units [IU]/ml and reaction to TB antigens minus nil control ≥ 0.35 IU/ml and >25% of nil value), negative (nil ≤ 8.0 IU/ml and mitogen minus nil ≥ 0.5 IU/ml and reaction to TB antigens minus nil control < 0.35 IU/ml or ≥ 0.35 IU/ml but <25% of nil value) or indeterminate (if nil > 8.0 IU/ml or mitogen minus nil < 0.5 IU/ml).[8]

Data analysis

Data were entered into an Access database (MicroSoft, Redmond, WA, USA) and checked for errors. Analysis was performed using STATA version 9.0 (Stata Corp, College Station, TX, USA). Dichotomous data

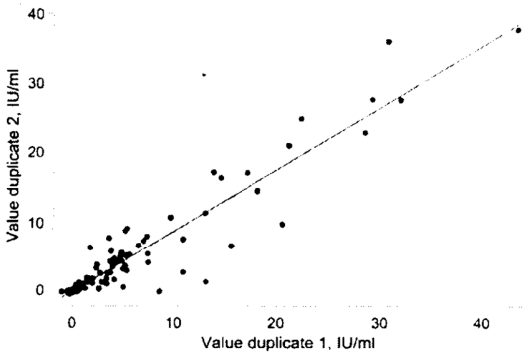

Figure 1 QFT-GIT intra-assay reliability. Interferon-gamma responses of duplicate 1 vs. duplicate 2 in the intra-assay reliability study. IU = international units; QFT-GIT = QuantiFERON®-TB Gold In-Tube.

were compared using the kappa (κ) statistic, while for continuous measures the intraclass correlation coefficient (ICC) was used. Strength of agreement was classified as poor for $\kappa < 0.4$, moderate for $\kappa = 0.4$–0.75 and excellent for $\kappa > 0.75$. Statistical analyses were significant for $P < 0.05$, and 95% confidence intervals (CIs) were used. Differences between medians were compared using Wilcoxon signed-rank test.

RESULTS

Subject characteristics

Only the characteristics of the subjects used in the reliability and delayed incubation studies are described: similar data were not collected from other studies. The median age of the 109 TB patients was 31 years (interquartile range [IQR] 25–35); 72 (66.1%) were male; 93 patients had HIV status recorded, of whom 58 (53.2%) were HIV-positive, 35 (32.1%) were negative and 16 (14.7%) had unknown results. The median CD4 counts of the HIV-positive and -negative patients were respectively 212 cells/μl (IQR 115–312, mean 265) and 542 cells/μl (IQR 450–700, mean 566).

Table 1 QFT-GIT results of duplicate samples (standard incubation 1 and 2)

	Standard incubation 2			
	Positive	Negative	Indeterminate	Total
Standard incubation 1				
Positive	77	0	6	83
Negative	0	10	1	11
Indeterminate	0	2	13	15
Total	77	12	20	109

QFT-GIT = QuantiFERON®-TB Gold In-Tube.

Intra-assay reliability of QFT-GIT

Agreement between duplicate samples was high using dichotomous (91.7%, $\kappa = 0.8$) and continuous (ICC 0.90, 95%CI 0.82–0.97) measures (Figure 1). Discordance was observed for nine patients, all of which included indeterminate results (Table 1). There was no discordance between positive and negative QFT-GIT results. Of the nine patients with discordant results, six were HIV-positive and three had unknown HIV status. Table 2 shows the IFN-γ responses of the nine patients with discordant results. There was evidence of an association between HIV status and discordant results (odds ratio [OR] 1.98, 95%CI 1.06–3.67, $P = 0.03$). CD4 counts of <200 cells/μl were associated with increases in indeterminate results for standard incubation 1 (Wilcoxon's, $P < 0.000$) and 2 (Wilcoxon's, $P < 0.000$).

The median IFN-γ responses of standard incubation 1 and 2 were respectively 2.06 IU/ml (IQR 0.39–5.31, mean 5.16) and 1.36 IU/ml (IQR 0.15–5.24, mean 4.52; Wilcoxon's, $P = 0.03$). Figure 2 shows box plots of standard incubation 1 and 2.

Effect of delayed incubation on IFN-γ response

When standard incubation 1 was compared to delayed incubation, agreement was moderate using dichotomous (77.1%, $\kappa = 0.54$) and continuous (ICC 0.51, 95%CI 0.17–0.85) measures. The median IFN-γ responses decreased from 2.06 IU/ml during standard incubation 1 to 0.52 IU/ml (IQR 0–1.61, mean 1.82)

Table 2 QFT-GIT results of nine discordant patients

Patient no.	Standard incubation 1 TB Ag-nil IU/ml	Standard incubation 2 TB Ag-nil IU/ml	Standard incubation 1/ standard incubation 2	HIV status	CD4 count cells/μl
1	0.5	0.00*	Positive/indeterminate	Unknown	120
2	0.4	0.14	Positive/indeterminate	Unknown	572
3	0.72	0.31	Positive/indeterminate	Unknown	1056
4	0*	0.10	Negative/indeterminate	Positive	75
5	0.14	0.04	Indeterminate/negative	Positive	26
6	8.6	0.00*	Positive/indeterminate	Positive	246
7	0.35	0.15	Positive/indeterminate	Positive	615
8	0.57	0.00*	Positive/indeterminate	Positive	342
9	0.07	0.02	Indeterminate/negative	Positive	238

* Negative values are reported as zero.
QFT-GIT = QuantiFERON®-TB Gold In-Tube; TB Ag-nil = TB antigen minus nil; HIV = human immunodeficiency virus; TB = tuberculosis.

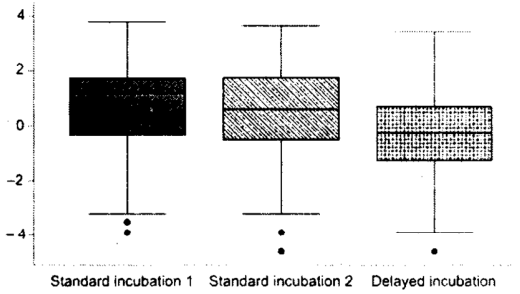

Figure 2 IFN-γ responses for standard and delayed incubations. Box and whisker plots (log transformations) showing range, interquartile range and median with dots representing outliers of IFN-γ responses (TB Ag-nil) of standard and delayed incubation. Median IFN-γ responses of delayed incubation are lower than those of either standard incubation 1 or standard incubation 2. IFN-γ = interferon-gamma; TB Ag-nil = TB antigen minus nil; TB = tuberculosis.

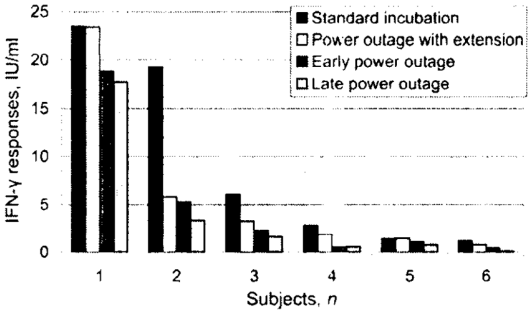

Figure 3 Effect of power outages during incubation on IFN-γ responses. Late power outages during incubation reduced IFN-γ responses to the greatest extent. IFN-γ = interferon-gamma.

when it was delayed (Wilcoxon's, $P < 0.0001$). Similar results were obtained when standard incubation 2 was compared to delayed incubation ($P < 0.0001$, $\kappa = 0.57$, 77.98%).

Delaying incubation of blood collection tubes by 24 h changed the results for 25 of the 109 (22.9%) patients. Of these, 16 (64%) changed from positive to indeterminate; 4 (16%) positive to negative; 4 (16%) negative to indeterminate and 1 (4%) indeterminate to positive (Fisher's, $P < 0.0001$). The median IFN-γ response of patients with changed results was 0.14 IU/ml (IQR 0.01–0.25, mean 0.35) compared to 0.9 IU/ml (IQR 0.33–2.4, mean 2.26) for those with unchanged results (Wilcoxon's, $P < 0.000$). These two groups of patients were similar in age (Fisher's, $P = 0.47$), sex (Fisher's, $P = 0.63$) and HIV status (Fisher's, $P = 1.0$). There was no evidence that HIV (OR = 1.04, 95%CI 0.65–1.65, $P = 0.87$) or CD4 count (OR = 1.00, 95%CI 0.99–1.00, $P = 0.99$) was associated with a changed result following delayed incubation.

Effect of power outage on IFN-γ responses

Late power outages during incubation reduced IFN-γ responses to the largest extent (Figure 3). Median IFN-γ responses obtained during the power outage experiments were: standard incubation 1.4 IU/ml (IQR 0.01–12.69, mean 6.02); early power outage with extension 1.44 IU/ml (IQR 0.02–4.58, mean 4.08); early power outage 0.57 IU/ml (IQR 0.005–3.8, mean 3.17) and late power outage 0.6 IU/ml (IQR 0–2.52, mean 2.69).

Six of the nine (66.7%) volunteers had positive results and three (33.3%) were negative. Of the six with positive results, five (Volunteers 1–5 in Figure 3) remained positive in all the power outage experiments, while one (Volunteer 6) became negative (0.12 IU/ml) during the late power outage experiment.

Effect of storage temperature on IFN-γ response

There was no difference in IFN-γ responses between blood collected in tubes stored at <25°C and those stored at 37°C for 3 months.

DISCUSSION

Our result of 91.7% intra-assay concordance of QFT-GIT has not been previously reported in this setting. There are limited data on reliability and reproducibility of IGRAs,[5,13–15] although a few studies have focused on within-person variability[8,14,16] which was not the focus of our study. A Cape Town study showed that QFT-GIT was consistent and seemed reliable, although the sample size was small.[8] Another study found that high test-retest concordance occurred when initial QFT-GIT values were <0.25 IU/ml or greater than the positive-test threshold of 0.35 IU/ml,[14] while discordance was observed when values were close to the cut-off point. In studies conducted by the manufacturer, the test-related coefficient of variation for QFT was 8.7% using replicate serum samples from well-characterised patients.[17,18]

The differences between duplicate samples could be due to differences in blood volumes in the antigen/mitogen-coated tubes, in the handling of the tubes (including shaking) as well as errors arising from use of manual rather than automated 'robotic' systems (such as pipetting).[8,14,19] In one study in San Francisco, duplicate testing of positive QFT samples resulted in 20% of samples with two negative tests, 12% with one positive and one negative test, and 68% with two positive tests.[20] Another study illustrated that within-person error was affected by Harvesters and ELISA operators, but this was significantly reduced after corrective measures were introduced.[19] Our study goes further to show that, in our setting, HIV infection may have an effect on reliability, although this needs to be confirmed in larger studies.

Our study has shown that a 24-h delay in incubation reduced IFN-γ responses by 3.34 IU/ml (mean difference with standard incubation 1) and changed

the results of 22.9% patients from either positive or negative to indeterminate. The decrease in IFN-γ responses due to delayed incubation has implications for resource-limited settings, where sample incubation may be delayed due to various factors. In a study investigating the effect of sample handling on cytokine response to *M. tuberculosis*,[21] delays in sample processing of as little as 2 h showed a decline in IFN-γ sensitivity and an increase in variability. Delayed incubation reduces the IFN-γ response due to decreased metabolism or cell death. In the reliability study, indeterminate results were due to failure to respond adequately to the positive control, reflecting underlying cellular immune suppression.[22] Many studies have shown that low CD4+ counts are associated with increases in indeterminate results.[6,23,24]

Power outages are a potential problem in many developing countries, and this may affect processing and storage of samples. The decreased IFN-γ response due to power outages has led to a minor modification of the QFT-GIT protocol in our sites. The incubation time is extended following power outages, with the aim of achieving an overall incubation period of 16–24 h, although this benefit may be limited. Although it may be possible to counteract the effect of power outages by using generators or solar energy, the costs need to be taken into consideration. As no such measures are currently in place at these sites, management forms are used to capture data on samples that experience power outages.

It is encouraging that our study demonstrated that storage of unused blood collection tubes at 37°C for 3 months had no effect on IFN-γ responses. In this setting, storage space with the recommended temperature of 4–25°C (air-conditioned room or refrigerator) is limited.

The results in our study re-emphasise the need for stringent sample collection and processing techniques for QFT-GIT. Although this test has been shown to be robust and highly reproducible,[8,14] these characteristics were determined using rigorous laboratory techniques that may be different under routine field conditions. This poses a challenge for resource-limited countries where laboratory quality assurance may not be systematically monitored.[19]

CONCLUSION

Although QFT-GIT seems reliable in our setting, we have identified some operational factors that affect its robustness. These factors may need to be taken into account in determining whether the test is appropriate for resource-limited settings. While most of these factors can be controlled, the effect of HIV infection may be a major limiting factor for the role of the test.

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RÉSUMÉ

CONTEXTE : Dans beaucoup de pays développés, les tests de libération de l'interféron-gamma (IFN-γ, IGRA) comme le QuantiFERON®-TB Gold In-Tube (QFT-GIT), deviennent une méthode préférée pour le diagnostic de l'infection tuberculeuse. Toutefois, les données sur l'efficience des IGRA dans les contextes à haute endémie de la tuberculose et du virus de l'immunodéficience humaine (TB-VIH) et à faibles ressources, comme la Zambie, sont limitées.

OBJECTIF : Déterminer la fiabilité et la robustesse du QFT-GIT au sein d'un essai dans un contexte de terrain en Zambie.

SCHÉMA : Entre juillet et octobre 2007, 109 adultes atteints d'une TB à bacilloscopie positive des crachats ont été recrutés pour déterminer la fiabilité du QFT-GIT ainsi que l'effet d'un délai de 24 h pour l'incubation. En outre, deux simulations expérimentales de laboratoire ont été réalisées en utilisant 9 à 14 volontaires pour explorer l'effet sur les réponses IFN-γ d'une coupure d'électricité au cours de l'incubation ainsi que celui de la température de conservation des tubes de collection.

RÉSULTATS : La concordance à l'intérieur de l'essai pour le QFT-GIT est de 91,7% ($\kappa = 0,8$). On a observé des discordances chez neuf patients, parmi lesquels six étaient séropositifs pour le VIH. Il y a des preuves d'association entre le statut VIH et la discordance des résultats (OR = 1,98 ; IC95% 1,06–3,67 ; $P = 0,03$). Un délai de 24 h avant l'incubation a modifié les résultats chez 25 des 109 patients (22,9%). Les ruptures de courant qui modifient la durée d'incubation ont diminué les réponses de l'IFN-γ.

CONCLUSION : Bien que le QFT-GIT semble fiable dans ce contexte, nous avons identifié des facteurs opérationnels qui affectent sa robustesse. Ces facteurs peuvent influencer l'efficience du test dans des contextes similaires à ressources limitées.

RESUMEN

MARCO DE REFERENCIA: Las pruebas de liberación de interferón gama (IFN-γ), como la prueba del QuantiFERON-TB Gold® En Tubo (QFT-GIT), se están convirtiendo en el método diagnóstico preferido de la infección tuberculosa en muchos países desarrollados. Sin embargo, no se cuenta con suficientes datos sobre la eficacia de este tipo de pruebas en entornos donde es alta la endemia de tuberculosis (TB) e infección por el virus de la inmunodeficiencia humana (VIH), como Zambia.

OBJETIVO: Determinar la fiabilidad y la consistencia de la prueba QFT-GIT en el terreno en Zambia.

MÉTODO: Entre julio y octubre del 2007, se incorporaron al estudio 109 pacientes adultos con TB y baciloscopia positiva con el fin de determinar la fiabilidad de la prueba QFT-GIT y el efecto de un retraso de la incubación de 24 h. Además, se simularon dos condiciones experimentales con las muestras de 9 y 14 voluntarios, a fin de examinar el efecto sobre la respuesta de liberación de IFN-γ de las interrupciones del suministro eléctrico durante la incubación y de la temperatura de almacenamiento de los tubos de recogida de muestras.

RESULTADOS: La concordancia intranalítica con el sistema QFT-GIT fue de 91,7% (índice $\kappa = 0,8$). Se observaron discordancias en nueve pacientes, de los cuales seis presentaban examen serológico positivo al VIH. Se encontró una asociación entre el estado de la serología del VIH y la discordancia de los resultados (OR = 1,98; IC95% 1,06–3,67; $P = 0,03$). Un retraso de 24 h en el comienzo de la incubación modificó los resultados en 25 de los 109 pacientes (22,9%). Las interrupciones de suministro del fluido eléctrico, que modificaron el tiempo de incubación disminuyeron las respuestas de liberación de IFN-γ.

CONCLUSIÓN: Si bien la prueba QFT-GIT parece fiable en este entorno, se detectaron factores operativos que afectan su consistencia. Estos factores pueden influir sobre la eficacia real de la prueba en medios similares, con escasos recursos.

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Annual Risk of Tuberculous Infection Using Different Methods in Communities with a High Prevalence of TB and HIV in Zambia and South Africa

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Abstract

Background: The annual risk of tuberculous infection (ARTI) is a key epidemiological indicator of the extent of transmission in a community. Several methods have been suggested to estimate the prevalence of tuberculous infection using tuberculin skin test data. This paper explores the implications of using different methods to estimate prevalence of infection and ARTI. The effect of BCG vaccination on these estimates is also investigated.

Methodology/Principal Findings: Tuberculin surveys among school children in 16 communities in Zambia and 8 in South Africa (SA) were performed in 2005, as part of baseline data collection and for randomisation purposes of the ZAMSTAR study. Infection prevalence and ARTI estimates were calculated using five methods: different cut-offs with or without adjustments for sensitivity, the mirror method, and mixture analysis. A total of 49,835 children were registered for the surveys, of which 25,048 (50%) had skin tests done and 22,563 (90%) of those tested were read. Infection prevalence was higher in the combined SA than Zambian communities. The mirror method resulted in the least difference of 7.8%, whereas that estimated by the cut-off methods varied from 12.2% to 17.3%. The ARTI in the Zambian and SA communities was between 0.8% and 2.8% and 2.5% and 4.2% respectively, depending on the method used. In the SA communities, the ARTI was higher among the younger children. BCG vaccination had little effect on these estimates.

Conclusions/Significance: ARTI estimates are dependent on the calculation method used. All methods agreed that there were substantial differences in infection prevalence across the communities, with higher rates in SA. Although TB notification rates have increased over the past decades, the difference in cumulative exposure between younger and older children is less dramatic and a rise in risk of infection in parallel with the estimated incidence of active tuberculosis cannot be excluded.

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Introduction

The annual risk of tuberculous infection (ARTI) is an epidemiological index derived from tuberculin skin test (TST) surveys among children to measure the extent of TB transmission in a community. It is the probability of acquiring new tuberculous infection or reinfection over a period of one year. ARTI trends are a critical indicator for progress, or lack thereof, in tuberculosis control in a community. In the recent past, ARTI estimates were used by TB control programmes to estimate the incidence of smear positive TB at a population level by using the Styblo rule [1]. However, it is now widely accepted that this assumed fixed mathematical relationship between ARTI and the incidence of TB is no longer valid [2]. In addition, ARTI trends have been used to assess the impact of the HIV epidemic on TB transmission [3,4] although it is still argued that the risk of infection in children allows little insight on the impact that HIV may exert on the burden of active tuberculosis in a population [5].

Despite the huge experience gathered over the past century, the operating characteristics of the TST ensure that there will always be a trade-off between sensitivity and specificity and that these will vary with the prevalence of infection with *M. tuberculosis* and other mycobacteria, including Bacille Calmette-Guerin (BCG) in the population. The predictive value of the test will also depend on the prevalence of tuberculous infection in the particular population [6]. Recent discussion has focused on the use of new technologies to determine who is infected [7] and on more sophisticated statistical methodologies in the interpretation of TST data to estimate the infection prevalence [8–10].

The most straightforward method is to use a predetermined cut-off value above which individuals are presumed to be infected with *M.tuberculosis*. The value proposed for the cut-off may take into

account the prior probability of being infected or the immune status of the individual [11]. A less arbitrary variant on this approach defines the cut-off in order to produce a high specificity among an unexposed population and then increases the calculated prevalence of infection by a factor derived from the observed sensitivity of the chosen cut-off in a population of patients with proven tuberculosis, who must therefore already have tuberculous infection [12].

Mirror methods are used to define prevalence in a population and do not assign all individuals to infected or non-infected states. The principal assumption is that the frequency distribution of indurations caused by tuberculous infection will be symmetrically distributed. The first approach assumes that the mode of the distribution reflects infection due to *M.tuberculosis* so that the prevalence of infection can be calculated by doubling the frequency of individuals whose induration is greater than the mode and adding those whose induration equals the mode [13]. A related approach is to use a value derived from previous data on the distribution in different populations and to assume this value to be the mode of the frequency distribution attributable to *M.tuberculosis* regardless of the observed distribution in the population [14].

Most recently, mathematical modelling approaches have been used which examine the likelihood of different theoretical populations being mixed together to produce the observed distribution [9,15,16]. Within such "mixture methods", it is then assumed that one population reflects infection, while one (or more) populations reflect non-specific reactions to BCG, environmental mycobacteria or background noise in the test's operating characteristics. There is also currently growing interest in another type of mixture model, called a latent class model, for analysing the results of multiple dichotomised tests [10].

We present data from two tuberculin skin test surveys conducted in 2005 as baseline studies of a large community randomised trial [17,18] called ZAMSTAR (Zambia South Africa TB and AIDS Reduction). The ZAMSTAR study evaluates two public health interventions that aim to reduce the prevalence of TB at community level across 16 communities in Zambia and 8 communities in the Western Cape Province of South Africa (SA). These tuberculin surveys served three objectives: to characterize ZAMSTAR communities with regards to tuberculous infection; to inform the randomization of the communities into the four intervention arms [18]; and to provide data for one of ZAMSTAR's secondary outcomes.

The primary objective of this paper is to estimate the prevalence of tuberculous infection and ARTI among school children aged 6–11 years in 24 communities in Zambia and South Africa using five methods. We also explore whether these methods alter the ranking of our communities, with regards to tuberculous infection. Finally, we examine the effect of BCG vaccination on prevalence of infection estimates.

Materials and Methods

Ethics statement

Written consent for tuberculin testing was obtained from the parent or guardian of every child. The study protocol was approved by the Ethics committees of the University of Zambia and Stellenbosch University as well as the London School of Hygiene and Tropical Medicine.

Study setting

The study was conducted in 24 selected communities in Zambia and Western Cape, South Africa. The term "community" (unit of randomisation) was defined as the population (minimum size of 25,000) accessing one TB diagnostic centre. Study communities were selected based on TB notification rates greater than 400/100,000 per annum, high HIV seroprevalence and proximity to a TB diagnostic centre. The communities selected were in five provinces of Zambia and in Western Cape Province of South Africa and included both urban and rural communities. Multi-stage purposive sampling for choosing communities was used.

ZAMSTAR study community ranking

The details of the design and randomization of the ZAMSTAR study are described elsewhere [17,18]. However, briefly for the randomization of the ZAMSTAR trial, we used stratification and restriction to randomize 24 clusters into four intervention arms in a 2×2 factorial design. To ensure that intervention effects were not distorted due to baseline imbalances between intervention groups, communities were ranked according to their TST prevalence estimates within country. Stratification was by country and tuberculous infection prevalence and restriction by tuberculous infection prevalence, HIV prevalence, urban/rural, social context, and geographical location.

Survey design and sample size

The primary schools that served the children within the community and closest to the TB diagnostic centre were selected. The TST surveys were conducted in 98 schools (56 in Zambia and 42 in SA), in the 24 communities. Our target sample size was 800 children aged between 5–9 years (grade 1–3) per community (19,200 in total). This target sample size was based on estimations for one of ZAMSTAR study secondary outcomes and has been explained in detail elsewhere [17].

Tuberculin skin testing

All children enrolled in grades 1 to 3 were eligible for inclusion in the survey. Survey staff were trained in the placement and reading of tuberculin skin tests according to the standard IUATLD protocol [19]. Training included exchange visits between Zambia and SA so that the trainers were using the same methods throughout. To standardise the reading of the tests healthy volunteers and TB patients were used. In SA, one team conducted the survey in all the 8 communities, while in Zambia, 6 teams located in the different geographical areas conducted the survey. Permission was obtained from the Ethics committees, departments of health and education, school authorities and community leaders.

Children were listed with their age, sex and address on a data collection form based on the school register. The size of the induration and the presence of a BCG scar as verified by a nurse were also recorded. Children were included in the survey whether they had a BCG scar or not. The skin testing was conducted using 2TU (Tuberculin Units) of PPD RT23 with Tween, supplied by the Statens Serum Institut (Copenhagen, Denmark). A single batch was prepared for these surveys. A dose of 0.1 ml was injected intradermally on the left forearm. Skin reactions were read using callipers 72 hours later. All pupils with reactions of $\geq$15 mm were referred to the clinic to be investigated for TB disease. In keeping with national guidelines, as the children were older than 5 years, they were not referred for prophylaxis. Data was dually entered.

Different methods to estimate prevalence of infection

Histograms of induration sizes were inspected for evidence of digit-preference and multi-modality of distributions. Tuberculous infection prevalence was calculated as the proportion of all children with a TST positive result over the total number of children with an administered and read skin test. TST positivity

was defined using cut-off, mirror and mixture analysis methods suggested by the literature, ranging from the simple cut-off value [12,14,20] approaches to the more sophisticated mixture analysis [9,15,16]. For randomization of the ZAMSTAR study, we determined the balance between the study arms using each method. Our sample size allowed us to dissect the data, when using mixture analysis, at the country but not the community level, because larger numbers of non-zero observations were required for the models to converge. With all other approaches, we investigated variation of infection prevalence at the community level. Tuberculous infection estimates at the country level were calculated as unweighted averages of community level estimates using the cut-off and mirror methods. In addition, 95% confidence intervals were calculated in order to account for the clustering effect at community level [21].

Fixed cut-off points at 10 mm or 15 mm. Infection prevalence was calculated using 10 mm and 15 mm cut-off points. The 10 mm criterion is the most widely used that considers all reactions ≥10 mm to be a marker of infection [20,22]. The 15 mm cut-off point has also been used elsewhere [20,23]. To adjust for sensitivity, a cut-off of 14 mm was used and the number multiplied by 1.22 to correct for false negatives [12,14]. The factor 1.22 has been established by data from population of infected individuals in Tanzania [12].

Mirror method. The mirror method was used to estimate prevalence using the mode as a mirror and a fixed mirror at 17 mm. The mode of the TST distribution was identified after smoothing (to adjust for obvious digit-preference bias) the crude count of TST indurations by a centred moving average of five successive reaction sizes. The total number of children with true reactions was calculated by adding the number of children showing reaction sizes equal to the mode to double the number with reaction sizes larger than the mode to determine the numerator [13,15]. The fixed mirror method considers all reactions of 17 mm counted once and indurations of ≥17 mm counted twice to obtain the estimated number of infections [12,14,24].

Mixture analysis. Three parametric models (normal, lognormal and Weibull distributions) describing infection with M. tuberculosis, and two (lognormal and Weibull distributions) describing those who reacted due to infection with environmental mycobacteria were tested to determine the best model.

A Bayesian Markov Chain Monte Carlo simulation approach [25] and programme codes for the R software were utilised for this analysis. The Metropolis-Gibbs sampler was used to calculate posterior distribution of mixture model parameters. The simulation programme initially ran for a burn-in period of 15,000 iterations the results of which were discarded. Following the burn-in period a thinned sample of 2,000 from 20,000 was used to summarise the posterior distribution of the model parameters. The validity of the model was assessable by how well it fitted the data. Models with maximum log likelihood values were used to quantify the fit. Comparisons between predicted and observed frequencies via posterior predictive model checks solidified the choice of model. For a model to be consistent with data, the posterior predictive failure rate was close to 5%. Tuberculous infection estimates at the country level were presented along with 95% confidence intervals unadjusted for the clustering effect of community level, as this was not possible using this method.

Estimating the annual risk of tuberculous infection

We calculated the annual risk of infection from the prevalence of infection estimates. We used the standard formula $R = 1-(1-\text{Prevalence})^{1/A}$ where R is the probability of being infected in any one year and A is the mean age [8,19]. Because the age (in full years) of each child at their last birthday was used, 0.5 was added to the mean age for the calculation of ARTI. Furthermore, two critical assumptions in the ARTI calculation were made. Firstly, we assumed that the ARTI was independent of the age of the person at risk of infection while exposure to TB is likely to change as people grow older. The second key assumption was that the ARTI was constant over time, which may not be the case. Because our surveys were done in children aged about 6 to11 years we estimated ARTI for each of the groups of 6, 7, 8, 9, 10 and 11-year-olds.

Impact of age and BCG

Indirectly standardised prevalence estimates using the total school children population whose consent was sought as the standard population, were calculated using the formula below:

$$TST_{indirect} = \frac{\sum_i r_i}{\sum_i n_i P_i}$$

+

Where

r_i is the number of children positive in the i^{th} age group

n_i is the number of children in the i^{th} age group

P_i is the proportion of children positive in the i^{th} age group in the standard population

The age specific prevalence of infection were stratified by country only and estimated using all methods apart from the mirror method due to inadequate numbers. In the mixture analysis, age was included as a covariate in the models.

The infection prevalence was also compared between children with a BCG scar and those without using all methods (apart from mixture analysis) and age group. A mixture model was not used to compare children with a BCG scar and those without because the data restricted us from including an additional factor in the model.

Results

Survey participation

A total of 49,835 eligible children aged between 4–18 years were registered in the TST surveys. Of those registered 25,048 (50%) had skin tests done and 22,563 (90%) of children tested were read. There was little difference in mean age and sex distribution among children sought, administered and read (Table 1). Analysis was restricted to 6–11 year-olds (94% of children read) because frequencies in the youngest and oldest age groups were small. In Zambia, this age range corresponds to primary school (7–13 years) gross attendance rate of 98.2% and 87.2% in urban and rural areas respectively [26] while in Western Cape an overall of 97% [27].

Despite the wide spread use of BCG, many children had no reaction to the TST (76% in Zambia and 69% SA). The frequency distribution of non-zero indurations is shown for each country (Figure 1). The Zambian distribution showed evidence of digit preference, but despite this, is still less symmetrical than the SA one, with a larger frequency of children with induration less than the mode than above it. The mode of the Zambian distribution, at 12 mm (discounting the 10 mm bin), was also 3 mm less than the SA 15 mm mode.

Infection prevalence and ARTI estimates

Table 2 compares the infection prevalence estimates, at the community and country level, using all methods. For two of the

Table 1. Children involved in the baseline tuberculin skin test surveys for all 24 ZAMSTAR communities.

Code	Geography	Consent sought N (% female) [mean age]	Administered N (% female) [mean age]	Read N (% female) [mean age]
Z1	Lusaka	2,914 (51) [9.4]	936 (56) [9.5]	806 (56) [9.5]
Z2	Copperbelt	2,287 (52) [8.2]	919 (53) [8.4]	903 (53) [8.4]
Z3	Copperbelt	3,011(52) [8.4]	1,145 (49) [8.2]	937 (49) [8.2]
Z4	Lusaka	2,901(50) [9.4]	956 (50) [9.5]	785 (51) [9.5]
Z5	Copperbelt	2,013(48) [9.5]	1,139 (47) [9.7]	927 (47) [9.7]
Z6	Lusaka	3,049(52) [9.6]	924 (52) [9.5]	806 (52) [9.5]
Z7	Lusaka	2,535(51) [9.2]	753 (51) [9.2]	704 (51) [9.2]
Z8	Southern	1,577(53) [7.9]	886 (55) [8.0]	788 (56) [8.0]
Z9	Southern	1,524(51) [8.0]	895 (51) [8.1]	759 (51) [8.1]
Z10	Central	2,171(51) [9.1]	1,286 (53) [9.1]	1,187 (53) [9.1]
Z11	Luapula	1,950(51) [8.8]	1,179 (52) [9.0]	1,064 (52) [9.0]
Z12	Copperbelt	2,115(51) [8.5]	936 (53) [8.6]	829 (54) [8.6]
Z13	Central	1,470(61) [8.3]	863 (63) [8.3]	830 (63) [8.3]
Z14	Southern	1,518(48) [8.7]	901 (48) [8.6]	872 (48) [8.6]
Z15	Luapula	1,663(51) [9.1]	933 (52) [9.2]	827 (52) [9.2]
Z16	Southern	1,732(50) [8.3]	949 (52) [8.2]	763 (53) [8.2]
SA1	Province	2,277(48) [7.5]	1,399 (49) [7.5]	1,319 (50) [7.5]
SA2	Province	1,678(46) [8.3]	913 (49) [8.2]	796 (47) [8.2]
SA3	Metropole	2,607(49) [8.1]	1,550 (50) [8.2]	1,438 (51) [8.2]
SA4	Metropole	1,626(48) [8.4]	1,028 (51) [8.3]	962 (51) [8.3]
SA5	Metropole	2,057(47) [7.5]	1,286 (48) [7.6]	1,232 (48) [7.6]
SA6	Province	890(48) [8.3]	561 (48) [8.4]	537 (48) [8.4]
SA7	Metropole	2,314(49) [8.2]	1,421 (49) [8.2]	1,292 (49) [8.2]
SA8	Metropole	1,956(46) [7.6]	1,290 (46) [7.6]	1,200 (46) [7.6]
TOTAL		**49,835 (50) [8.6]**	**25,048 (51) [8.5]**	**22,563 (51) [8.5]**

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communities with few non-zero readings, the frequency distribution did not have a single mode above 10 mm, so no value was calculated for the mirror method. Using these infection prevalence estimates, we calculated the ARTI for the Zambian communities to be as follows: 0.8% (15 mm); 2.0% (10 mm); 1.3% (14 mm*1.22); 2.8% (mirror); 0.8% (fixed mirror); 1.2% (mixture). The ARTI for the SA communities were as follows: 2.5% (15 mm); 4.2 % (10 mm); 3.8% (14 mm*1.22); 4.2% (mirror); 2.5% (fixed mirror); 4.2% (mixture).

For the mixture analysis method for the Zambian population, the best model fit was given by distributional assumption made for the

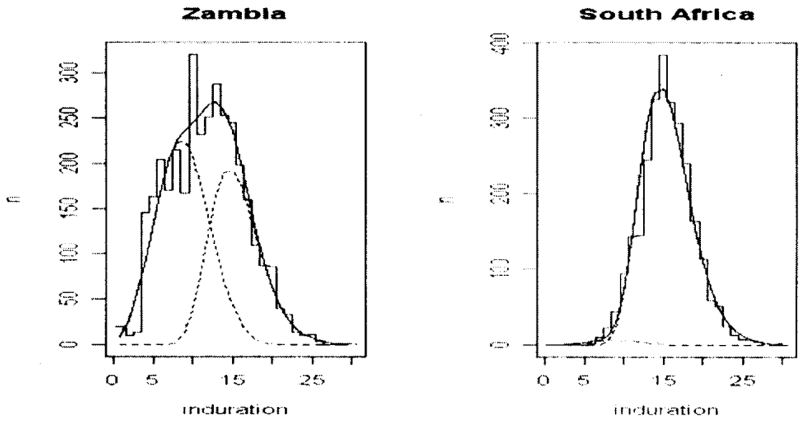

Figure 1. Histogram of frequencies of observed non-zero indurations as reactions to TST by country.
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Table 2. Tuberculous infection prevalence estimates by community and country.

	Community code	15 mm *Crude*	15 mm *Adjusted*+	10 mm *Crude*	10 mm *Adjusted*+	14 mm*1.22	Mirror	Fixed Mirror	Mixture
	Z16	1.8	1.8	6.0	6.1	3.0	N/A*	0.7	N/A*
	Z15	2.8	2.8	6.1	6.0	4.5	8.2	4.1	N/A*
	Z13	2.8	2.8	14.1	14.2	7.3	19.5	0.7	N/A*
	Z14	3.6	3.6	8.1	8.1	5.7	N/A*	3.5	N/A*
	Z12	3.9	3.9	14.9	14.9	6.7	18.1	4.0	N/A*
	Z10	4.8	4.8	19.3	19.2	8.7	25.2	2.1	N/A*
	Z11	5.1	5.1	9.6	9.5	6.3	10.3	4.3	N/A*
	Z9	6.4	6.5	16.9	17.2	10.3	21.9	4.7	N/A*
	Z8	7.6	7.6	17.3	17.5	11.4	20.8	6.4	N/A*
	Z3	8.5	8.6	19.8	20.0	13.4	24.3	7.9	N/A*
	Z2	9.7	9.7	21.3	21.3	13.8	29.5	10.0	N/A*
	Z1	10.8	10.7	22.1	21.7	16.0	33.2	11.5	N/A*
	Z5	11.4	11.3	23.9	23.4	15.9	31.5	13.3	N/A*
	Z6	11.9	11.8	21.5	21.0	17.1	22.6	17.0	N/A*
	Z7	12.0	12.0	23.8	23.4	17.6	26.5	5.7	N/A*
	Z4	13.0	12.9	21.9	21.4	18.8	24.2	15.6	N/A*
Zambia (95%CI)		**7.3 (5.4–9.1)**	**7.2 (5.5–9.0)**	**16.7 (11.9–21.4)**	**16.5 (12.0–21.1)**	**11.0 (7.8–14.3)**	**22.5 (15.5–29.6)**	**7.0 (3.8–10.2)**	**10.8 (9.1–12.1*)**
	SA8	14.2	14.3	26.0	26.5	21.6	24.8	10.8	N/A*
	SA7	14.7	14.8	24.8	24.9	22.1	18.6	13.8	N/A*
	SA5	17.0	17.0	28.5	29.0	24.9	23.8	18.1	N/A*
	SA6	17.1	17.2	26.3	26.3	23.9	31.8	18.7	N/A*
	SA2	18.7	18.7	31.7	31.8	29.6	32.8	17.0	N/A*
	SA3	22.3	22.4	32.0	32.1	32.4	29.4	19.9	N/A*
	SA1	22.6	22.7	30.4	31.1	30.8	33.8	27.4	N/A*
	SA4	27.1	27.1	42.4	42.6	39.0	47.8	27.9	N/A*
South Africa (95%CI)		**19.2 (14.4–24.0)**	**19.3 (14.4–24.1)**	**30.3 (22.6–38.0)**	**30.5 (22.9–38.2)**	**28.0 (19.2–36.9)**	**30.3 (11.6–49.1)**	**19.2 (10.5–28.9)**	**30.3 (29.4–31.1*)**

* Non-assessable.

+ Indirect age-standardized estimate using the total as the standard population

Country level estimates are calculated as unweighted averages of community estimates and 95% confidence intervals (CI) account for the clustering effect at the community level. Communities are ordered in ascending 15 mm cut-off method order.

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observed cross-reaction/ tuberculous infection of Weibull/Lognormal and the prevalence was 10.8% (95% credible interval (CI): 9.1–12.1). For the SA population the Lognormal/Weibull and Normal/Weibull assumption both fit the model well and the prevalence was 30.3% (95% CI: 29.4–31.1).The distribution of observed (histogram), mixture distribution and component distribution of tuberculous infection and cross-reactions by country is shown in Figure 1. The mode of the distributions for presumed tuberculous infection given by the mixture analyses is 15 mm for both datasets.

For each method there was considerable variation in infection prevalence (Table 2) and ARTI (Figure 2) estimates among the communities and between countries. The mirror methods showed the highest and lowest infection prevalence estimates for Zambia. Infection prevalence estimates for the Zambian communities varied from 7.0% using the fixed mirror method to 22.5% using the mirror method. Similarly, for the SA communities, infection prevalence estimates varied from 19.2% using the fixed mirror method to 30.3% using mirror, mixture, or the 10 mm cut-off methods.

The differences in infection prevalence between the Zambian and SA communities varied from 7.8% using the mirror method to 19.5% using the mixture method. All methods suggested that the prevalence of infection was considerably higher in the SA communities than the Zambian ones (Table 2). However, with the methods using a cut-off, there was no overlap in the range of estimated prevalences, whereas when the mirror methods were used, many Zambian communities were estimated to have prevalences similar to those found in the SA communities (Table 2).

For the Zambian communities, there was little variation in the ARTI estimates across the different age groups (Figure 2) However, for the SA communities there was a downward trend in estimated ARTI with increasing age until the final (11 year old) cohort.

Using a linear regression model with ARTI as calculated by the fixed mirror method the slope of the line was −0.1% (95% CI:−0.3%, 0.1%; p = 0.3), indicating a small downward, but inconclusive, trend. Exploring the data further, and recognising the limitation of sub-group analyses, when we excluded 11 year-olds (as the smallest group) the slope was −0.2% (95% CI:−0.3%, −0.1%; p = 0.02), with clear evidence supporting a steeper

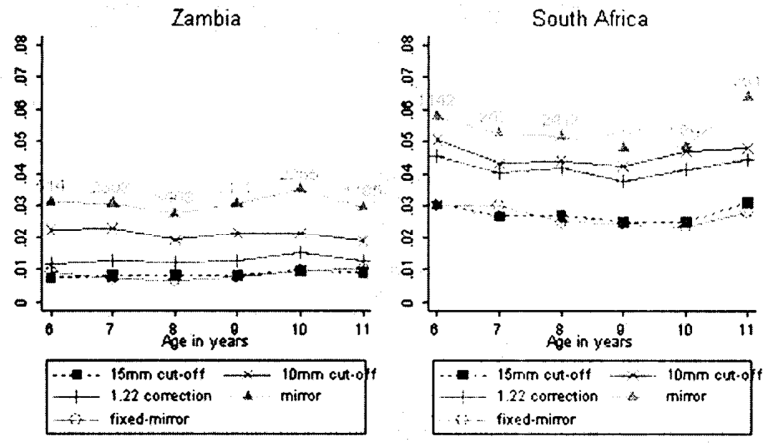

Figure 2. Annual risk of tuberculous infection, as calculated using five methods, by country and age.
doi:10.1371/journal.pone.0007749.g002

downward trend. Broadly similar results were drawn when using ARTI estimates calculated from the other four methods.

Effect of BCG

Scars typical of BCG vaccination were recorded in 74% of Zambian and 85% of SA children. Similar proportions of children within each country of both sexes (data not shown) and all ages had BCG scars.

There was a 1–3% difference in infection prevalence estimates among children with a BCG scar and those without using country level estimates when different methods were compared (Table 3).The 10 mm cut off method showed the largest difference in infection prevalence estimates between children with a BCG scar and those without for SA whereas for Zambia and overall the largest respective difference was given by the mirror method. There was no convincing evidence of a greater effect in younger children in either country for any of the methods used (Figure 3 and Figure 4).

Impact of different methods on community ranking in the ZAMSTAR study

Regardless of the approach used for the determination of tuberculous infection prevalence, there was little difference between the ZAMSTAR study intervention arms produced by the randomization procedures (Table 4). Absolute differences between approaches were present. To evaluate the association of community ranks by method, a correlation matrix of the ranks according to the five different methods was used (data not shown). Cut-off methods ranks tended to have very high correlation ($r = 0.960$ for ranks 10 mm vs. 15 mm; $r = 0.987$ for ranks 15 mm vs. 14 mm*1.22), whereas their comparison with mirror methods was not so ($r = 0.681$ for ranks 15 mm vs. mirror; $r = 0.757$ for ranks 10 mm vs. mirror).

Discussion

This is the first time tuberculin survey data of this magnitude have been collected and presented from Zambia whereas, our data from Cape Town, largely agree with previous studies from the Western Cape of SA [28,29]. These surveys have reported ARTI estimates ranging from 0.8% to 2.8% and 2.5% to 4.2% for Zambia and SA communities respectively, depending on the method we used. These ARTI estimates confirm that TB transmission is high in these communities, irrespective of the method used to define it. In addition, they remain comparatively higher than those reported in other African countries where TST

Table 3. Tuberculous infection prevalence estimates (95% confidence intervals) by country and overall.

	Zambia	Zambia	South Africa	South Africa	Overall*	Overall*
	BCG present (N = 8,453)	*BCG absent (N = 3,043)*	*BCG present (N = 6,745)*	*BCG absent (N = 1,181)*	*BCG present (N = 15,198)*	*BCG absent (N = 4,224)*
15 mm	7.4 (5.4–9.4)	7.3 (4.5–10.1)	19.6 (15.0–24.2)	17.7 (8.5–26.9)	11.5 (7.3–15.6)	10.8 (6.7–14.9)
10 mm	17.0 (12.0–22.0)	16.6 (8.9–24.4)	31.0 (22.8–39.2)	27.8 (11.0–44.7)	21.7 (15.0–28.3)	20.4 (13.1–27–7)
14 mm*1.22	11.2 (7.9–14.4)	10.9 (5.1–16.7)	28.6 (19.5–37.7)	25.7 (11.5–39.9)	17.0 (8.9–25.0)	15.8 (7.9–23.8)
Mirror	23.2 (14.5–31.9)	22.4 (10.9–33.9)	25.9 (0.7–51.1)	24.1 (0.0–49.9)	26.0 (19.1–32.9)	24.1 (16.3–32.0)
Fixed mirror	6.5 (4.0–9.1)	8.2 (0.3–16.2)	19.4 (12.3–26.6)	16.8 (0.0–42.0)	10.8 (5.8–15.8)	11.1 (3.7–18.5)

* 2,135 observations with unknown or doubtful BCG status
Country level and overall estimates are unweighted averages of community estimates and 95% Confidence intervals.
doi:10.1371/journal.pone.0007749.t003

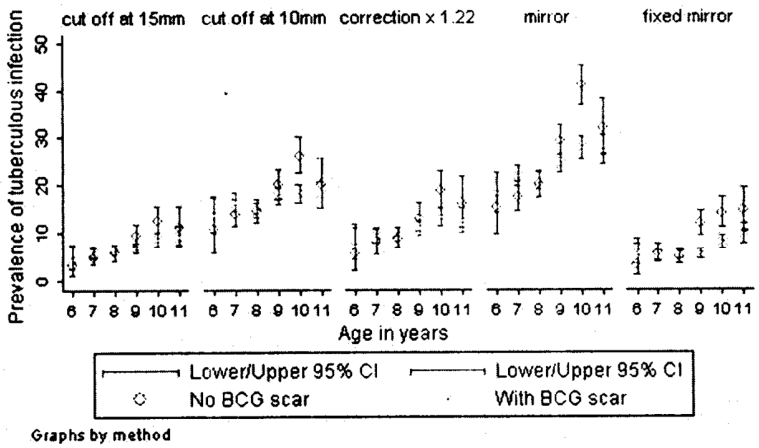

Figure 3. Prevalence of tuberculous infection with 95% binomial exact CIs, as calculated using five methods, by BCG and age for Zambia.
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surveys have been conducted [3,4,30] or worldwide [31–33]. Two recent TST surveys in Cape Town both showed an ARTI of 4.1% [28,29] using the 10 mm cut off, largely agreeing with findings in this study.

Asymmetrical distributions, such as that seen in the Zambian communities have been reported from Tanzania [12] and are presumed to be due to a larger number of children being sensitised to environmental mycobacteria as found in places where the climate is more tropical. The mixture methods of analysis are designed to provide a better estimate of prevalence of infection in such situations than a simple cut-off based approach. A recent large prevalence survey of tuberculosis disease done in Zambia identified a large number of non-tuberculous mycobacteria [34].The rather symmetrical distribution seen in the cooler temperate areas of Cape Town has also been reported in other TST surveys [29]. In this situation, mixture methods are redundant since the distinction between infected and uninfected children is easily made and there are few children with intermediate results.

These TST surveys also confirm the difficulty in estimating absolute prevalence rates for tuberculous infection and highlight the danger in making comparisons across countries with different geographical and environmental conditions. The estimated absolute prevalence for each community or country varied widely. Our results are similar to other studies showing that estimates of infection prevalence vary widely depending on the method used to

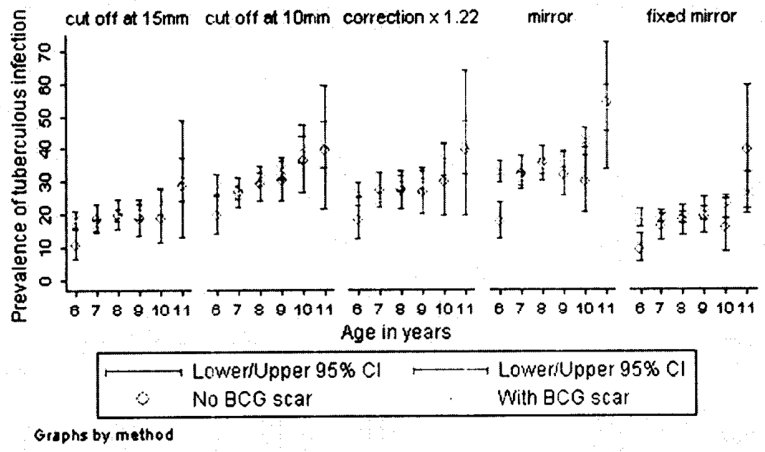

Figure 4. Prevalence of tuberculous infection with 95% binomial exact CIs, as calculated using five methods, by BCG and age for South Africa.
doi:10.1371/journal.pone.0007749.g004

Table 4. Average tuberculous infection prevalence estimates by intervention arm based on estimated prevalence calculated by various published methods.

ZAMSTAR Intervention arm [18]	15 mm	10 mm	14 mm*1.22	Mirror	Fixed mirror
Arm A	11.3	22.0	16.9	28.7 (n = 5)[+]	11.2
Arm B	11.4	20.5	16.8	25.8 (n = 5) [+]	11.6
Arm C	11.8	21.5	17.4	23.9	11.2
Arm D	10.5	20.7	15.7	23.7	10.1

[+]These estimates are based on five and not six prevalence estimates as all other estimates in the table. This is because for two communities we could not clearly define a "true" mode of TST induration. Both these communities have low prevalence estimates as calculated by all other methods and would most probably produce low infection prevalence estimates for the mirror method too. We believe the observed differences in between intervention arms averages are an artefact of what we describe here.

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calculate them [10,16]. Although various methodological approaches for the analysis of TST data have been suggested, more work on how to best interpret the results comparatively across different populations is needed.

The variability between populations in patterns of exposure and reactivity to different mycobacterial species means that judgement will remain an important element in the analysis and interpretation of such data [35].The conventional method of analyzing such data consists of presentation of tuberculin reaction sizes as a frequency distribution curve and locating an anti-mode on the curve, which is considered as the cut-off point for identification of the sub-group infected with *M.tuberculosis* [36]. However, a clear anti-mode is not always evident, especially in communities with high prevalence of cross-sensitivity to tuberculin. In such situations, mirror-image technique is used for estimating the prevalence of infection. However, identification of this mode poses further problems in communities with low prevalence of infection and high prevalence of cross-reactors [36]. Other statistical techniques for estimating the proportion of individuals infected with *M.tuberculosis* can then be employed such as mixture model analysis. The mixture model has been proposed as a possible solution to overcome problems in the interpretation of tuberculin surveys due to difficulties commonly encountered in the identification of modes and anti-modes of reactions due to infection with tubercle bacilli on the frequency distributions of reactions [37]. However, lack of supporting epidemiological evidence to show that mixture analysis is better than traditional methods has been raised by others [35].

When the communities are aggregated by country, the difference in tuberculous infection prevalence estimates between the Zambian and SA communities using mixture analysis is greater compared to other methods and the mode of the distribution of presumed infection is the same for both countries. Unfortunately, our non-zero induration data at the community level were not enough for conclusive results. As expected from the frequency distribution of induration in the SA children with very little cross-reaction, the mixture, the mirror and 10 mm cut-off methods give very similar results. Whereas in Zambia, with evident cross-reaction of environmental mycobacterial, the mixture method estimates are only similar to the 14 mm*1.22 method. Infection prevalence estimates obtained from mixture models have been shown to be lower [9,32].or fairly concordant [9] with those from cut-off methods depending on the presence of environmental mycobacterial.

The mirror method shows the highest estimates of infection prevalence when countries are compared. When community level estimates are used, for both countries at the 15 mm cut-off point and at the 10 mm for the Zambian communities, the mirror method still showed higher estimates. At the 10 mm cut-off point, the SA communities show higher estimates by the mirror method in 50% of communities. The mirror method shows higher estimates than the 15 mm cut off method, for instance, since it counts as infected almost exactly twice the number of subjects when the assumed mode of the underlying distribution is 15 mm. In a study [15], using a range of chosen modes ,the mirror method yielded a wide variation in the estimates of the prevalence, a well-recognised problem. For the three cut off methods, infection prevalence was highest and lowest for the 10 mm and 15 mm cut off methods for each country. Several reasons have been raised for challenging the traditional cut-off approach to estimate infection prevalence [9].

The different methods did not greatly alter the ranking of the ZAMSTAR communities, and when used to stratify randomisation for the ZAMSTAR study, the infection prevalence estimates within each intervention arm differed very little, irrespective of method used. We have clearly demonstrated that the differences in method do not interfere with using any one method across 24 communities for randomization.

There was little difference among children that had BCG scar and those without, adding to the growing literature that when BCG is given at birth, little difference can be detected when using tuberculin skin test in children, adolescents or adults [22,28,38]. However, the 1–3% difference in infection prevalence estimates among children with a BCG scar and those without may be significant in countries with a low prevalence of tuberculous infection.

There is a lively debate about whether the rising incidence of active tuberculosis seen over the past decades in Southern Africa has led to higher risks of infection among children [39,40]. It has been postulated that this rise in TB notification rates in adults is not well reflected by a corresponding increase in ARTI probably due to the less infectiousness of HIV positive individuals compared to HIV negative ones [40]. The youngest children in our surveys were exposed to tuberculosis from 1999, when they were born, until 2005 when the survey was performed. The oldest children were exposed from 1994 to 2005. Since the ARTI is calculated from the cumulative incidence of infection over the lifetime of the child, the differences in expected cumulative exposure are less dramatic than the changes in the incidence of tuberculosis. The WHO estimates that the incidence of tuberculosis reached a peak in Zambia in 2003, whereas it was still rising in South Africa in 2007 [41]. For the Zambian communities all methods agreed that the estimates of ARTI hardly vary for children of different ages, whereas there is a tendency for younger South African children to have higher estimates of ARTI. This is what would be expected if there was a direct relationship between cumulative incidence of

tuberculosis and risk of infection. However, despite the much larger sample size in this study than the study that sparked the debate [29], we are not able to confirm or reject the hypothesis that 5-fold rise in tuberculosis rates in the whole of South Africa is reflected in higher infection risks for the younger cohorts in Cape Town.

Our study has some limitations. There is clear evidence of digit preference in the Zambian data despite efforts to standardise training and reading across the two country teams which may lead to under or overestimation of prevalence of infection. The use of six teams in Zambia may have also contributed to inter-reader variability. In addition, not all children vaccinated with BCG leave a scar and the scar may wane with time. Since the surveys were primarily conducted to obtain estimates to rank communities they were not necessarily a representative sample of the district or province from which they were drawn nor can the differences be extrapolated to compare Zambia and SA at the country level.

Although only 50% of registered children were injected and read, we believe that this has not lead to major bias in the interpretation of our results since there was no difference by age and sex among children registered, administered and read (Table 1). In Zambia, surveys of this nature were virtually unknown before ours and therefore our teams had initial difficulties in recruiting children which improved with increased community sensitization. Furthermore, since most large tuberculin surveys are often an integral part of the National Tuberculosis programs in countries with a high incidence of tuberculosis [24,42], these do not require written consent, unlike ours. Parental written and informed consent often necessitates parents attending meetings where the study can be explained and this is difficult to achieve.

Conclusion

Estimates of the annual risk of tuberculous infection are heavily dependent on the method used for calculating prevalence of infection and quantitative comparisons, particularly across large distances and different climate zones are likely to be flawed. Among the communities selected for the ZAMSTAR study, there are large variations in the prevalence of tuberculous infection, with substantially higher estimates in the SA communities than in the Zambian ones. TB transmission remains very high in these communities. We cannot exclude the possibility that the increasing tuberculosis notification rates, fuelled by the HIV epidemic in sub-Saharan Africa, have led to an increased risk of infection among school-children.

Our data add to the consensus that in settings where BCG is given at birth, results of tuberculin skin tests are not much affected by whether a child has been vaccinated or not. In this regard, the loss of specificity that is often cited as a reason to move to IGRAs [7] may be less important in typical African settings [43].

Acknowledgments

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Author Contributions

Conceived and designed the experiments: CS HA NB PGF. Performed the experiments: KS AB. Analyzed the data: KS CS HA NB AS KAL PGF. Wrote the paper: KS CS HA NB AS PGF. Revised the manuscript critically for intellectual content: HA NB PG-F.

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The Effects of HIV on the Sensitivity of a Whole Blood IFN-γ Release Assay in Zambian Adults with Active Tuberculosis

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Abstract

Background: Interferon gamma release assays (IGRA) are replacing the tuberculin skin test (TST) as a diagnostic tool for Mycobacterium tuberculosis infection. However research into the test's performance in the high HIV-TB burden setting is scarce. This study aimed to define the sensitivity of an IGRA, QuantiFERON-TB® Gold In-Tube (QGIT), in adult Zambian patients with active smear-positive tuberculosis. Secondary outcomes focussed on the effect of HIV on the test's performance.

Principal Findings: Patients attending government health clinics were recruited within 1 month of starting treatment for TB. Subjects were tested with QGIT and TST. T lymphocyte counts were estimated ($CD3^+$, $CD4^+$, $CD8^+$). QGIT was performed for 112 subjects. 83/112 were QGIT positive giving an overall sensitivity of 74% [95%CI: 66,82]. A marked decrease in sensitivity was observed in HIV positive patients with 37/59 (63%) being QGIT positive compared to 31/37 (84%) HIV negative patients [chi^2 p = 0.033]. Low $CD4^+$ count was associated with increases in both indeterminate and false-negative results. Low $CD4^+$ count in combination with high/normal $CD8^+$ count was associated with false-negative results. TST was recorded for 92 patients, 62/92 were positive, giving a sensitivity of 67% [95%CI: 58,77]. Although there was little difference in the overall sensitivities, agreement between TST and QGIT was poor.

Conclusions: QGIT was technically feasible with results in HIV negative subjects comparable to those achieved elsewhere. However, where under-treated HIV is prevalent, an increased proportion of both indeterminate and false-negative QGIT results can be expected in patients with active TB. The implications of this for the diagnosis of LTBI by QGIT is unclear. The diagnostic and prognostic relevance of IGRAs in high burden settings needs to be better characterised.

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Introduction

Infection with *Mycobacterium tuberculosis* (MTB) results in nine million new cases of tuberculosis disease (TB) and just under two million deaths a year [1] The vast majority of TB is found in South-East Asia, Africa and the western Pacific regions. However, it is only in the African region and particularly in eastern and southern Africa that the incidence continues to rise. This increase is fuelled by the dual epidemic of TB and HIV and poses considerable challenges for TB control [2]. Strengthening DOTS and improving anti-retroviral therapy (ART) services are essential [3]. Improving detection and treatment of latent tuberculosis infection (LTBI) is also important.

The natural history of TB is altered in the presence of HIV infection. The risk of both primary progressive disease and reactivation of latent tuberculosis infection (LTBI) is increased, resulting in a high incidence of active disease in this population [4,5]. There is also an increased proportion of smear negative disease [6]. The detection and treatment of LTBI with isoniazid has proven efficacy in reducing incidence of active disease but as a policy is poorly implemented [7,8]. One barrier to the effective management of LTBI is a lack of accurate diagnostic tools.

MTB is a slowly multiplying, intracellular pathogen that is capable of surviving for many years in an immunocompetent human host. As bacterial load is low in LTBI, conventional microbiology has little to offer to aid diagnosis [9]. The tuberculin skin test (TST) has been the most extensively used immune-based test. A positive TST has clear association with an increased risk of developing active tuberculosis. If used as a screening test, isoniazid preventative therapy has greatest effect in a population who are

TST positive [10]. However many factors including infection with non-tuberculous mycobacteria, use of BCG and immunosuppression have been identified as influencing the test outcome [11]. In particular the test has a much lower sensitivity in HIV positive patients [12].

Western medicine has incorporated interferon-gamma release assays (IGRAs) into everyday practice and there is a growing literature characterising their performance in this setting [13]. In contrast, there is a scarcity of data from low income, high burden countries. In the absence of a gold standard LTBI diagnostic, active TB has been used as a surrogate in order to estimate the sensitivity of IGRAs. Only two studies from Africa have been published that use this approach. In a township in Cape Town where between 55 and 61% of TB patients are thought to be HIV infected, 100/154 (65%) culture positive TB patients were QuantiFERON-TB® Gold In-Tube (QGIT) positive [14]. Only 41 patients knew their HIV status, of these, 17/26 (65%) HIV positive subjects had a positive IGRA result compared with 11/15 (73%) HIV negative. Those that were HIV positive had lower responses to TB antigens and all 5 indeterminate QGIT results occurred in the HIV positive group. As part of a larger study in The Gambia, 80 recently diagnosed culture positive TB patients were recruited [15]. 7/80 (8.8%) were HIV positive. The authors report results for 75 subjects of whom 48 were QGIT positive giving a sensitivity of 64.0% (95%CI: 51,73). The only other study from a high burden area reports QGIT results for 60 pulmonary TB patients of whom 97% were culture positive and 5% HIV positive [16]. Their interferon-gamma responses were then followed through the course of TB treatment. Prior to treatment, 44/60 (73%) were QGIT positive. Responses fluctuated over the following six months but with no significant change in overall sensitivity.

In this study, we aimed to define the sensitivity of QGIT in Zambian adults with smear positive TB, focussing on the effect of HIV infection on the test's performance.

Methods

Study Population

The study was conducted in the government clinics of the four Zambia and South Africa tuberculosis and AIDS reduction study (ZAMSTAR) sites in Lusaka. In 2004, Zambia reported the sixth highest notification rate in Africa with 471 new or relapsed cases per 100 000 population. The true incidence is estimated to be over 600 per 100 000. HIV prevalence among pregnant women in urban areas is greater than 20%, with an estimated prevalence of HIV among adult TB cases of 54% [17]. The study protocol was approved by London School of Hygiene and Tropical Medicine ethics committee and by the University of Zambia ethical research committee.

Eligibility & Recruitment

Recruitment occurred over the period of July to October 2007. The target population was all patients recorded as smear positive over the age of 18 within one month of commencing treatment. Patients were defined as smear positive if acid-fast bacilli were detected by direct light microscopy in at least one sputum smear. These patients are required to attend clinic daily for observed therapy. Eligible patients were identified through the review of patient-held treatment cards on arrival at clinic. In exceptional circumstances due to infirmity or work commitments a surrogate collects daily drugs for the patient or a week's supply is given. No efforts were made to trace non-attenders or defaulters, however, we attempted to identify and capture weekly attenders by reviewing clinic-held treatment cards.

Venepuncture

Venous blood was obtained by needle and syringe and immediately transferred to evacuated blood collection tubes that were inverted 8 times to thoroughly mix contents. Nursing staff in Lusaka had previously reported problems with slow and incomplete filling of QGIT tubes (manufactured by Becton, Dickinson and company - BD, Franklin Lakes, USA, supplied by Cellestis, Carnegie, Australia) using the vacutainer system alone. This is presumably due to the small volume and relatively low negative pressure in the tubes compared to the ambient pressure in Lusaka at around 1300 m above sea level. By using a syringe, correct filling was ensured so that each of the QGIT tubes received 1 ml and the K_2EDTA tube (BD) for T lymphocyte estimations at least 1 ml. The full set of three QGIT tubes was used comprising one coated with each of TB RD1 test antigens, PHA as a positive control or heparin for the negative control. Blood collection occurred between 08:00 and 12:00 with all QGIT samples placed simultaneously at 12:00 in a portable incubator (Cellestis) preheated to 37°C.

TST

TST was placed according to the Mantoux method using two tuberculin units PPD RT23 in 0.1 ml Tween-80 (Statens Serum Institut, Copenhagen, Denmark) [18]. Transverse induration was measured in millimetres using callipers on one occasion between 48 and 164 hours after placement. This wide window for reading was used in an attempt to improve capture and has been shown to give reasonably reliable results [19]. In patients with TB and HIV, cutaneous anergy is thought to be an all or nothing effect and so there may be little gain in terms of reducing false-negative TST results by lowering the cut-off from 10 mm to 5 mm [20]. However standard practice and is to use the 5 mm cut-off in HIV positive patients. As all of our subjects had active TB and the majority were expected to be HIV positive, the 5 mm cut-off was used to define a positive TST in the main analysis. A secondary analysis applied the 10 mm cut-off for comparison.

Laboratory Procedures

T lymphocyte estimations were performed within eight hours of sampling. $CD3^+$, $CD4^+$ and $CD8^+$ were measured by flow cytometry (FACSCount, BD) according to the manufacturer's instructions using standard reagents (BD). On arrival in the laboratory, and after no more than 2 hours from the start of incubation, QGIT samples were transferred to a Jouan incubator (Thermo Fisher Scientific, Waltham, USA) to complete 24 hours at 37°C. Samples were then returned to room temperature and, after no more than 2 hours, centrifuged for 10 mins at 2200 RCF (Thermo). Plasma was extracted and transferred to −20°C. ELISA was performed precisely according to manufacturer's instructions using standard kits (Cellestis). Data acquired was transferred to QuantiFERON-TB® Gold analysis software (Cellestis) for results calculation.

Analysis of QGIT cut-off value

To calculate QGIT results, a value is calculated for the concentration of IFN-gamma in the TB antigen tube minus the corresponding concentration in the nil tube to take into account background IFN-gamma level. The cut-off value for positive results is set by the manufacturer at ≥0.35 IU/ml. It is possible that this cut-off is not appropriate in all populations. In particular it has been suggested that a lower cut-off may be appropriate in populations where TB is prevalent and in HIV infected patients with reduced T lymphocyte counts [21]. A lower cut-off value was

suggested for South Korea by these investigators following receiver operating characteristic (ROC) analysis of their data. Although none of their subjects were HIV positive 29 of 87 were classified as immunocompromised. A study of untreated culture-confirmed cavitary pulmonary tuberculosis patients in Turkey also found that reducing the cut-off to between 0.05 and 0.10 IU/ml improved sensitivity with little effect on specificity [22]. The main analysis for this study uses the manufacturer's suggested cut-off of ≥0.35 IU/ml. A sub-analysis was performed to assess the impact of lowering the cut-off to ≥0.13 IU/ml.

Data Handling

Data were analysed in STATA v.10 (StataCorp LP, Texas, USA). When calculating sensitivity some authors have been tempted to disregarded indeterminate results labelling them as uninterpretable [14]. This definition artificially inflates the test sensitivity and is less applicable on the population level. Furthermore, if better understood, indeterminate results may also be able to provide important clinical information on a personal level. Sensitivity was therefore defined as number of positive results over total number tested.

Results

Subject characteristics

112 adults with smear-positive tuberculosis were recruited (table 1). 71 (63%) were male, median age was 31 (range: 18,58; IQR: 25,36). 40 (36%) had sputum smear recorded as 1+, 18 (16%) as 2+ and 53 (47%) as 3+, 1 (1%) was unknown. 20 (18%) were relapse cases. 30 (27%) had received no treatment, 60 (54%) were in the first two weeks of treatment and the remaining 21 (19%) had had between 2 and four weeks. 96 subjects had HIV status recorded, of these, 59 (61%) were HIV positive. 105 subjects had T-lymphocyte estimations performed. Median $CD4^+$ count overall was 316 cells/μl (range: 6,1708; IQR: 170,594), dropping to 212 cells/μl (range: 6,1015; IQR: 109,332) in those recorded as being HIV positive and with a broad range in the HIV negative subjects (median 542; range: 84,1708, IQR: 437,698). Median body mass index (BMI) was 19 (range:13,25; IQR: 17,21).

Table 1. Summary demographic and clinical characteristics of subjects.

Variable	Subcategory	n/N (%) or Median (IQR)
Sex	Male	71/112 (63)
Smear grade	1^+	40/111 (36)
	2^+	18/111 (16)
	3^+	53/111 (48)
	Unknown	1/112 (1)
BCG scar	Present	71/109 (65)
	Unknown	3/112 (3)
HIV status	Positive	59/96 (61)
	Unknown	16/112 (14)
ART	Started	14/112 (13)
TB treatment period	None	30/111 (27)
	1 to 14 days	60/111 (54)
	15 to 31 days	21/111 (19)
Relapse case		20/112 (18)
Age, years		31 (25,36)
BMI, kg/m^2		19 (17,21)
$CD3^+$, cells/μl		1112 (808,1687)
$CD8^+$, cells/μl		715 (344, 1028)
$CD4^+$, cells/μl	Overall	316 (170,594)
	HIV negative	542 (437,698)
	HIV positive	212 (109,332)

ART, antiretroviral therapy; BMI, body mass index.
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QGIT

83/112 were QGIT positive giving an overall sensitivity of 74% (95%CI: 66,82). Of the remainder, 13 (12%) had negative and 16 (14%) indeterminate results (table 2). In subjects recorded as being HIV negative, 31/37 were QGIT positive giving a sensitivity of 84% (95%CI: 71,96). Among the HIV positive subjects, sensitivity was significantly reduced to 63% (95%CI: 50,75), 37/59 being QGIT positive with a marked increase in the proportion of negative results (Pearson's chi^2. 2df, $p = 0.033$).

A low $CD4^+$ count has previously been shown to reduce the sensitivity of QGIT [5,23]. To explore this effect, data were stratified according to $CD4^+$ count. <100 cells/μl represents a very low $CD4^+$ count, <200 is AIDS defining, 350 is the lower end of the normal range for men. With falling $CD4^+$ count there was a decrease in sensitivity of QGIT with relative increases in both negative and indeterminate results (Pearson's chi^2, 6df, $p<0.001$). This was particularly marked at counts less than 100 cells/μl, where only 3/13 (23%) had a positive QGIT result (figure 1).

The time on treatment was not seen to alter the proportion of results in each category (Pearson's chi^2, 2df, $p = 0.377$; table 2).

For further analysis of factors affecting sensitivity, QGIT result was transformed to a binary variable by combining negative and indeterminate results. In order to increase statistical power, continuous variables were redefined as dichotomous variables

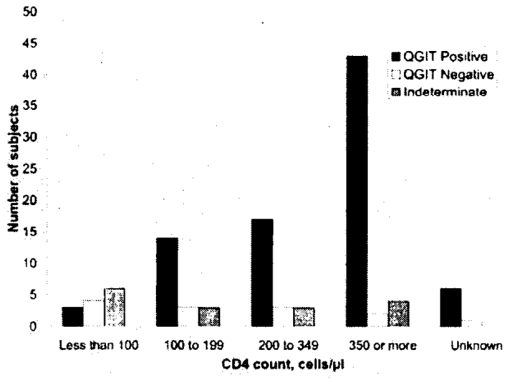

Figure 1. Distribution of QuantiFERON-TB® Gold In-Tube results by $CD4^+$ lymphocyte count. Proportion of both negative and indeterminate results increased with falling $CD4^+$ lymphocyte count. QGIT, QuantiFERON-TB® Gold In-Tube.
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using the following arbitrary cut-off values: age 31 years (median of study population), BMI 18.5 kg/m^2 (World Health Organisation definition of malnutrition in adults), $CD4^+$ count 200 cells/μl (AIDS defining), TB treatment period of 2 weeks.

In univariate analysis, a CD4 count greater than 200 cells/μl was the only variable to show a significant association with positive

Table 2. Distribution of QuantiFERON-TB® Gold In-Tube results by HIV status, $CD4^+$ lymphocyte count, treatment period and TST result.

		QGIT result, n/N_{row} (%)		
		Positive	**Negative**	**Indeterminate**
Overall		83/112 (74)	13/112 (12)	16/112 (14)
HIV Status	Negative	31/37 (84)	1/37 (3)	5/37 (13)
	Positive	37/59 (63)	12/59 (20)	10/59 (17)
	Unknown	15/16 (94)	0/16 (0)	1/16 (6)
$CD4^+$ count, cells/μl	Less than 100	3/13 (23)	4/13 (31)	6/13 (46)
	100 to 199	14/20 (70)	3/20 (15)	3/20 (15)
	200 to 349	17/23 (74)	3/23 (13)	3/23 (13)
	350 or more	43/49 (88)	2/49 (4)	4/49 (8)
	Unknown	6/7 (86)	1/7 (14)	0/7 (0)
Treatment period	0 days	21/30 (70)	6/30 (20)	3/30 (10)
	1 to 14 days	46/60 (77)	4/60 (7)	10/60 (16)
	15 to 31 days	15/21 (72)	3/21 (14)	3/21 (14)
TST	≥5 mm	51/62 (82)	6/62 (10)	5/62 (8)
	<5 mm	22/30 (74)	4/30 (13)	4/30 (13)

QGIT, QuantiFERON-TB® Gold In-Tube; TST, Tuberculin Skin Test.
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QGIT result and this persisted when adjusted for age, sex, smear status, relapse cases, treatment period and BMI (OR 4.71; 95%CI: 1.74,12.60; table 3).

Although a low $CD4^+$ count was associated with both negative and indeterminate results (median 144, 181 cells/μl respectively; Wilcoxon p = 0.710), $CD8^+$ count was high/normal in those with negative results but low in those with indeterminate results (median 999, 369 cells/μl respectively; Wilcoxon p = 0.017; figure 2).

TST

92 subjects had a TST result recorded (figure 3). 81/92 were recorded within the standard 48–72 hour period, 9/92 at 96 hours and 2/92 at 120 hours. Using a cut-off of 5 mm or more, 62/92 were TST positive, giving a sensitivity of 67% (95%CI: 58,77; table 4). There was no evidence of loss of TST sensitivity at >72 hours with 10/11 late readings being positive. 78 subjects had both known HIV status and a TST result. The test was impaired in HIV positive subjects with only 26/47 (55%) having a positive TST compared to 25/31 (81%) in the HIV negative group (Pearson's chi^2, 1df, p = 0.021). A $CD4^+$ count of less than 200 was clearly associated with negative TST (p<0.001). Presence of a BCG scar was not associated with variation in TST result (p = 0.264).

There was no significant difference between the overall sensitivities of TST and QGIT (McNemar's, p = 0.055). Although clearly affected by some of the same factors, agreement between the two tests was poor (agreement 64%, kappa 0.09; table 4). There was no improvement if only HIV negative subjects or those with $CD4^+$ count>200 were included (agreement, kappa: 68%, −0.18; 77%, −0.01 respectively). Negative TST result was not particularly associated with either negative or indeterminate QGIT results (Pearson's chi^2, 2df, p = 0.599; table 2).

Using a 10 mm cut-off, 48/92 were TST positive giving a reduced overall sensitivity of 52% (95%CI: 42,63; table 4). This sensitivity was significantly lower than QGIT's (McNemar's, p<0.001) and agreement remained poor (agreement 55%, kappa 0.08).

Cut-off value for QGIT

Applying the cut-off suggested by Harada et al of ≥0.13 IU/ml to our data [21], 5 negative and 4 indeterminate results would have been reclassified as positive. All but one of these subjects were HIV positive. This adjustment would bring the overall sensitivity up to 82% (95% CI: 75,89). With the high background prevalence of LTBI in Zambia, it was deemed inappropriate to attempt to estimate IGRA specificity. Population specific ROC analysis could not be performed to determine the cost in terms of reduced specificity. No adjustment to the mitogen cut-off defining indeterminate results has been suggested.

Discussion

This study investigated the performance of QGIT in a population with high TB incidence and HIV prevalence. As no gold standard exists for the diagnosis of LTBI, Pai et al recommend the use of microbiologically confirmed tuberculosis cases as an essential step in the assessment of new diagnostic tests [24]. This approach was taken, recruiting adult smear-positive TB patients into a cross-sectional study. QGIT was compared to TST as well as smear microscopy. The aim of the study was not to evaluate QGIT as a diagnostic test for active TB, but to increase understanding of the test's performance in this population by using active TB as a surrogate for LTBI.

Table 3. Analysis of patient characteristics to determine association with positive QuantiFERON-TB® Gold In-Tube result.

	Univariate Analysis			Multivariate Analysis, n = 100	
	n	**Odds Ratio [95%CI]**	**p**	**Odds Ratio [95%CI]**	**p**
$CD4^+$≥200 cells/μl	105	4.71 [1.87,11.83]	0.001	4.68 [1.74,12.60]	0.002
Smear grade	111	1.28 [0.80,2.04]	0.297	1.12 [0.65,1.94]	0.691
Male sex	112	1.60 [0.67,3.78]	0.288	1.10 [0.29,3.07]	0.861
BMI<18.5	102	1.29 [0.53,3.18]	0.574	1.59 [0.58,4.38]	0.368
First TB episode	112	1.29 [0.44,3.73]	0.644	1.22 [0.33,4.46]	0.762
TB treated<2 weeks	111	1.17 [0.40,3.36]	0.777	1.71 [0.48,6.18]	0.408
Age>31 years	112	1.20 [0.51,2.81]	0.672	1.13 [0.41,3.10]	0.810

BMI, body mass index.
doi:10.1371/journal.pone.0002489.t003

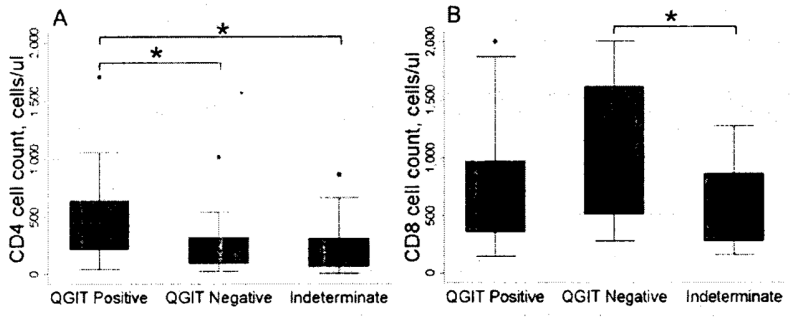

Figure 2. T lymphocyte counts by QuantiFERON-TB® Gold In-Tube result. A. Low $CD4^+$ lymphocyte counts were associated with negative and indeterminate results whereas B. $CD8^+$ lymphocyte counts were high in those with negative but low in those with indeterminate results. Box and whisker plots show range, inter-quartile range and median with dots representing outliers; QGIT, QuantiFERON-TB® Gold In-Tube; * significant difference, $p<0.05$ by Wilcoxon rank sum test.
doi:10.1371/journal.pone.0002489.g002

The study estimates QGIT sensitivity at the lower end of the range of all other studies in active TB [13], but at a level similar to other studies in high burden settings [14,15,16]. As previously observed [14,25], HIV infection was associated with decreased sensitivity with point estimates for HIV positive and negative groups being respectively well below and above the pooled sensitivity. As the sensitivity in the HIV negative patients was equivalent to those found in low burden countries, this study has shown that use of QGIT in the clinic setting in Zambia is technically possible, highlighting the utility of the portable incubator. There was, however, a significant change in the performance of the test in the population with $CD4^+$ counts less than 100 cells/μl. Using QGIT to diagnose LTBI in Denmark and in San Francisco, the test was also found to perform poorly when $CD4^+$ counts dropped below 100 cells/μl [5,23].

It was expected that immunosuppression, whether due to severe TB or advanced HIV, would lead to a shift from QGIT positive to indeterminate results. Surprisingly, in the subgroups with lower QGIT sensitivity, an increase in not only indeterminate but also false-negative results was seen, with 20% of HIV positive subjects receiving a false-negative result.

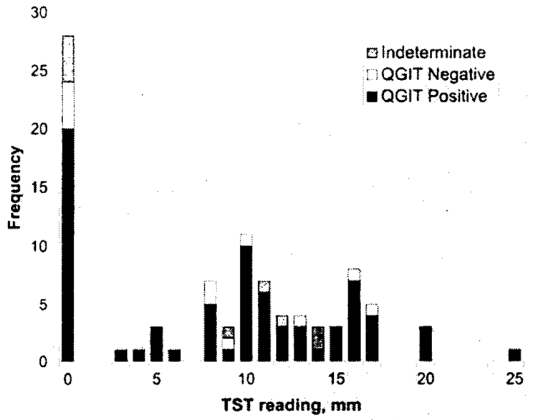

Figure 3. Frequency plot of tuberculin skin test readings showing QuantiFERON-TB® Gold In-Tube result. Concordance between TST and QGIT was poor with negative and indeterminate QGIT results occurring throughout the range of TST results. TST, tuberculin skin test; QGIT, QuantiFERON-TB® Gold In-Tube.
doi:10.1371/journal.pone.0002489.g003

It is possible that false-positive sputum smears would be associated with apparently false-negative QGIT. However the prevalence of non-tuberculous mycobacteria identified by sputum smear is thought to be very low, especially in high TB prevalence settings where specificity of a positive smear for MTB is excellent [26,27]. A second explanation is that of specific anergy due to a predominance of regulatory TB specific T lymphocytes [28]. This would be unlikely to affect only HIV positive patients.

An alternative explanation is suggested by looking at the difference in $CD4^+$ and $CD8^+$ counts between those with negative and indeterminate results. Both $CD4^+$ and $CD8^+$ cells can respond to stimulation with phytoheamagglutin [29], used as the positive control in QGIT. However, because of their length, the overlapping peptides used as the TB test antigen are essentially MHC class II restricted and so only $CD4^+$ cells respond. Subjects with low $CD4^+$ in conjunction with high/normal $CD8^+$ counts will therefore react to the positive control but not the test antigen and generate negative QGIT results, whereas suppression of both cell lines will lead to indeterminate results. As moderately advanced HIV is characterised by loss of number and function of $CD4^+$ cells but intact antigen presenting and $CD8^+$ cells [30], and TB is a feature of advancing HIV disease, this paradigm should be expected wherever TB and moderately advanced HIV are prevalent. An alternative positive test antigen targeting specifically CD4+ cells may eliminate this problem of false-negative results in such populations.

TST performed reasonably well overall but poorly in HIV positive subjects and especially in those with low $CD4^+$ counts. Both QGIT positive-TST negative discordant results and a significant number of QGIT negative-TST positive results occurred. The interpretation of this is impossible within the limits of this study. The presence of typical BCG scarring did not seem to affect either test.

In their meta-analysis, Menzies and colleagues draw attention to the fact that all studies of QGIT to-date have used a cross-sectional design with many using active TB as a surrogate for LTBI [13]. Acknowledging practical, financial, and ethical reasons why this approach is so favoured, they emphasise that long-term prospective trials would be useful to inform the prognostic interpretation of the IGRA. That is not to say that the cross-sectional approach

Table 4. Tuberculin skin test results showing both 5 mm and 10 mm cut-offs with subgroup analysis according to HIV status and $CD4^+$ lymphocyte count and agreement with QuantiFERON-TB® Gold In-Tube.

		TST 5 mm cut-off, n/N_{row} (%)		TST 10 mm cut-off, n/N_{row} (%)	
		Positive	**Negative**	**Positive**	**Negative**
Overall		62/92 (67)	30/93 (33)	48/92 (52)	44/92 (48)
HIV Status	Positive	**26/47 (55)**	**21/47 (45)**	19/47 (40)	**28/47 (60)**
	Negative	25/31 (81)	6/31 (19)	19/31 (61)	12/31 (39)
	Unknown	**11/14 (79)**	**3/14 (21)**	**10/14 (71)**	**4/14 (29)**
$CD4^+$, cells/μl	<200	9/28 (32)	19/28 (68)	6/28 (21)	22/28 (79)
	≥200	**51/60 (85)**	**9/60 (15)**	**41/60 (68)**	**19/60 (32)**
	Unknown	2/4 (50)	2/4 (50)	1/4 (25)	3/4 (75)
QGIT	Positive	**51/73 (70)**	**22/73 (30)**	**40/73 (55)**	**33/73 (45)**
	Non-positive†	11/19 (58)	8/19 (42)	8/19 (42)	11/19 (58)

QGIT, QuantiFERON-TB® Gold In-Tube; TST, tuberculin skin test.
†combined QGIT negative and QGIT indeterminate.
doi:10.1371/journal.pone.0002489.t004

using active TB is not useful, but the results must be generalised with due caution.

As the test is measuring immunity, the diversity in the aetiology and natural history of disease states, both in terms of mycobacterial activity and the host's immune response, must be considered. There are clear changes between latent infection and active disease and differences between smear positive and smear negative disease. The transition from latent infection to active disease represents a break down in immune control, similarly smear negative disease is often associated with more extensive, less well contained infection than smear positive. Immunity is further impaired in severe TB possibly as cause or effect, but the patients in the majority of studies are ambulatory with mild to moderate disease. On a population level, ethnic differences in immune response and the effects and prevalence of immunomodulatory factors such as helminths and non-tuberculous mycobacteria are unclear [31]. The effect of TB treatment on the immune response is inconsistent although treatment up to one month was not seen to have any significant effect in the present study [16]. Interpreting these data must therefore be done with a clear idea of the population involved and their disease state. In the present study the subjects were similar in many ways to those in previous studies, they were adults with active, smear positive TB of mild to moderate severity. However, being set in Zambia, it is one of few studies in a sub Saharan African population with a high incidence of TB and high prevalence of HIV.

Although it is possible that false-positive smear results could lead to underestimation of QGIT sensitivity, using positive sputum smear as a gold standard probably overestimates the true overall sensitivity of QGIT in active TB. When mycobacterial culture was used in South Africa, sensitivity in smear negative disease was 65% compared to 80% in smear positive [14]. This is important as around two-thirds of patients treated in one of the clinics in Lusaka were registered with a smear negative diagnosis, the proportion being even higher in those with HIV (unpublished survey). As we know that active TB is associated with immunosuppression and develops via a breakdown of the immune control of latent infection, directly extrapolating the sensitivity in active disease to LTBI is conceptually challenging. If we assume that people with positive results in the active disease state would have had also been positive in the latent phase, the focus must be on understanding the causes of negative and indeterminate results.

Conclusions

A tool to improve diagnosis of LTBI could be useful to direct isoniazid preventive therapy in high burden settings. Present barriers to the implementation of isoniazid preventative therapy policies are sited as cost, logistic problems and the difficulty of excluding active TB [8]. Introducing an IGRA would incur direct costs and also require considerable logistical and laboratory resources.

This study has shown that use of QGIT in the clinic setting in Zambia is technically possible. However, the accuracy of QGIT in populations with high prevalence of disease remains to be confirmed. A policy of providing isoniazid preventative therapy to all people living with HIV therefore continues to be an evidence based, safe, low-cost option [32]. Further research into IGRA in high burden settings is required both in terms of prognostic and programmatic issues.

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Author Contributions

Conceived and designed the experiments: ER. Performed the experiments: ER AD MM PD. Analyzed the data: ER. Wrote the paper: ER. Other: Supervised the project in London: PG. Supervised the project in Zambia: HA. Facilitated and Coordinated research within the ZAMSTAR team: JB.

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