ASSESSMENT OF THE QUANTIFERON TB GOLD IN-TUBE TEST FOR

THE DIAGNOSIS OF PULMONARY TUBERCULOSIS IN

NAMIBIAN PATIENTS

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ABSTRACT

Namibia had a tuberculosis (TB) case notification rate of 589 per 100 000 population in 2010. TB diagnostic methods, especially the conventional microbial culture to detect *Mycobacterium tuberculosis* (*M. tuberculosis*), takes long to produce results and those with active TB can spread the infection and treatment is delayed. Immunodiagnosis of TB by interferon-gamma (IFN-ɣ) release assays is relatively very rapid, in comparison to culture, as results can be obtained within 24 hours. The *in-vitro* Quantiferon TB Gold In-Tube (QFT-IT) test is an enzyme-linked immunosorbent assay (ELISA)-based test employing specific antigens of *M. tuberculosis*. In this thesis, the usefulness of this blood based test was validated in Human Immunodeficiency Virus (HIV) positive and negative Namibian patients. Furthermore, the levels of IFN-ɣ in QFT-IT supernatants of the TB cases were evaluated longitudinally to ascertain if the test would be useful as a tool for monitoring TB treatment response. One hundred (100) individuals suspected of having TB disease who were ≥ 18 years old, HIV positive and negative, from Katutura State Hospital were recruited in this study. Sputum for direct microscopy and culture, and blood for QFT ELISA were collected. Follow-up samples were collected from individuals in whom pulmonary TB was confirmed at 2 months on treatment, and on completion of treatment (month 6). The QFT-IT test ascertained TB disease at recruitment with a sensitivity of 90.5%, [95% confidence interval (CI) 69.6 – 98.55%] and specificity of 57.8%, [95% CI 44.8 – 70.1%], and a positive predictive value (PPV) of 41% and a negative predictive value (NPV) of 95% when Mycobacteria Growth Indicator Tube (MGIT) culture was used as gold standard. A statistically significant

difference was observed between QFT-IT and MGIT culture (chi-square = 18.4, $p <$ 0.05); and also between QFT-IT test and HIV status of participants (chi-square = 12.2, $p < 0.001$). The ANOVA test showed a statistically significant difference, ($p < 0.05$) within the IFN-ɣ levels between recruitment (before initiation of treatment) and at completion of treatment. The QFT showed no agreement with MGIT culture test and it indicated a better test in ruling out pulmonary TB caused by *M. tuberculosis* in patients; and it is not influenced by the HIV status. Interferon-gamma levels in QFT-IT supernatants declined when compared between recruitment and to completion of treatment, suggesting a role in monitoring TB treatment.

Key words: tuberculosis, *Mycobacterium tuberculosis*, interferon gamma release assays, quantiferon, immunodiagnostics, pulmonary tuberculosis, latent tuberculosis, active tuberculosis, tuberculosis suspects, patients, immune response, cytokines, clinical diagnosis, laboratory diagnosis, microbiological tests, direct microscopy, Mycobacterium Growth Indicator Tube, treatment response

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ABBREVIATIONS

AE-TBC   African European Tuberculosis Consortium

AFB   Acid-Fast bacilli

AIDS   Acquired Immune Deficiency Syndrome

ANOVA   Analysis of Variance

BCG   Bacille Calmette-Guėrin

BSC   biological safety cabinet

BMI   Body Mass Index

CDC   Centers for Disease Control and Prevention

CFP   Culture Filtrate Protein

CNR   Case Notification Rate

DOTS   Directly Observed Treatment Short-course

EMB   Ethambutol

EDCTP   European and Developing countries Clinical Trials Partnership

ELISA   Enzyme-Linked Immunosorbent Assay

ESAT   Early Secreted Antigenic Target

FDA	Food and Drug Administration
INH	Isoniazid
HIV	Human Immunodefieciency Virus
IFN-ɣ	Interferon Gamma
IGRAs	Interferon Gamma Release Assays
IU/mL	International Units per milliliters
IUATLD	International Union Against Tuberculosis and Lung Disease
MDR-TB	Multi-Drug Resistance Tuberculosis
MGIT	Mycobacteria Growth Indicator Tube
MoHSS	Ministry of Health and Social Services
MOTT	mycobacteria other than *M. tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
NALC	N-acetyl L-cysteine
NaOH	sodium hydroxide
NPV	Negative Predictive Value
NTLP	National Tuberculosis & Leprosy Programme

NTM   Non-Tuberculous Mycobacteria

OD   Optical Density

PBMC   peripheral blood mononuclear cells

PBS   phosphate buffer saline

PCR   Polymerase Chain reaction

PO$_4$ Phosphate

PPD   Purified Protein Derivatives

PPV   Positive Predictive Value

QFT   Quantiferon

QFT-IT   Quantiferon TB Gold In-Tube

RIF   Rifampicin

RD   region of difference

STM   Streptomycin

SPSS   Statistical Package for Social Sciences

TB   Tuberculosis

TST   Tuberculin Skin Test

WHO   World Health Organisation

X^2 chi-square

XDR-TB   Extremely-Drug Resistance Tuberculosis

PAZ   Pyrazinamide

ZN   Ziehl-Neelsen

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DEDICATIONS

This thesis is dedicated to my beloved grandmother "Kuku" Hilya Nampweya and my dearest parents for their inspirations and believes in education.

DECLARATIONS

I, Josefina N. Nelongo, declare hereby that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

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Josefina N. Nelongo

CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction

This study validated the usefulness of Quantiferon TB Gold In-Tube test to diagnose pulmonary tuberculosis (TB) a disease caused by *Mycobacterium tuberculosis* (*M. tuberculosis*), in the Namibian patients. This chapter provides the general introduction of the study which includes background of the study, the statement of the problem, hypotheses, research objectives, the significance and the organisation of the study.

1.2 Background of the study

The lungs play a role in gas exchange, yet it is the prime target for infections and other injuries (Nelson & Martins, 2000) to human. Tuberculosis is an infectious bacterial disease that affects humans and many other mammals (Greenwood, Slack & Peutherer, 2000). Tuberculosis (TB) infection that could eventually lead to TB disease is caused by *M. tuberculosis* and it claims the life of many people in the world. Although the causative agent for TB was discovered more than 100 years ago, it continues to be the leading cause of morbidity and mortality worldwide, killing more than 2 million people per year (Peabody, Shimkhada, Tan & Luck, 2005).

In the world, TB burden is highest in Asia and Africa geographically; India and China accounts for almost 40% of the world's TB cases of which about 60% of the cases are in the South-East Asia and western Pacific regions. The African region has 24% of the world's cases and the highest rates of cases and death per capita (World Health Organisation [WHO], 2012). The WHO estimated that there were almost 9 million new TB cases in 2011 and 1.4 million TB deaths of which 990 000 were among Human Immunodeficiency Virus (HIV) negative people and 430 000 HIV associated TB deaths globally (WHO, 2012).

Namibia in particular was recorded having a high case notification rate (CNR) of TB in 2009. CNR is defined as the number of TB patients per 100 000 population during a particular period. The record showed 13 332 cases of TB notified, equivalent to a CNR of 634 per 100 000 population (MoHSS, 2011). The high case load is attributed mainly to the HIV epidemic as reflected by the HIV prevalence of 17.8% among ante-natal clinic attendee and an HIV prevalence rate of 58% among TB patients. In 2010, 12 625 cases of TB were notified, with a CNR of 589 per 100 000 population. The HIV prevalence was 18.8% among the ante-natal clinic attendees and an HIV prevalence rate of 56% among TB patients (MoHSS, 2012). The Ministry of Health and Social Services (MoHSS) in Namibia routinely screens all TB patients for HIV and they are encouraged to know their HIV status and vice versa (MoHSS, 2012). This is to ensure appropriate care and treatment.

The distribution of the TB burden as by the records of National Tuberculosis & Leprosy Programme (NTLP) of 2009, varies from region to region (Figure 1); the most affected regions were Khomas and Kavango (MoHSS, 2011).
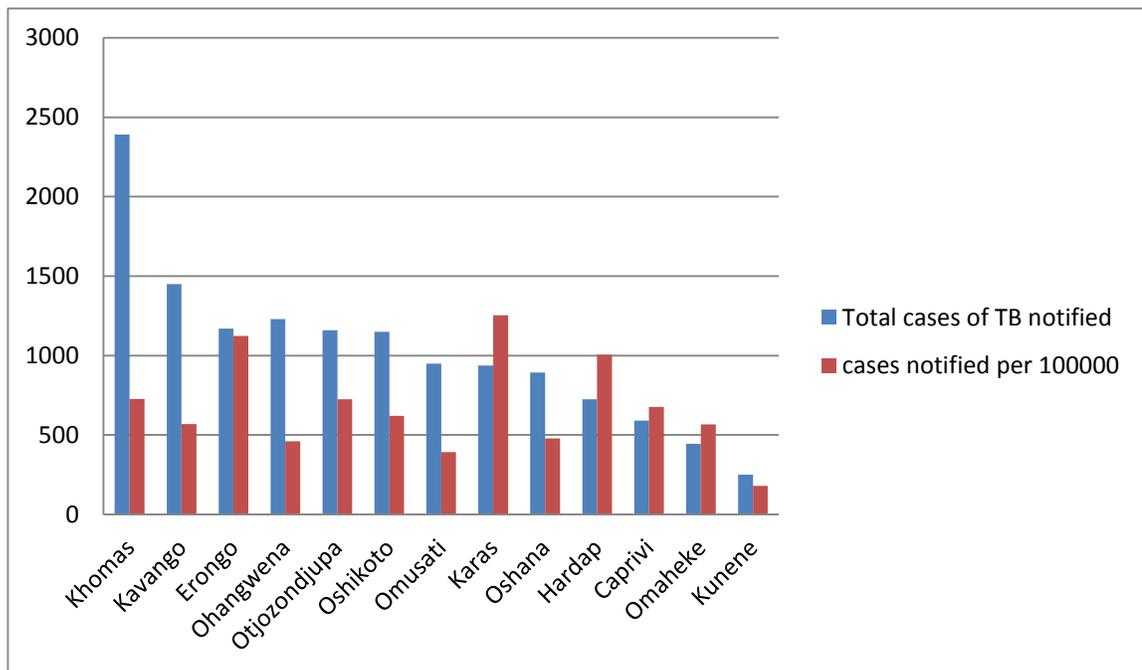
Figure 1: Burden of tuberculosis in Namibia by region in 2009 (adopted from MoHSS, 2011)

Although, there was a decrease in the number of notified cases noted in 2010 compared to those noted in 2009, the figure is still among the highest in the world.

Tuberculosis is reported more commonly among men than women and affects mostly adults in the economically productive age group (WHO, 2012). The age group between 15-45 years remained the most heavily TB affected for new pulmonary TB smear positive cases in both males and females (Namibia's Country Co-ordinated Proposal to The Global Fund to fight AIDS, Tuberculosis and Malaria, 2002). This affected age group is the most economically active because they are supposed to be studying, employable and prospective leaders of the countries.

It raises a lot of questions as to why TB still continues to be among the top diseases that affects human and it is among the number one human microbial killer in the 21 century while there are control measures in place. Pulmonary TB can be cured and prevented when infrastructures are in place but without adequate diagnosis TB cases can advance unnoticed (Hamilton et al., 2007).

Prompt and proper diagnosis of TB is a prerequisite for an effective therapy and has good treatment outcomes. The first line anti-TB medicines are: rifampicin (RIF), isoniazid (NIH), pyrazinamide (PZA), streptomycin (STM) and ethambutol (EMT) (MoHSS, 2012). An effective recovery of TB patients can be reached by the standard therapy consisting of two (2) months of four drugs: RIF, NIH, PZA and EMT daily in the initial phase followed by four (4) months of RIF, NIH and EMT daily in the continuation phase (MoHSS, 2012). This is recommended for all patients with new onset

of uncomplicated pulmonary or extrapulmonary tuberculosis. The eradication of pulmonary TB through treatment and prevention had been and is a serious challenge for the MoHSS in Namibia due to the fact that the correct diagnosis of TB infection and disease is delayed or never done at all in some clinical cases.

Tuberculin Skin Test (TST) is a test used to assess whether a person has acquired *M. tuberculosis* following exposure and is useful in persons not immunised with Bacille Calmette-Guėrin (BCG) (Mandal, Wilkins, Dunbar & Mayon-White, 2004. p. 193). Although TST has been the standard diagnostic test for latent tuberculosis infection, it has been found to be unreliable, providing false negative results in high-risk groups such as immunosuppressed, immunocompromised patients and young children globally (Van Rooyen & Brink, 2007). Its specificity is also low with false positive results occurring frequently in those vaccinated with BCG and in those infected by non-tuberculous mycobacteria (NTM).

The Quantiferon TB Gold In-Tube method is a commercial T-cell based on interferon gamma (IFN-γ) release assays (IGRA). It is an *in vitro* diagnostic test using a peptide "cocktail" simulating early secreted antigenic target 6 (ESAT-6), culture filtrate protein 10 (CFP-10) and TB7.7 (p4) proteins to stimulate cells in heparinised whole blood. Detection of IFN-γ by Enzyme-Linked Immunosorbent Assay (ELISA) is used to identify *in-vitro* responses to these peptide antigens that are associated with *M.*

tuberculosis infection (Cellestis Limited. Quantiferon TB Gold (In-Tube Method) [Package insert]). The assay is used in the screening of infection associated with *M. tuberculosis*.

The conventional diagnosis of mycobacterial disease such as active pulmonary TB depends mainly on laboratory diagnostic methods such as direct microscopy of the stained smear, microbiological culture on media using Lowenstein Jensen or other methods, clinical evaluation and chest radiograph (Forbes, Sahm & Weissfeld, 2007). Although these methods are cost effective, the conventional microbiological culture method which is considered as a laboratory 'gold standard' is time consuming. By the time culture-positive cases are detected the TB suspects may have already infected many of their close contacts (Perkins, 2000). The direct microscopy has been reported with rising cases of smear negative in those with TB disease because of the co-infection with HIV (Perkins, 2000). Furthermore, direct microscopy and chest radiograph simply cannot distinguish between the different mycobacteria strains and it is of no help in categorising Multi-Drug Resistance TB (MDR-TB) and Extremely-Drug Resistance TB (XDR-TB). In addition, molecular diagnostic methods of tuberculosis are too expensive for many countries.

The medical scientists developed interferon–gamma release assays (IGRAs) in which Quantiferon TB Gold In-Tube and ELISPOT tests are parts. Therefore, the study

validated the usefulness of Quantiferon TB Gold In-Tube test for the diagnosis of *M. tuberculosis*, the causative agent for pulmonary TB in Namibian patients.

1.3 Statement of the problem

The conventional methods used to diagnose pulmonary TB in Namibia are believed to be effective, but these methods have many shortcomings. Direct examination of unconcentrated sputum smears by microscopy is relatively rapid and inexpensive (Caviedes et al., 2000) but suffers from poor sensitivity and specificity. The conventional microbiological culture method takes a long time for the results to be available, as the sample has to be incubated and observed daily for up to 42 days before a sample can be declared negative for TB (Runa et al., 2011). This leads to delays in the initiation of treatment and consequently, while patients are waiting to get results, it is possible for them to spread the infection (Perkins, 2000). Most importantly, these conventional methods which rely on sputum examination are not suitable for individuals who have difficulties in providing sputum, such as children and those with extra-pulmonary TB (Huggett, MucHugh & Zumla, 2003 and Lighter, Rigaud, Eduardo, Peng & Pollack, 2009). Immunological methods which rely on blood specimens might be more beneficial.

The Quantiferon TB Gold In-Tube assay is a relatively novel assay that has been investigated widely, especially in low burden, high income countries and is currently the gold standard for the diagnosis of *M. tuberculosis* infections in some settings, but has not been validated in Namibia. Similar studies in other countries on IFN-γ levels with treatment arrived at different findings, whereby some are not changing, increasing or decreasing.

Although scientists developed the Quantiferon TB Gold In-Tube test which shortens the time taken for test results to be available, its usefulness in terms of diagnosing pulmonary TB or *M. tuberculosis* infection in Namibia has not been assessed. In addition, studies for detecting and diagnosing *M. tuberculosis* have been carried out in developed countries and few in developing countries; however, there are no published studies on interferon-gamma release assays especially the Quantiferon TB Gold In-Tube test in Namibia.

1.4 Hypotheses of the study

Given that the Quantiferon TB Gold In-Tube test is a whole blood assay based on the detection of IFN-γ produced in response to *M. tuberculosis* specific antigens, it is hypothesised that the quantiferon test is a better test for the diagnosis of pulmonary TB caused by *M. tuberculosis* than MGIT culture test and that quantiferon test is influenced

by HIV status of the patient. It is also hypothesised that IFN-γ responses will change in TB patients during treatment and at the end of treatment as compared to pre-treatment, implying that the test can be used to measure if the patient is responding to TB treatment or not.

1.4.1 Null hypotheses

i.   There is no agreement between the 'gold standard' MGIT culture and quantiferon test results in diagnosing pulmonary TB caused by *M. tuberculosis*.

ii.   The quantiferon test is not influenced by the HIV status of the patients.

iii.   There are no significant differences in the baseline, month 2 and month 6 levels of IFN-γ in quantiferon supernatants of TB patients during TB treatment.

1.5 Objective of the study

1.5.1 General objective

The study validated the usefulness of the Quantiferon TB Gold In-Tube test in the diagnosis of pulmonary TB in Namibian patients and also the utility of IFN-ɣ levels detected in the quantiferon supernatants as a measure of response to anti-tuberculosis therapy.

1.5.2 Specific objectives

i. To validate the usefulness of the Quantiferon TB Gold In-Tube test in the diagnosis of pulmonary TB in Namibian patients.

ii. To evaluate the influence of HIV on Quantiferon TB Gold In-Tube test results.

iii. To evaluate the levels of IFN-γ in quantiferon supernatants of pulmonary TB patients undergoing treatment as a measure of response to TB chemotherapy.

1.6 Significance of the study

The rapid and accurate diagnosis of pulmonary TB disease is one of the most important measures that are needed by TB control programs worldwide for the effective control of the disease. The findings of the study could inform the Ministry of Health and Social services to diagnose *M. tuberculosis* infection early (within 24 hours after the patient presents at the hospital) and early treatment can be embarked on while the disease is at its initial stage.

Additionally, since an effective diagnosis can lead to the proper treatment and management of pulmonary TB in Namibia, it can eventually lead to the reduction in new cases of pulmonary TB. This could be of great help to public health care because results are provided without the need of a patient having to wait longer as it is the case with

conventional microbiological culture method. This particular study did not only contribute to data on the diagnosis of pulmonary TB using QFT in the Namibian context with specific reference to Katutura State Hospital, but it also benefited the TB patients as they were encouraged to finish the course of treatment during follow up time points until the end of TB treatment.

1.7 Organisation of the study

This thesis consists of six chapters, a reference list and appendices; chapter one introduced the study by presenting the background of the study, the statement of the problem, hypothesis of the study, the research objectives, and the significance of the study.

Chapter two provides the literature review, where it covers what has been done on pulmonary TB diagnosis. Apart from an introduction to the chapter; a historical background of TB, tuberculosis in early times, symptoms of pulmonary TB, tuberculosis and immune response, methods of diagnosing pulmonary TB both clinically and in the laboratory are presented. The TB case definitions, burden of TB on individual and nationally, treatment (management) of TB are included. Monitoring of TB treatment response and IFN-ɣ levels in HIV infected patients are also presented.

Chapter three presents the research methodology which includes the research design, population and sampling strategies, materials and methods of the study, data analysis, limitations of the study and research ethics.

Chapter four deals with results and chapter five interprets the results, which is presented as discussions. Finally, chapter six presents the conclusion reached and it where the main findings are summarised, and the recommendations are also given.

1.8 Conclusion

This chapter focused on the overview of the background of TB in general where it gave alarming data that showed the seriousness of TB disease in the world and Namibia specifically; an emphasis was given to TB diagnostic methods. It elaborated on the need for intervention in order to reduce pulmonary TB cases. The core objectives of the study as informed by the statement of the problem were stated, and it further pointed out the significance of the study.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

This chapter provides an analysis of existing knowledge about pulmonary TB infection and disease and its diagnostics. It consists of the historical background of TB, tuberculosis in early times, its symptoms, TB and immune response, the diagnosis, TB case definitions, its burden on individual and national, and treatment of tuberculosis. It also presents the monitoring of TB treatment response and the levels of IFN-ɣ in HIV infected patients.

2.2 Historical background of tuberculosis

Tuberculosis is an infectious bacterial disease which is caused by the bacillus *M. tuberculosis*; it affects humans and many other mammals (Greenwood et al., 2000). It spreads from one person to another by aerosol route; the lung is mainly the first site of infection for pulmonary TB (Gillespie & Bamford, 2007) and it can also affect other sites as well for extrapulmonary TB (WHO, 2012). The disease is spread in the air when a person who is sick with pulmonary TB expels the bacilli (Figure 2), for example through coughing and sneezing. Tuberculosis can either be latent or active; latent TB compared to active TB does not present with symptoms.

A person does not feel sick, chest X-ray and sputum test are normal and an individual with latent TB cannot spread it to others. The incubation period of the disease ranges from weeks to years, although it is rarely more than 5 years and after this point any incidence is usually due to further contact with an infectious source (Grimard & Harling, 2003). In early days, TB was generally referred to or commonly known as consumption (Murray, 2004).
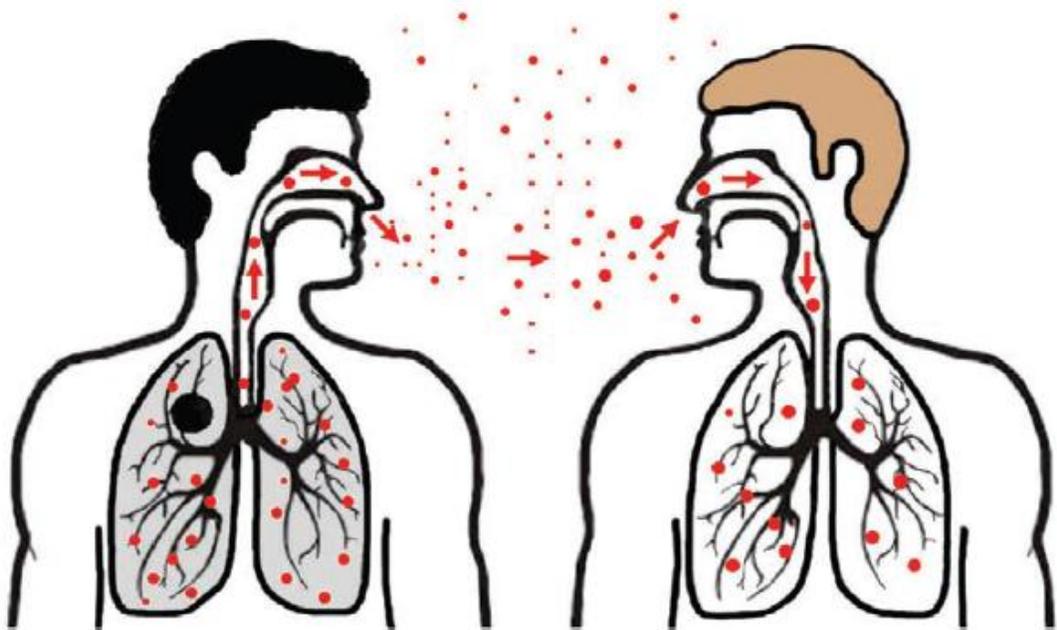
Figure 2: An example of the transmission of tuberculosis (http://www.darkgovernment.com/news/completly-drug-resistant-tuberculosis-spread)

There are several facts that are involved in the rise in incidence of TB such as: the ability of the bacteria to subvert the host immune response, poor TB management programmes, poor treatment adherence, co-infection with human immunodefieciency virus (HIV) and the emergence of drug-resistant *M. tuberculosis* strains (Siawaya, Beyers, van Helden and Walzi, 2009).

2.3 Tuberculosis in early times

Tuberculosis has been in existence since long. The understanding of its pathogenesis began with the work of Theophile Laennec at the beginning of the 19th century and it advanced by the demonstration of the transmissibility of *M. tuberculosis* infection by Jean-Antoine Villemin in 1865 (Daniel, 2006). *M. tuberculosis* was first identified in the laboratory by the German Scientist Robert Koch in 1882; therefore, laboratory diagnostics today are based on his work. The disease complex of organisms which can cause human disease consists of *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. microti* and *M. canetti* (Lawn & Zumla, 2011). In 1895, Wilhelm Konrad Rontgen discovered chest X-rays (Murray, 2004) and in 1907 Clemens von Pirquet developed the tuberculin skin test (Daniel, 2006), and then later, the TB treatment and control was the news by the discovery of streptomycin in 1944 and isoniazid in 1952. In the late 19th and early 20th centuries, treatments of patients who were infected with tuberculosis were based on sanatoria (Daniel, 2006).

2.4 Symptoms of tuberculosis

Tuberculosis may present with or without symptoms and presentations have different intensities. The most common signs and symptoms of pulmonary TB that makes up the medical history are: persistent cough for 2 weeks or more, haemoptysis, chest pain, night sweats, dyspnea, loss of appetite and loss of weight (MoHSS, 2012). Similar characteristic symptoms of pulmonary tuberculosis as mentioned early in approximate decreasing of frequency (Davis, 2008): cough, malaise, fever, weight loss, night sweats, breathlessness and chest pain.

The cough presents being constant and irritating, it can be unproductive or productive of sputum. Rupture of a blood vessel may leads to hemoptysis which may be dramatic and rarely life-threatening. Malaise is of gradual onset but likely to demotivate the patient so that after a few weeks of the disease, they want to do nothing but rest. Fever is normally of low grade, rarely rising above 40°C and characteristically low in the morning and peaking at night. Weight loss is gradual but if treatment is not started it may become dramatic over the space of a few months, and the patient can go on even to lose 50% or more of body mass. Night sweats may be profuse, they may continue for some time even months after treatment has been started. Breathlessness occurs later when a substantial part, probably more than 20% of the lungs has been destroyed. In preexisting lung disease, this symptom occur earlier (Davis, 2008).

2.5 Tuberculosis and immune response

Tuberculosis is a disease of the lungs, a lung being the port of entry of gases and it is also the major site of the disease. Minute bacterial pathogens including *M. tuberculosis* are engulfed by the alveolar macrophages and drained into the lymph nodes where small granulomatous lesions are formed (Kaufman, 2002). The infected majorities develop the disease later because the bacteria are not eradicated by the initial means of immune response. The risk of the disease development is on the severity of immuno-deficiency especially in HIV infected individuals. Therefore, those who are latently infected are at risk because once the immune system is weakened, outbreak may occur owing to reactivation. Bacterial containment is focused on the granulomatous lesion, and different T-cell populations (CD4 T-cells, CD8 T-cells, ɣδ T-cells and CD1 restricted T cells) are involved in the protective immune response.

M. tuberculosis has the ability to persist in early phagosomal compartment (Kaufman, 2001 cited in Kaufman, 2002) and survive a stage where many bacterial pathogens are killed by inhibiting phagolysosome fusion. Some bacteria are killed or at least prohibited from replication through anti-bacterial mechanisms including reactive oxygen and nitrogen intermediates produced by activated macrophages. The T-cell populations produce interferon gamma (IFN-ɣ) and hence are of the T helper 1 (Th1) type. IFN-ɣ synergises with tumor necrosis factor α (TNFα) in activating

macrophages. IFN-ɣ is a cytokine which is the central mediator of macrophage activation; it has shown activation of anti-mycobacterial macrophage than TNFα *in vitro* (Flesch & Kaufman, 1993 cited in Kaufman, 2002). Cytokines are a large heterogeneous group of potent polypeptide mediators of intercellular communication with diverse biological actions (Nelson & Martin, 2000). Cytokine networks are extraordinarily complex and they operate in a delicate balance to restore homeostasis.

2.6 Diagnosing pulmonary tuberculosis

2.6.1 Clinical diagnosis

The medical history is the first step towards the diagnosis of TB (MoHSS, 2012), it is based on the symptoms that a patient presents with and states to the medical health personnel and the signs that the medical health personnel observes on the patient.

2.6.2 Laboratory diagnosis

The WHO reported that some cases of pulmonary TB notified, were not bacteriologically confirmed using recommended laboratory methods and a portion of the patients whom TB was clinically diagnosed without laboratory confirmation may not

had TB (WHO, 2012). Therefore, it is very important that TB be confirmed by both clinically and mainly by laboratory tests.

Tuberculosis diagnosis has been based on the use of sputum and other specific specimens but blood testing was never given much attention and it is only recent that little attention is given to the *in vitro* immunodiagnostics to detect TB infections and disease. Diagnostic methods using commercial, antibody-based serodiagnostic test and commercial interferon-gamma release assays (IGRAs) as a public intervention to detect latent TB infection and active TB disease in low and middle income settings received negative critic statements by the WHO (WHO, 2012). Therefore, its use must be thoroughly assessed.

In most resource-limited countries; direct sputum smear microscopy is the primary means for pulmonary TB diagnosis, and automated liquid culture systems such as Bact/ALERT MB (bioMerieux Inc, Durham, NC, USA) or BD BACTEC MGIT (Becton Dickinson, Sparks, MD, USA) are currently considered the 'gold standard' approaches for isolating mycobacteria (Pai, Minion, Sohn, Zwerling & Perkins, 2009). In Namibia, the confirmation of pulmonary TB disease in the laboratory is also dependent on direct sputum smear microscopy of Ziehl-Neelsen or o-Auramine and conventional culture media which is an automated culture system BD BACTEC MGIT.

In addition, there are molecular laboratory techniques that are used worldwide that can detect TB better or at almost the same level as the above mentioned; among them includes nucleic acid amplification tests using polymerase chain reaction assay (PCR) (Knechel, 2009); Namibia has also started using the molecular tests currently. The method can differentiate *M. tuberculosis* from other mycobacteria based on the genetic information and provides rapid results within few hours. Although, it can provide rapid confirmation of *M. tuberculosis* in sputum specimen positive for acid fast bacilli, it has limitations such as high cost, low sensitivity and low availability.

Therefore, in this study, the quantiferon test was assessed parallel to the direct microscopy and automated culture liquid system in order to find out how they compare. This immunological based test (quantiferon) using blood was compared to the 'gold standard' test MGIT culture and as well as to the direct microscopy using sputum.

2.6.2.1 Microbiological tests

A diagnosis of TB is not complete without a bacteriological examination which is the only reliable confirmation of the presence of bacilli causing TB disease (MoHSS, 2011).

2.6.2.1.1 Direct microscopy

Direct microscopy of sputum smear for acid fast bacilli (AFB) microscopy is known to be inexpensive to perform, very specific in high prevalence settings and detects the most infectious subset of patients. However, there are drawbacks as incidences of smear-negative disease in countries where HIV infection is prevalent such as in sub-Saharan Africa is rising (Perkins, 2000). A positive sputum direct microscopy is described when at least 5000 organisms/ ml of sputum is found, fewer than this, the smear is unlikely to be positive. In the United Kingdom (UK), 56% of patients diagnosed as having pulmonary TB are sputum smear-negative (Davis, 2008). Therefore if TB diagnosis happened to be done based on direct smear microscopy, some individuals with TB infections and disease may be left undiagnosed and those without a disease will be diagnosed with a disease unnecessarily. Huggett, MucHugh & Zumla, (2003) reported that only 50-80% of patients with untreated pulmonary TB will have positive smear and only 5% of children with pulmonary TB will have positive smear while HIV positive patients are more likely to be negative.

2.6.2.1.2 Conventional culture media

Definitive diagnosis of tuberculosis requires the identification of *M. tuberculosis* in a culture of a diagnostic specimen; the most frequent sample used in a patient with a persistent and productive cough is sputum (Knechel, 2009). The use of automated liquid culture systems are being substantially faster and have a 10% greater yield than solid

media (Lawn & Zumla, 2011). However, the sample in the instrument has to be observed daily up to 6 weeks for any growth (Runa et al., 2011) and before a sample can be declared negative for TB, and it can lead to a reliable definitive confirmation of TB infection by *M. tuberculosis*.

Current TB diagnostics are really in need of improvement; especially that 5-10% of infected individuals with latent TB will have a chance to progress to active TB (Frahm et al., 2011). It is therefore important that there must be early detection of TB infection, and means of differentiating latent TB from active TB need to be attended to. Although it is a fact and it has been indicated in many studies that QFT TB Gold In-Tube test is used in the diagnosis of infection by *M. tuberculosis*, it is not useful in high TB incidence areas as IFN-ɣ cannot distinguish between latent and active TB disease (Chegou, Walzi, Bolliger, Diacon & Van den Heuvel, 2008; and Van Rooyen & Brink, 2007). The preliminary report of a study done in South Africa by Chegou, Black, Kidd, van Helden & Walzi, 2009 using Luminex, suggested that active TB may be accurately differentiated from latent TB, as Luminex can explore on additional multiple biomarkers that can help to distinguish between latent and active TB. Furthermore, biomarkers combination; IL-15 and MCP-1 identified 86% of active and latent TB patients. These biomarkers (IL-15 and MCP-1) response pattern could either be a marker for the presence of active TB or instead could represent a response associated with greater susceptibility to development of active TB after infection (Frahm et al., 2011).

2.6.2.2 Immune-based tests for tuberculosis

Immune-based tests have appeal for the diagnosis of TB, because the disease is difficult to diagnose. There is a challenge in diagnosing active disease, because of the protean clinical manifestations, radiological tests are insensitive and / or not specific, while microbiological tests tends to be slow and labour intensive (Menzies, Schwartzman & Madhukar, 2009). Since the rates of TB disease have fallen, the emphasis is shifted to identification of latent TB infection and treatment to prevent later reactivation.

Immune-based tests include: tuberculin skin test (TST) and interferon-gamma release assays (IGRAs). Latent TB infection measures cell-mediated response to TB antigens. TST measures a complex *in vivo* response to intradermal injection of fairly crude mixtures of protein antigens derived from cultures of *M. tuberculosis*, while IGRAs measures IFN-ɣ produced in-vitro by sensitised lymphocytes after *in vitro* exposure to antigens found in *M. tuberculosis* (Menzies, Schwartzman & Madhukar, 2009). Interferon-gamma release assays (IGRAs) are a class of ex-vivo assays that measure (IFN- ɣ) released by sensitised T-cells after stimulation by *M. tuberculosis* antigens. Early version of the IGRAs used purified protein derivatives (PPD), but now replaced by newer versions that uses antigens that are more specific to *M. tuberculosis* than PPD. The antigens included are: early secreted antigenic target 6 (ESAT-6), culture filtrate protein 10 (CFP-10) and TB7.7 (Rv2654). ESAT-6 and CFP-10 are encoded by genes located within the regions of difference 1 (RD1) segment of the *M. tuberculosis* genome

(Menzies, Schwartzman & Madhukar, 2009). The antigens are more specific and not shared with any neither of the BCG vaccine strains nor by several species of non-tuberculous (environmental) mycobacteria including *M. avium*.

Two IGRAs are available as commercial kits: the Quanti-FERON-TB Gold (Cellestis Ltd, Carnegie, Australia) assay and the T-SPOT.TB test (Oxford Immunotec, Oxford, UK). They differ from each other with respect to the technique of IFN-ɣ detection. The Quanti-FERON-TB Gold is an ELISA-based assay that uses whole blood, and it is available in two formats. A 24-well culture plate format (second-generation test, approved by the US Food and Drug Administration (FDA)), and a newer, simplified In-Tube format (FDA approved). T-SPOT.TB is based on enzyme linked immunospot (ELISPOT) and uses peripheral blood mononuclear cells (PBMC) (Adetifa et al., 2007).

2.6.2.2.1 Quantiferon TB Gold In-Tube

The *in vitro* T-cell interferon gamma (IFN-ɣ) release assays including Quantiferon TB Gold In-Tube has been introduced and studied for the diagnosis of *M. tuberculosis* infections in developed and in few developing countries, however none has been performed in Namibia. Quantiferon TB Gold In-Tube test is an enzyme–linked immunosorbent assay (ELISA) that uses whole blood and more specific antigens of *M. tuberculosis* (Madariaga, Jalali & Swindles, 2007). It is an innovative blood test based

on the principle of detecting the release of INF-ɣ by *M. tuberculosis* specific circulating effector memory T-cells (Bocchino et al., 2010). It is a qualitative test that is reported as positive, negative or indeterminate after performing a quantiferon ELISA and analysing the result with the QFT software (Cellestis Limited. Quantiferon TB Gold (In−Tube Method) [Package Insert] and CDC, 2011).

According to Cellestis package insert, the overall sensitivity of Quantiferon TB Gold In-Tube for active TB from TB suspects from USA, Australia and Japan who were confirmed to have *M. tuberculosis* infection by culture was 89% [95% CI 83-93%]. Meanwhile the overall specificity of QFT-G was 99.1% [95% CI 98-100%] for persons with no reported risk for *M. tuberculosis* infection by TST.

In a reviewed study that looked at the role of IGRAs in diagnosing active TB; Quantiferon TB Gold indicated the sensitivity of the test ranging from 55% to 88%, with a mean of 75% (Pai & Menzies, 2007). In a systematic review and meta-analysis of interferon-gamma release assays for the diagnosis of extrapulmonary tuberculosis; a pooled sensitivity of 72% [95% CI 65-79%] for QFT GIT was recorded. The sensitivity in high income countries was 79%, [95% CI 72−86%], while in low income countries it was 29%, [95% CI 14-48%]. The pooled specificity was 82% [95% CI 78−87%]; QFT GIT conducted in high-income countries had a specificity of 83%, [95% CI 78−87%],

and in low/middle-income countries it was 71%, [95% CI 48–89%] (Fan, Chen, Hao, Hu & Xiao, 2012). In another review; the pooled sensitivity for the diagnosis of active TB was 80% [95% CI 75-84%] for quantiferon TB Gold In-Tube, meanwhile specificity was 79% [95% CI, 75-82%] (Sester et al., 2011). The two reviews showed sensitivities and specificities closer to each other.

2.6.2.2.2 T SPOT-TB

T SPOT-TB is another IGRA that can aid in diagnosing *M. tuberculosis* infection and it is commercially available in the U.S.A. Its use has also been studied worldwide. Its measurement is based on the number of IFN-ɣ producing cells (spots) (CDC, 2011), and it is reported as positive, negative, indeterminate or borderline.

In one study that compared Quantiferon-TB Gold (QFT-G) and T SPOT-TB for diagnosing *M. tuberculosis* infection; indicated that QFT-G was more convenient that T SPOT (Lee et al., 2006). It further noted QFT-G being superior in terms of specificity while T SPOT was superior in terms of sensitivity. However, the overall findings suggest that the ex vivo IFN-ɣ assays are superior in diagnosing *M. tuberculosis* infection compared with TST.

2.6.2.2.3 Tuberculin Skin Test (TST)

The tuberculin skin test has been in use for many years, it is a test that detects previous infection with *M. tuberculosis* (Davis, 2008). TST recalls the delayed-type hypersensitivity response to the intradermal inoculation of protein purified derivative (PPD). Tuberculin skin test uses PPD which contains a mixture of more than 200 antigens that are widely shared by mycobacteria other than *M. tuberculosis* (MOTT); it includes the vaccinal strain of *M. bovis* BCG and many NTM. A positive TST may suggest active TB, past infection, BCG vaccination, or sensitisation by environmental mycobacteria. A negative result in this test may not necessarily exclude tuberculosis (Tiwari et al., 2007). This resulted in individuals sensitised by previous exposure to NTM or BCG vaccine responding immunologically to PPD (Cerezales & Benitez, 2011). The TST's limitations are also accounted on sensitivity in certain groups of individuals who are immunosuppressed, immunocompromised patients and young children (Van Rooyen & Brink, 2007 and Horsburgh, 2004). It is unfortunate that the current study did not do any test on TST to compare it to the Quantiferon TB Gold In-Tube test, because TST is not used as a routine test in the Namibian health settings.

2.6.2.3 Advantages and disadvantages of quantiferon TB Gold In-Tube over TST

The use of IFN-ɣ assays have shown improved advantages over TST (Van Rooyen & Brink, 2007):

- Increased specificity as there are fewer false positives that would result from BCG vaccination of infection with NTM. IFN-ɣ utilises TB specific antigens that reduce cross-reactivity with other mycobacteria, which include BCG (Frahm et al., 2011). With this information, therefore only few false positive would be expected when using quantiferon as the Namibian population was vaccinated with BCG at birth.

- Improved sensitivity for detecting latent TB in children infected with HIV,

- Similar sensitivities to or better than the TST in active TB,

- Only requires one patient visit, no follow up needed. The return of the patient the next day has limited the effectiveness of TST (Frahm et al., 2011).

- Results are available within 24-48 hours.

- Reproducible and objective; eliminating subjective measurement of skin duration that could differ from one personnel to another, possible to do serial testing without the boosting phenomenon.

- Tests have a positive internal control assessing the patient's IFN-ɣ response to a mitogen, thereby eliminating false negative results due to T-cell anergy or immune suppression.

In addition; there are only few disadvantages of INF-ɣ assays:

- The assay's sensitivities decrease in severely immunocompromised patients. In this study, knowing the HIV status of the participants was necessary in order to compare the assay's response in HIV positive and negative participants.

A study on quantiferon by Ferrara et al. (2005) indicated that immunosuppression has been reported negatively affecting the test performance with a significant rate of indeterminate results in the most vulnerable population. So, very young or very old patients (younger than 3 or older than 80 years), those receiving immunosuppressive therapy, cancer patients, HIV infection or renal failure also reported indeterminate (Madariaga et al., 2007). The test sensitivity also raised concerns in children who are less than 2 years of age in a study conducted in the United States of America (USA) (Lighter et al., 2009). A study conducted by Latorre et al. (2010) in Spain on IFN-γ response on T-cell based assays in HIV infected patients for detection of tuberculosis infection concluded that IFN-γ tests have the benefit over TST which is influenced by BCG vaccination, but it cautioned that the assays are influenced with the level of immunosuppressant. It is for this reason, that this study found it necessary to consider the adult group only; who are 18 years of age and older and to exclude children. In addition, only individuals with symptoms of TB (TB suspects) were considered to avoid people that might be too immunocompromised and/or immunosuppressed.

- INF-ɣ TB assays cannot distinguish between active and latent TB; this could be a challenge especially in the Namibian setting where the measurements of other biomarkers using the Luminex assay technology is limited.

Luminex assay is based on xMAP Technology for quantification and detection of cytokines and signal transduction. It possess the high possibility to discriminate between latent and active TB infection and disease because it contains a combination of potential markers and it is achieved by using a Human Cytokine multiplexed bead array (Frahm et al., 2011).

Therefore, the use of IFN-γ assays could be a helpful method for diagnosing pulmonary tuberculosis infection in HIV infected population like Namibia. The IFN-γ assays were reported that they cannot discriminate active from latent TB, but in a preliminary report done in South Africa, it suggested that active TB may be accurately differentiated from latent TB infections utilising adaptations of the commercial quantiferon test that includes measurements of EGF, sCD4OL, MIP-1β, VEGF, TGF-α or IL-1α in supernatants from quantiferon assays (Chegou et al., 2009).

2.6.2.4 Molecular diagnostic methods

Molecular diagnostic methods have been developed for direct detection of *M. tuberculosis* from clinical specimens, species identification, and drug susceptibility testing of mycobacteria (Soini & Musser, 2001). They are nucleic acid amplification methods such as the enhanced *M. tuberculosis* Direct Test (Gen-Probe) and the amplicor *M. tuberculosis* test (Roche Diagnostic Systems). Xpert MTB/RIF is a latest method which WHO endorsed its use (Theron et al., 2011). It is an automated real-time PCR assay for rapid detection of *M. tuberculosis* and rifampin resistance (Hillemann, Russch-Gerdes, Boehme & Richter, 2011; Moure et al., 2011; Theron et al., 2011). PCR can be performed on a single specimen and yields a result within a day; the diagnostic process is shorter and more patient friendly, which may reduce dropout levels and contribute to reduced transmission (Kivihya-Ndugga et al., 2004) and requires minimal hands on (Boehme et al., 2010).

The sensitivity of GeneXpert was reported to be 98.2% with smear-positive and 72.5% with smear-negative tuberculosis. The test was specific at 99.2% in patients without tuberculosis and it identified 97.6% with rifampin-resistant bacteria and 98.1% with rifampin-sensitive bacteria (Boehme et al. 2010). The overall sensitivity of GeneXpert was reported early to be 89% for direct identification of *M. tuberculosis* from sputum and bronchial specimens and rose to 98% in smear-positive specimens (Marlowe et al. 2011).

2.6.3 Identity of *M. tuberculosis*

M. tuberculosis is an obligate intracellular pathogen which is aerobic, acid fast, non-motile, non-encapsulated, and non-spore forming bacillus. It grows successfully in tissues with high oxygen content such as the lungs (Lawn & Zumla, 2011). Its cell wall is lipid-rich, therefore relatively impermeable to basic dyes unless combined with phenol. *M. tuberculosis* is neither gram positive nor gram negative; therefore it is referred to as acid fast. Other NTM also contain mycolic acids; they are also acid fast and therefore cannot be distinguished from *M. tuberculosis* on microscopic sputum smear examination (Lawn & Zumla, 2011). *M. tuberculosis* has the ability to enter a dormant state under adverse metabolic conditions and delays its multiplication for years, enabling long-term survival in humans (Cardona & Ruiz-Manzano, 2004 cited in Siawaya, Beyers, van Helden and Walzi, 2009).

2.7 Tuberculosis cases definitions

An accurate diagnosis is very important in order to avoid subjecting individuals to unnecessary treatment. TB cases as defined by the WHO are used to classify patients as either TB or non-TB case (WHO, 2012).

Definite case of TB

A patient with *M. tuberculosis* identified from a clinical specimen by culture or by newer methods of molecular assays. Whenever culture is not available; a pulmonary case with one or more initial sputum specimens positive for acid-fast bacilli (AFB) is also considered.

Case of TB

A patient in whom TB has been confirmed by bacteriology or diagnosed by a clinician and a decision is made to be put on full course of anti-TB treatment.

Case of pulmonary TB

A patient with TB disease involving the lung parenchyma.

Smear-positive pulmonary case of TB

A patient with one or more initial sputum smear examinations (direct smear microscopy) are AFB positive; or one sputum examination is AFB positive and a radiographic abnormalities consistent with active pulmonary TB as determined by a clinician.

Smear-negative pulmonary case of TB

A patient with pulmonary TB who does not meet the above criteria for smear-positive disease is considered for this class. Diagnostic criteria should include: at least two AFB negative sputum smear examinations; radiographic abnormalities consistent with active pulmonary TB; no response to a course of broad-spectrum antibiotics (expect in a patient

for whom there is a laboratory confirmation or strong clinical evidence of HIV infection); and a decision by a clinician to treat with a full course of anti-TB chemotherapy. A patient with a positive culture but negative AFB sputum examination is also a smear-negative case of pulmonary TB.

Extra-pulmonary case

A patient with TB of organs other than the lungs (e.g pleura, lymph nodes, abdomen, genitourinary tract, joints and bones, meninges) is classified as extra-pulmonary case. Diagnosis in this class should be based on one culture-positive specimen or histological or a strong clinical evidence consistent with active extra-pulmonary disease, followed by a decision by a clinician to treat with full course of anti-TB chemotherapy. A patient in whom both pulmonary and extra-pulmonary TB has been diagnosed should be classified as a pulmonary case.

With the above definitions of cases, the question remains as to whether an individual who is diagnosed as a TB case is latently infected or actively infected, and whether the diagnosis is correct. For the purpose of this study, a diagnosis in relation to the above definitions, patients were only considered TB case or non-TB case without further categorisation.

2.8 Burden of TB on individual and national

Tuberculosis is known to be one of the first opportunistic infection striking Acquired Immune Deficiency Syndrome (AIDS) patients (Grimard & Harling, 2003). HIV has had a catastrophic impact on the tuberculosis control efforts, especially in sub-Saharan Africa; it has seriously complicated TB case detection and disease control (Perkins & Cunningham, 2007). Illness costs are incurred by caregivers and the sick person, and the cost fall on the household budget (Berman, Kendall & Bhattacharyya and Sauerborn, Ibrango, Nougatar, Borchert, Hien, Benzler, Koob & Diesfeld, as cited in Russell, 2004, p. 147). Illness costs includes direct costs such as expenditure linked with seeking treatment, including non-medical expenses for example transport or special food and indirect costs such as loss of household productive labor time for patients and caregivers (Russell, 2004). Indirect costs of TB were high because of the long duration of the disease and long delays before proper diagnosis. When a woman is sick or dies from TB, the household not only struggles from loss of earnings; in addition, there will be reduction on the activities that a woman routinely performs in the household (Ahlburg, 2000).

Individuals and members of the households infected and affected by TB disease do suffer psychologically due to discrimination because TB patients are rejected and

receive less social support during treatment or they may lose their jobs (Ahlburg, 2000).

The economically active age groups of those who are supposed to be studying, employable and prospective leaders of the countries are mostly affected by TB. As stated in the background of the study, many of those infected with new TB cases are between 15-45 years of age amongst both males and females (Namibia's Country Co-ordinated Proposal to The Global Fund to fight AIDS, Tuberculosis and Malaria, 2002).

2.9 Treatment (management) of pulmonary TB

Proper diagnosis of TB is necessary in order to prevent further spread or reoccurring of TB as well as to prevent resistance to drugs. The anti-TB drugs have three main desirable properties; it has bactericidal activity, sterilising activity and prevention of resistance (Donald & McIlleron, 2009). The anti-TB agent should eliminate the bulk of actively metabolising bacilli, sterilise TB lesion by killing dormant or intermittently metabolising organisms that are responsible for relapse and prevent the emergence of resistance to companion drugs. As stated in the background of the study, each individual diagnosed with the pulmonary TB disease is managed wisely. An effective recovery of TB patients can be reached by the standard therapy consisting of two (2) months of four drugs mainly RIF, NIH, PZA and EMT daily in the initial

phase followed by four (4) months of RIF NIH and EMT daily in the continuation phase (MoHSS, 2012); this makes a successful TB treatment to be completed in six months.

2.10 Monitoring of pulmonary TB treatment response

Sputum smear and culture results after 2 months of treatment are currently the only path for monitoring TB treatment response (Denkinger, Pai, Patel & Menzies, 2013). Ziehl-Neelsen (ZN) test for AFB in the sputum two (2) months after the start of therapy was recommended by the International Union Against Tuberculosis and Lung Disease (IUATLD) as a marker of early treatment response for patients undergoing directly observed treatment short-course of anti-TB chemotherapy (DOTS) (Enarson, Rieder, Arnadottir & Trebucq, 2000 cited in Siawaya, Beyers, van Helden & Walzi, 2009).

Studies conducted on IGRAs shed light on TB treatment monitoring, since IFN-ɣ responses are associated with mycobacterial antigenic load, which declines with treatment (Lalvani, 2004). A study by Siawaya et al. (2009) indicated that markers such as fractalkine, G-CSF, IFN-ɣ, IL-1ra, TGF-α and VEGF were detectable in the plasma of healthy latent TB infected individuals and active TB patients. However, there was no significant difference at any time during treatment. This could be due to the localization of the disease to the lung and compartmentalized expression of some cytokines, and as

well as the nature and function of some cytokines could have prevented their involvement in the immunity of TB.

In a study that assessed plasma levels of IFN-ɣ inducible protein 10 (IP-10/CXCL10) and/or PTX3 for monitoring ongoing inflammation and disease activity in *M. tuberculosis* infected individuals; associated active TB with increased levels in the plasma (Azzurri et al., 2009). It further elucidate that cured patients had significant reduction in other cytokines such as CXCL10 levels at the end of treatment. Consequently non-cured patients had their levels progressively increased at the end of treatment. Meanwhile in another study that determined serum concentrations of some cytokine levels including IFN-ɣ in patients with active and inactive pulmonary TB; serum levels of IL-10, IL-12p40, TNF-α and IFN-ɣ were found raised in patients with active pulmonary TB (Deveci, Akbulut, Turgut and Muz, 2005). Similarly, median plasma levels of IL-4 and IP-10 were reported significantly decreased while the level of IFN-ɣ, MCP-3 and MIP-1β increased significantly after treatment in a study by Mihret et al. (2012).

Kabeer et al. (2011) demonstrated a decrease of IP-10 secreted response to selected RD1 peptides during specific treatment in patients with active TB between baseline and end of treatment of TB, accompanied by a significant decrease in the positive rates of the test. A significant decline in the qualitative and quantitative results of both IGRAs with

treatment was reported on a study that evaluated the effect of TB treatment on T-cell responses as measured by the T-SPOT.TB and Quantiferon TB Gold In-Tube in pulmonary TB patients (Chee et al., 2010).

Pai et al. (2007) indicated that effects of TB treatment on IFN-ɣ responses, have shown inconsistent results; declining, unchanging or fluctuating over treatment. However, an assessment as to whether QFT reversion or a decline in quantitative IFN-ɣ levels after 2 months of treatment was associated with smear or culture conversion to negativity observed large variability of sequential IFN-ɣ measurements within individual persons independent of whether the smear or culture results for the patients converted (Denkinger, Pai, Patel & Menzies, 2013). With much variation in literature on monitoring pulmonary TB treatment response regarding IFN-ɣ levels using Quantiferon TB Gold In-Tube test, it was necessary to find out how it performs in our setting.

2.11 IFN-ɣ response in HIV-infected patients

As stated early that reactivation of latent TB infection and the likely of progression to active TB are attributed to immunosuppression especially in individuals infected with HIV (Corbett, Charalambous, Moloi, Fielding, Grant, Dye, De Cock, Hayes, Williams & Churchyard, 2004 cited in Latorre et al., 2010). In people with compromised immune

system; for example it is the case in HIV patients, TB disease develops easily after primary infection (Kaufman, 2002).

Low CD4+ T-cell levels are concerned impairing the sensitivity of IGRAs in Quantiferon TB Gold In-Tube assay (Kaplan et al., 2009). A metanalyses reported that HIV associated immunosuppression, measured as CD4+ T-cell count, negatively affects the performance of Quantiferon TB Gold In-Tube (Santin, Munoz, Rigau, 2012 cited in Cheallaigh et al., 2013). In low TB prevalence setting, HIV positive individuals with advanced immunosuppression measured as low CD4+ T-cell counts are more likely to have negative QFT-IT results (Cheallaigh et al., 2013). It was of interest to evaluate QFT's performance in the Namibian setting which is hit by both TB and HIV.

2.12 Conclusion

The IGRAs have been done in many countries; variations and similarities in findings occurred, therefore given the main findings in literature it was necessary to conduct the study in Namibia. A study of this nature was never done in Namibia, its finding will add to the body of knowledge and data will be available for our setting.

The next chapter focuses on the research methodology; where the details regarding to the procedures that guided the study are elaborated.

CHAPTER 3: RESEARCH METHODOLOGY

3.1 Introduction

This chapter presents the research design, population and sampling strategy, data collection and data analysis methods, limitations of the study and the research ethics. The main aim of this chapter is to address how the research was conducted.

3.2 Research design

The study was guided by a quantitative and qualitative, longitudinal, contextual and experimental design. It provided details for the research to be replicated and there were assumptions that needed to be proven. Creswell (2009, p. 12) indicated that, experimental research seeks to determine if a specific treatment influences an outcome.

Whole blood was collected from study participants directly into a set of three tubes provided by the manufacturer (Qiagen, Germany). One of the blood collection tubes had been pre-coated with a "cocktail" of *M. tuberculosis* specific antigens (ESAT-6, CFP-10 and TB7.7), the other with phytohaemagglutinin (the mitogen tube/ positive control), and the other with normal saline (the nil tube/ negative control). The study compared the

conventional MGIT culture and direct smear microcopy tests to the Quantiferon TB Gold In-Tube test as diagnostic tools for pulmonary TB.

The diagram below gives an outline of the steps and experimental design that were followed towards the assessment of Quantiferon TB Gold In-Tube test to diagnose pulmonary TB in Namibian patients.
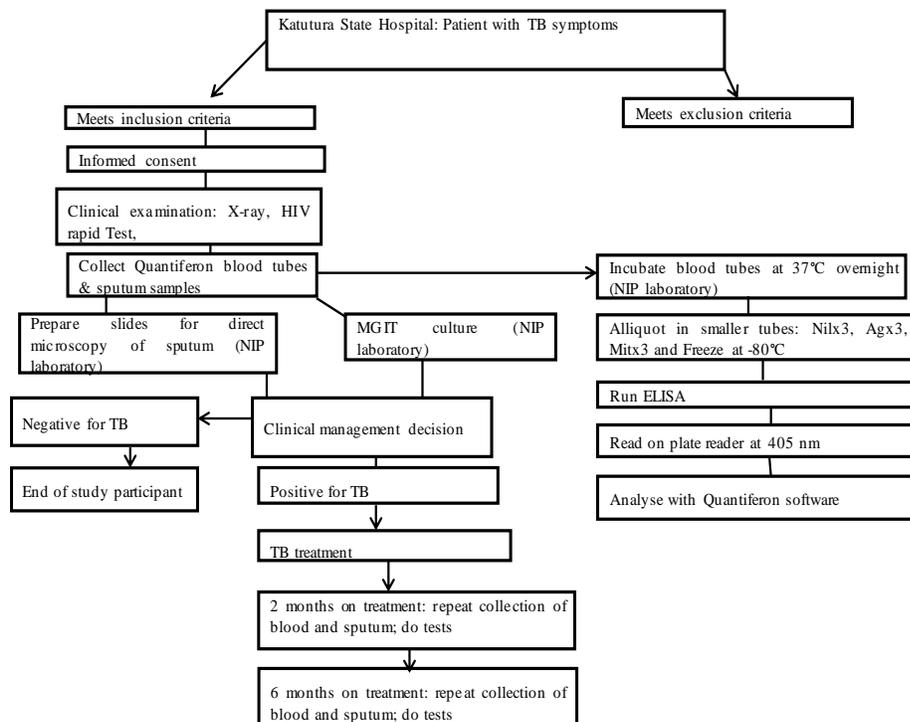
Figure 3: Flow chart showing steps used towards the assessment of the Quantiferon TB Gold In-Tube to diagnose pulmonary TB

3.3 Population

The targeted population of the study was adults with pulmonary TB symptoms (TB suspects) who were 18 years of age and older, including HIV non-infected and infected individuals that were living permanently in Windhoek and visited Katutura State hospital between June 2011 to June 2012.

Katutura State Hospital was chosen because it is easily accessible; it is near by the laboratory where the specimens were to be processed and could be transported within 2 hours of collection.

3.4 Sample

The study used the purposive sampling technique. Only patients who were suspected of having TB were relevant to the study. A total number of 100 patients who were suspected of having TB disease on the basis of symptoms and met all the inclusion criteria were considered for the study. The study was part of a large consortium; the African European Tuberculosis Consortium (AE-TBC) that involved six other African institutions and five European partners. At the beginning of the large project, sample size calculations were done and it was estimated that a total of 100 patients would be sufficient to achieve the project's objectives in Namibia. This work was done as a sub-

study of this AE-TBC main study where harmonised protocols were used in collection and processing of specimens across the consortium partners.

The following inclusion and exclusion criteria were used: Only participants with symptoms suggestive of pulmonary TB, i.e. persistent cough (generally ≥ 3 weeks) and at least one of other symptoms such as: fever, malaise, recent weight loss, night sweats, contact with active case, hemoptysis, chest pain and loss of appetite were eligible. Only individuals that were 18 years or older and willing to give written informed consent, willing to have their blood samples tested for HIV and willing to have their HIV infection status confidentially disclosed to the study field workers were eligible.

Participants were excluded if they were on anti-TB treatment, or had received anti-TB treatment in the past 90 days and must have not received quinolone or aminoglicozide antibiotics in the past 60 days. Moreover, patients with a history of previous TB but who had not received anti-TB therapy in the previous 90 days were included.

Hemoglobin (Hb) was checked if it was more than 10g/l for the patients to be eligible. In addition, women of fertile age who were pregnant had to wear a lead apron during chest radiography examination.

3.5 Research nurse

A research nurse who was well conversant with a number of languages such as English, Afrikaans, Oshiwambo, and Herero administered the consent form and collected the specimens. A provision for an interpreter was made where it was necessary. The nurse did consent (Appendix D) the participants and they agreed on the suitable day and time to draw blood and provide sputum specimen. There were those who were ready to provide the specimens on the day of consent. The research nurse was full time on the research project because she was collecting samples for the AE-TBC project.

3.6 Materials and methods

3.6.1 Specimen collection

Blood was drawn by venipuncture to a volume of 1 ml directly into Quantiferon® tubes; the tubes consisted of the Nil, TB Antigen and Mitogen; the vacutainer tubes are made to resist blood flowing into them once they have reached a volume of 1 ml. The tubes were shaken 10 times to ensure that the blood properly mixed with entire content of the tube. They were properly labeled with a number which was linked to the form leading to a patient identification prior to blood drawing. They were transported to the laboratory at room temperature within 2 hours of collection for processing (16 hours recommended by manufacturer).

After collection of blood samples from all study participants, sputum samples were collected into a screw capped container that was pre-labeled with a patient identification number. All samples were then transported to the laboratory for processing.

3.6.2 Plasma processing: Quantiferon TB Gold In-Tube assay

The processing of quantiferon tubes was done according to the manufacturer's package insert (Cellestis Limited. Quantiferon TB Gold (In–Tube Method) [Package Insert]). The Quantiferon TB Gold (In-Tube Method) includes a set of three blood collection tubes namely: Nil control (Grey cap), TB Antigen (Red cap) and Mitogen control (purple cap).

The Quantiferon TB Gold ELISA kit consisted of the following reagents and components: ELISA plate strips, freeze dried standard, freeze dried conjugate 100x concentrate, green diluent, wash buffer 20x concentrate, enzyme substrate and enzyme stopping solution.

3.6.2.1 Incubation and centrifugation of the quantiferon blood tubes

The quantiferon blood tubes were mixed by shaking ten times to ensure entire inner surface of the tube is coated with blood to solubilise the antigens on the walls of the tubes. The tubes were incubated upright at 37°C for 21 hours (overnight) and

centrifuged for 15 minutes at 3000 RCF (g); re-centrifugation was considered at higher speed if there was no proper separation into a supernatant.

3.6.2.2 Harvesting of plasma from quantiferon tubes

The plasma was aliquoted into small micro-centrifuge tubes for storage at -80°C before ELISA analysis. Three aliquots were made for each tube (3x Nil, 3x Mitogen and 3x TB Antigens) in volumes of 80-120µl depending on the volume resulted after centrifuging, but only one aliquot was used for the analysis.

3.6.2.3 IFN-γ ELISA procedure

The quantiferon plasma supernatants and reagents were brought to room temperature (25°C), at least for 60 minutes before use for equilibration, except for the conjugate 100x concentrate. The quantiferon standards were assayed in triplicates. Freeze dried kit standard was reconstituted with the volume of deionised or distilled water as indicated on the label of the vial to produce a solution of 8.0 IU/ml and it was mixed gently to minimise frothing and ensuring solubilisation.

Four tubes were labeled for the standards (S): S1, S2, S3 and S4 to produce a 1 in 4 dilution series in a green diluent. An amount of 150µl green diluent was added to each

tube of the labeled tubes and 150µl of reconstituted kit standard [8.0 IU/ml] was added

to the tube labeled S1. Fifty (50) µl of S1 [4IU/ml] was transferred to S2 and a 50µl of

S2 [1IU/ml] was transferred to S3 making it [0.25IU/ml], mixing thoroughly was

considered before each transfer. S4 served as a zero standard and contained only a green

diluent.

 The conjugate 100x concentrate was reconstituted with 300µl of distilled water and a

working strength which is 1x conjugate was prepared using reconstituted conjugate 100x

concentrate with the green diluent. The 1x working strength wash buffer was prepared

by adding 50ml of the wash buffer 20x concentrate to 950ml of distilled water.

An amount of 50µl of 1x conjugate was added to each of the 96-well plate and 50µl of

plasma samples which were well vortexed to ensure that IFN-γ is evenly distributed and

standards were added to the wells appropriately. Figure 4 displays the layout of the

samples and standards in a 96-well ELISA plate. The conjugate with the plasma

supernatant samples and the standards were mixed thoroughly for 1 minute using a

micro plate shaker. The 96-well plate was covered and incubated at room temperature

for 2 hours out of direct sunlight and away from an environment with deviating

temperature.

Using the multi-channel pipettes, the wells were washed manually with 400μl of 1x wash buffer for at least 10 cycles. An enzyme substrate solution of 100μl was added to each well and tapping the sides carefully. The plate was further incubated at room temperature and after 30 minutes; 50μl of enzyme stopping solution was added to each well in the same order and speed as the substrate. The optical density of each well was measured within 5 minutes of stopping the reaction on a plate reader using a 450nm filter and with reference filter between 620nm and 650nm. The raw data were analysed using the QUANTIFERON® TB Gold analysis software version 2.50.2. in order to get the qualitative results.

Row	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

Figure 4: Standards (S) and samples layout for Nil (N), TB antigen (A) and Mitogen (M) supernatants for 28 patients in a 96-well plate (adapted from Cellestis, Package insert)

3.6.3 Sputum processing

All sputum processing work were carried out in the microbiology laboratory level 3 in the aseptically clean biological safety cabinet (BSC). The sputum sample was decontaminated using the N-acetly-L-cysteine sodium hydroxide (NALC-NaOH) method by Kent and Kubica, 1985 as described in Peres et al. (2009); Sharma et al. (2012) in order to prepare specimen for MGIT culture. The decontamination removes unwanted bacteria from the specimens. To a MGIT tube, 0.8mL of MGIT supplement has been added in advance and 0.5mL of the supernatant mixture was inoculated to the MGIT tube. The mixture of the sputum sediment was inserted in the MGIT tubes and incubated into the MGIT machine (Bactec MGIT 960 instrument). The MGIT tubes were monitored on an hourly basis for up to 42 days for growth of the mycobacteria; tubes without growth signaled negative.

On the MGIT culture flagging positives; Ziehl-Neelsen (ZN) was performed to confirm whether the AFB was present on the samples and it was further identified using the BD TBc (MPT64 antigen) rapid identification test to differentiate between *Mycobacterium tuberculosis* complex (MTBC) and MOTT (Said, Ismail, Osman, Velsman and Hoosen, 2011). The positive cultures were also visually inspected for turbidity and inoculated on a blood agar plate to rule out contaminations. A final positive MGIT culture result was given after a microscopic ZN stained smear from culture confirms presence of AFB and an identification of the *M. tuberculosis* isolate.

3.6.3.1 NALC-NaOH decontamination method

A volume of 5mL NALC-NaOH solution in a ratio of 0.25g0:50mL was added to 5mL of sputum sample into a 50mL sterile plastic capped centrifuge tube. It was vortexed for < 20 seconds at a moderate speed and was let to stand for 15 minutes at room temperature (25°C). 20mL of Phosphate buffer saline (PBS) at 0.067M and pH 6.8 was added to reduce the continued action of NaOH and lower viscosity of the mixture. The tube was tightly recapped and inverted several times to mix the contents. The specimen was centrifuged at 4°C and 3000 x g for 15 minutes to concentrate the mixtures. The sediment obtained after centrifuging was re-suspended in 2mL of phosphate buffer (0.067M, pH 6.8) and vortexed for 20 seconds to re-suspend the sediment. The sediment was allowed to sit for 5 minutes and a smear for AFB was prepared and the MGIT tube was inoculated.

3.6.3.2 Preparing smears from positive cultures using Ziehl-Neelsen staining

Microscopic slides were prepared in advance in the BSC and dried for at least two hours by dipping a cotton swab in albumin and making one Namibian dollar size coin on the slide. A drop of culture from the bottom of the tube was put on the albumin portion using a sterile pipette and it was spread over the entire surface of albumin. The smear was let to dry completely and heat fixed on a hot plate at 65-75°C. Carbol fuchsin was applied to stain the slides smeared with cultures from positive MGIT culture tubes. The slides were heated with a Bunsen flame until steam rises from the stain. The time was set

for 5-7 minutes and the stain was not let to boil and dry on the slides. Excess stain was drained off and washed away from the slides with a stream of tap water. 3% of acid alcohol was applied for 2-3 minutes to decolorise the smeared slides, which were washed with deionised water and drained off. The slides were covered with 0.1% malachite green stain for 5 minutes to counter stain, which was drained and washed off with tap water. They were air dried and examined for AFB on the microscope.

3.6.3.3 Ziehl-Neelsen (ZN) staining for direct microscopy

The slide smeared with unconcentrated sputum was heat fixed and placed on the staining rack where it was flooded with filtered carbol fuchsin. It was further steamed with a Bunsen flame and left to stand for 5 minutes. The slide was rinsed with clean tap water and drained, it was flooded with acid-alcohol and let stand for 2 minutes. It was rinsed again and flooded with methylene blue which stood for 2 minutes. The slide was rinsed and allowed to air dry and it was finally examined for AFB using the light microscope and a score was given as either positive or negative accordingly.

3.6.3.4 TBc identification test

The MGIT culture broth was mixed by inverting several times using a sterile pipette and tip to remove 100μL of the broth culture and dispense in the sample well of the TBc identification device. After 15 minutes of waiting for the reaction to occur; the MTBC

was identified by the presence of a pink-red line at the test and control position while the MOTT was identified by no pink-red line at the test position and a line at the control position.

3.7 Data analysis

The findings of this thesis are presented using tables, charts and graphs. The sensitivity, specificity and predictive values were calculated as diagnostic accuracy measures of quantiferon test as compared to the gold standard methods: MGIT culture and direct microscopy. The indeterminate results of the quantiferon test were not included in the calculations of sensitivity and specificity. The decontaminated, lost viability and MOTT of MGIT culture test results were also not included in the calculation of the diagnostic accuracy tests. The online MedCalc statistical software was used for these calculations as: Sensitivity = a / (a + b), Specificity = d / (c + d), Positive predictive value (PPV) = a / (a + c) and Negative predictive value (NPV) = d / (b + d)

Where: a = true positive

b = false negative

c = false positive

d = true negative

The chi-square (X^2) test was used to measure the agreements between different diagnostic methods in which the main compared tests were quantiferon and MGIT culture. Furthermore, it was used to find out if the quantiferon test results were influenced by the HIV status of the participants.

Additionally, to evaluate if the quantiferon test could be used as a tool to monitor TB treatment response, IFN-γ levels detected in QFT supernatants at recruitment (the time before initiation of TB treatment) and during follow-up time point at 2 months on treatment and on completion of the treatment (at month 6) were evaluated by repeated measures analysis of variance (ANOVA). This is to evaluate whether and how IFN-γ levels would change with mycobacterial antigenic load as affected by anti-TB treatment.

Differences were considered significant if the *p*-value was ≤ 0.05 for both statistical tests. The data were analysed using statistical software packages, namely: Statistica 9, statsoft, Ohio, USA and Statistical Package for Social Sciences (SPSS) version 21 for ANOVA and chi-square respectively.

3.8 Limitation of the study

Due to limited financial resources, the study was limited to patients visiting one hospital in Namibia which was Katutura State Hospital and the sample size was estimated based on the affordability of laboratory consumables. It also considered that the patients who were to be classified as TB cases were to be repeated two times making it three runs per TB case. This was not a true representative of the population as some people go to the health centers and clinics other than Katutura State Hospital, and the study was only carried out in one region.

In order to achieve the objectives; the study depended on the willingness of the participants to take part in the study. It was also depended on the confidence of participants to discuss and disclose their health related issues especially HIV status with the research nurse and research technologist, therefore this might have affected the willingness of individuals to participate in the study. It further depended on the participants who were on TB treatment coming back for follow-up. In this study; the 39 TB cases that were diagnosed with pulmonary TB disease and classified as TB cases; and only 19 of them completed the study.

The time between the collection of blood samples and analysis of specimen was critical and should be adhered to, otherwise the results could not reflect true infection and disease nature of TB.

The study did not collect specimens for CD4 cell count, which could have been of a great help in interpreting the relationship between the quantiferon results and HIV status of participants. It did not also perform the TST which has been a screening test for latent TB infection and it could have been the best test to compare with quantiferon. TST is known in inducing antigen specific T-cell responses by PPD (Pai et al., 2007), omitting it made it necessary not to disturb the T-cell response which might affect performance of the QFT.

3.9 Research ethics

The participant's written informed consent was obtained after proper discussion and explanation of the purpose of the study. The participant's information was not let into the hands of unauthorised people as this is a definite risk that could not be ignored. No name of any participant or information such as physical address that could lead to them being identified was revealed on the sample tubes or in the presentation of results in this study. Only numbers allocated to them during sample collection and were linked to the form leading to a patient identification were used for data analysis and presentation.

As it was mentioned early in this chapter; the study was part of the African European Tuberculosis Consortium (AE-TBC) project which was funded by the European and Development countries Clinical Trials Partnership (EDCTP). It obtained the permission from the Ministry of Health and Social Services in Namibia on research policy and ethics (Appendix C). Permission was also granted by the project's principal investigator at the University of Namibia for the study to be carried out for the master's degree (Appendix B).

3.10 Conclusion

The methodology and the design that guided this research were dealt with in this chapter in order to reach the objectives of the study. The inclusion and exclusion criteria used to recruit participants were stated. Furthermore, the data analysis and research ethics were presented.

CHAPTER 4: RESULTS

4.1 Introduction

In this chapter, the research findings are presented; it analyses the findings in order to distill the meanings out of them and at the same time trying to address the research objectives.

The specific objectives of the study were to:

i.   Validate the usefulness of the Quantiferon TB Gold In-Tube test in the diagnosis of pulmonary TB in Namibian patients.

ii.   Evaluate the influence of HIV on Quantiferon TB Gold In-Tube results.

iii. Evaluate the levels of IFN-γ in quantiferon supernatants of TB patients undergoing treatment as a measure of response to TB chemotherapy.

4.2 Participant selection and realisation of the study

A total of hundred (100) patients with the suggestive symptoms of pulmonary TB "tuberculosis suspects", and who met the inclusion criteria and agreed to give written informed consent formed part of the study. These participants included both HIV

positive and negative, their age ranged from 18 to 61 years. The mean age was 36.15 and the standard deviation was 9.37. Table 1 and Table 2 have the details of the demographic and clinical information collected on the participants. Of the 100 participants, 54 were males and 46 females; Figure 5 shows the age distribution of the participants and the gender based on the HIV status. Amongst the HIV positive group; 52% were males and 48% were females. The HIV negative participants were 39, whilst 61 samples were from the HIV positive participants.

Table 1: Clinical and demographic characteristics of study participants

	All
Number of participants	100
Age, mean years ± SD	36.15 ± 9.37
Male/Female ratio	54/46
HIV positive	61%
QFT positive	51%
QFT negative	44%
QFT Indeterminate	5%
MGIT culture positive	22%
MGIT culture negative	67%
MGIT contaminated	5%
MGIT lost viability	3%
MGIT MOTT	3%
TB case	39%
BMI, mean ± SD	21.06 ± 4.70
Hb, mean ± SD	12.54 ± 1.58

Table 2: HIV status of participants

		HIV status of participants		Total
		Positive	Negative	
Gender of	Male	32%	22%	54%
participants	Female	29%	17%	46%
Total		61%	39%	100%

The majority of participants were males compared to females and the study was formed up by many patients who were HIV positive.
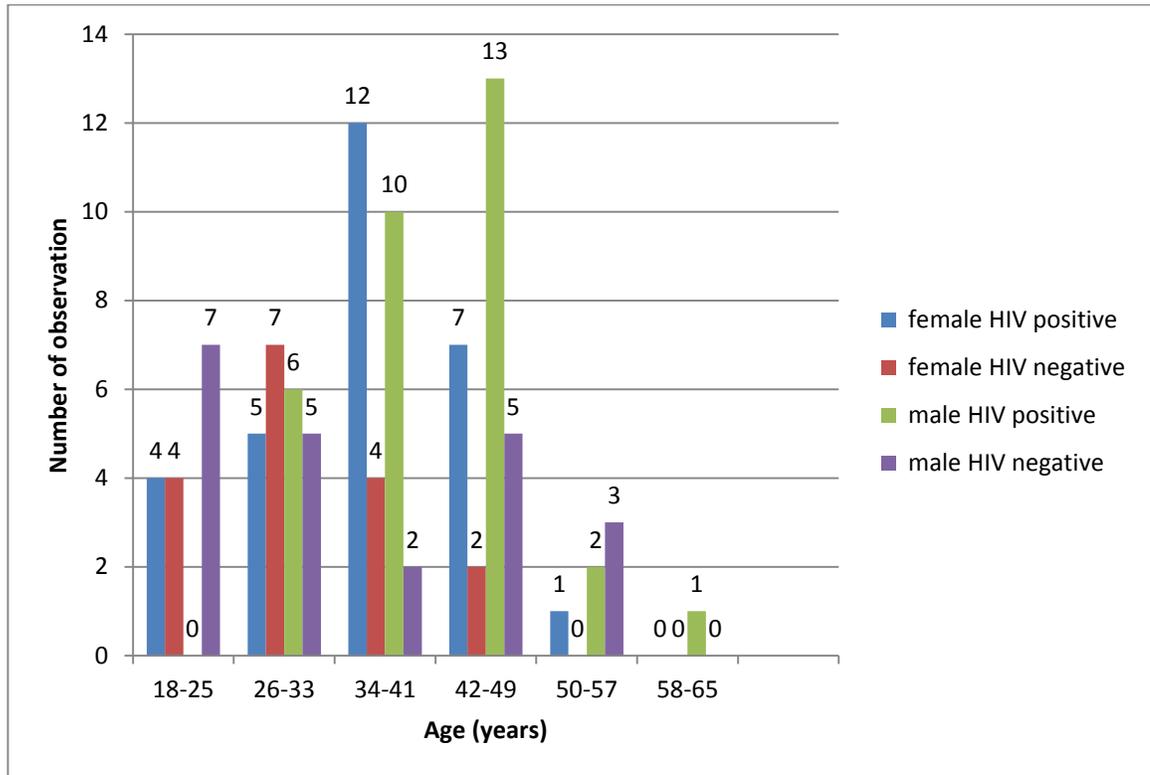
Figure 5: Participants age distribution categorised by gender and HIV status

Classification of TB or non-TB cases was based on a combination of clinical evaluation, chest radiograph and with evidence from the laboratory; however it also used the participant's recent routine samples from the hospital that were done on them. Amongst the participants; (39/100) 39% were classified as TB cases and were eligible for TB treatment and follow-up, meanwhile (61/100) 61% were non-TB cases (Figure 6).
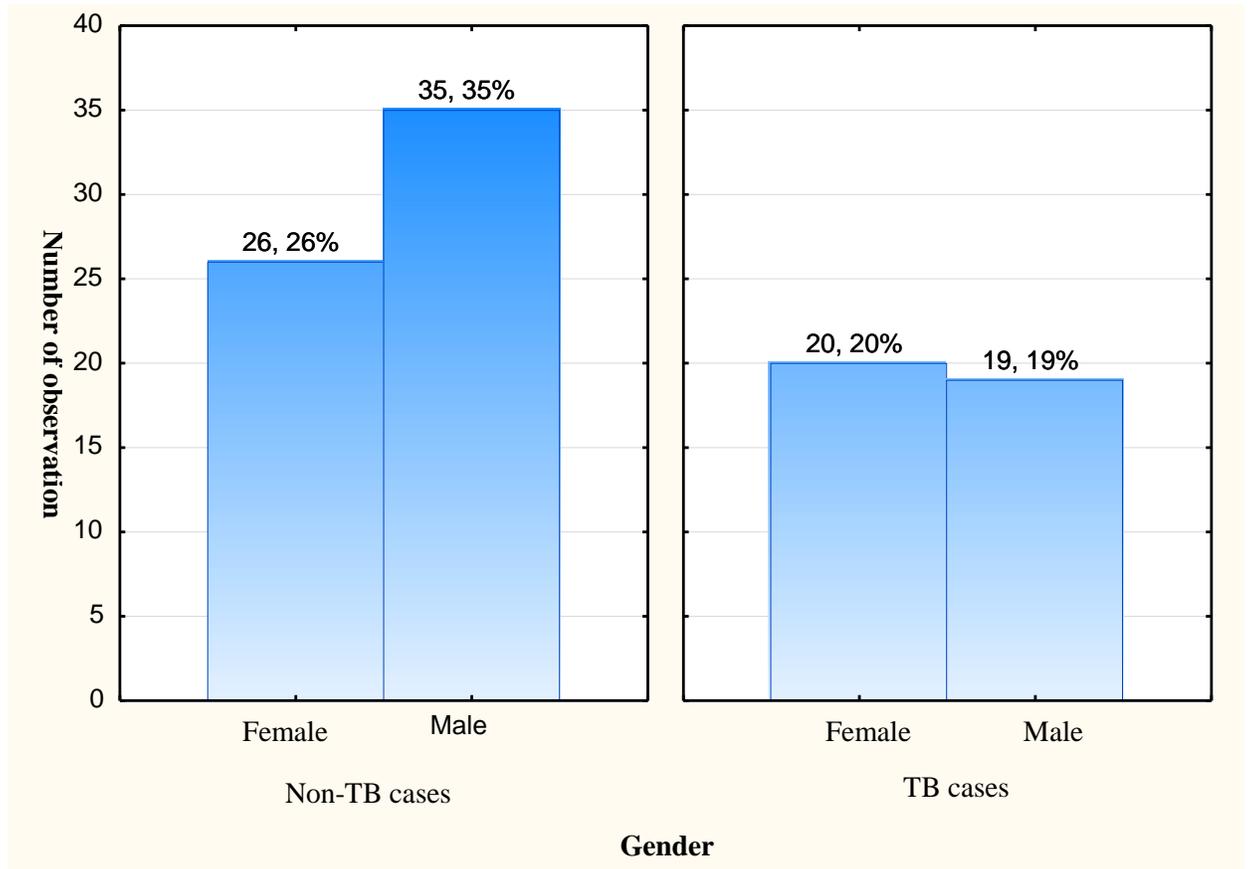
Figure 6: Gender of participants categorised by TB classification

4.3 Usefulness of QFT TB Gold In-Tube test in diagnosing TB

It was hypothesised that QFT is a better test for the diagnosis of pulmonary TB caused by *M. tuberculosis* than MGIT culture test.

4.3.1 Quantiferon TB Gold In-Tube compared with MGIT culture

Overall, out of the 100 TB suspects; (22) 22% were diagnosed as TB positive by MGIT culture, 67% negative for TB, and 3% mycobacterium other than *M. tuberculosis* (MOTT). The others, 5% and 3% were not identified because they were contaminated and lost viability respectively; therefore they were to be repeated (Table 3).

Table 3: MGIT culture test and Quantiferon TB Gold In-Tube test

	MGIT culture test					
Quantiferon test	Positive	Negative	Contaminated	Lost viability	MOTT	**Total**
Positive	19%	27%	2%	1%	2%	51%
Negative	2%	37%	2%	2%	1%	44%
Indeterminate	1%	3%	1%	0%	0%	5%
Total	22%	67%	5%	3%	3%	**100%**

The quantiferon ELISA test displayed raw optical density (OD) (Appendix E) that depicted IFN-ɣ values which is measured in international units per milliliters (IU/ mL) and were interpreted as either quantiferon positive, negative or indeterminate by the quantiferon software (Appendix F). The evaluated test; Quantiferon TB Gold In-Tube screened 51% positive, 44% negative and 5% indeterminate. The figures are given in

Table 3. In this study, a total of 22 participants with MGIT culture confirmed TB positive; the Quantiferon TB Gold In-Tube test detected (19/22) 86% of the cases (true positive), and classified (2/22) 9% as negative (false negative) and (1/22) 5% indeterminate. A total of 67 participants with MGIT culture TB negative; the Quantiferon TB Gold In-Tube test found (37/67) 55% of the cases negative (true negative) and (27/67) 40% were positive (false positive) and (3/67) 4% indeterminate. All the samples that gave indeterminate are from HIV infected participants.

One of the aims of the study was to assess the QFT test compared with the 'gold standard' MGIT culture to detect pulmonary TB disease and infections caused by *M. tuberculosis*. Evaluation of QFT compared to MGIT culture showed a sensitivity of 90.5%, [95% confidence interval (CI) 69.6 – 98.55%) and a specificity of 57.8%, [95% CI 44.8 – 70.1%], and a positive predictive value (PPV) of 41% and a negative predictive value (NPV) of 95%. However, 15 participants were not included in the calculation of the sensitivity and specificity due to the indeterminate of the quantiferon results as well as the contaminated, lost viability and MOTT given on the MGIT culture results.

In order to find if there is an agreement between the 'gold standard' MGIT culture test and Quantiferon TB Gold In-Tube test; the chi-square test found a statistically

significant difference with a $X^2 = 18.4$, $p < 0.05$ (0.02). There was no agreement between MGIT culture and the QFT test.

It was also of an interest to find out if there were agreements between others variables measured in the study such as direct microscopy of the sputum smear and TB classification by the clinician, each compared with QFT and the findings will follow next.

4.3.2 Quantiferon compared with direct microscopy

The sensitivity and specificity of QFT when compared to direct microscopy smear as a gold standard test to diagnose pulmonary TB disease and infections is 75% [95% CI 50.89 – 91.25%) and 52% [95% CI 41.15– 63.69%) respectively.

Table 4: Quantiferon and direct microscopy results

	Direct microscopy results		
Quantiferon results	Positive	Negative	**Total**
Positive	15%	36%	51%
Negative	5%	39%	44%
Indeterminate	2%	3%	5%
Total	22%	78%	**100%**

A statistically significant difference between direct microscopy of the sputum smear and the quantiferon test results was not observed, a chi-square of 5.5 and $p > 0.05$ (0.07), the data used are taken from Table 4.

4.3.3 Quantiferon compared with TB classification

The sensitivity and specificity of QFT test calculated in comparison with clinical TB classification as the gold standard test were 65.7%, [95% CI 47.7-80.3%] and 53.3%, [95% CI 40-66.1%] respectively.

Table 5: Quantiferon results compared with TB classification

	TB Classification		
Quantiferon test	**TB cases**	**Non-TB cases**	**Total**
Positive	23%	28%	51%
Negative	12%	32%	44%
Indeterminate	4%	1%	5%
Total	39%	61%	**100%**

Quantiferon test analysed against TB classification by the clinicians; a statistically significant difference was observed, $X^2 = 6.9$ and $p < 0.05$ (0.03) (Table 5). There was no agreement between them.

4.3.3.1 TB classification and MGIT culture

The results given by MGIT culture as compared to diagnosing a participant as having TB by the clinician showed a statistically significant difference, a chi-square value = 36.0 and $p < 0.001$; the data used are displayed in Table 6. Diagnosis of participants by the clinicians as a TB case or non-TB case was not in agreement with MGIT culture outcome.

Table 6: MGIT culture results compared to TB classification

MGIT results	TB Classification		
	TB case	Non-TB case	Total
Positive	18%	4%	22%
Negative	13%	54%	67%
Contaminated	4%	1%	5%
Lost viability	3%	0%	3%
MOTT	1%	2%	3%
Total	39%	61%	**100%**

4.4 Quantiferon test and HIV status

The other objective of the study was to find out how quantiferon test performed in participants with different HIV status (being either HIV positive or negative), because it was hypothesised that QFT test is influenced by HIV status of the patients. The chi-square (X^2) test value of 12.2, and $p < 0.001$ observed a statistical significant difference as to how the quantiferon test categorised an individual based on the HIV status (Table 7). The quantiferon test was not influenced by the HIV status of an individual.

Table 7: Quantiferon test evaluated on HIV status

HIV status	Quantiferon results			Total
	Positive	Negative	Indeterminate	
Positive	23%	33%	5%	**61%**
Negative	28%	11%	0%	**39%**
Total	**51%**	**44%**	**5%**	**100%**

Considering 61 participants with HIV positive status; amongst which 23 (38%) gave positive QFT results, the majority (33/61) 54% had negative QFT results and (5/61) 8% were indeterminate. On the other hand, the 39 participants with HIV negative status; 28

of them (72%) were QFT positive and only (11/39) 28% were QFT negative. Therefore, QFT responses are not influenced by HIV positive status.

4.4.1 QFT test on followed up participants

Eleven out of the nineteen (11/19) 58% and 8/19 42% of the participants followed up were HIV positive and negative respectively. The 11 HIV positive of those followed up were 4 positive, 5 negative and 2 indeterminate on the QFT test at recruitment (Appendix G).

Nineteen (19) of patients that were followed-up; 9 patients had QFT positive results at recruitment and 4/9 (44.4%) remained positive meanwhile 5/9 (55.6%) became QFT negative at completion of anti-TB treatment. 1/8 of those who were QFT negative at recruitment became QFT positive at end of treatment. Overall; at recruitment, at 2 months on treatment and after completion of treatment: 9, 9 and 5 participants had QFT positive results respectively and 8, 9 and 13 participants had QFT negative results respectively (Table 8). At month 2; one participant did not attend clinic for follow-up (N/A), but attended at month 6, while one who attended clinic at month 2 did not attend at month 6. For each of the follow-up time points (month 2 and month 6) there was one participant who failed to attend clinic.

Table 8: Quantiferon results of the followed up participants on anti-TB treatment

	T0 (recruitment)	T2 (2 months on treatment)	T6 (completion of treatment at end of month 6)
QFT positive	9	9	5
QFT negative	8	9	13
QFT indeterminate	2	0	0
Total	**19**	**18 + N/A**	**18 + N/A**

4.4.2 TB classification by the clinician and HIV status

The overall classification of TB case or non TB case compared with HIV status shows no statistical significance difference with $p > 0.05$ ($p = 0.55$) on Fisher's Exact test. Classifications of TB cases were influenced by HIV status; Figure 7 presents a chart with the classification of TB by the clinicians related to HIV status of the participants.
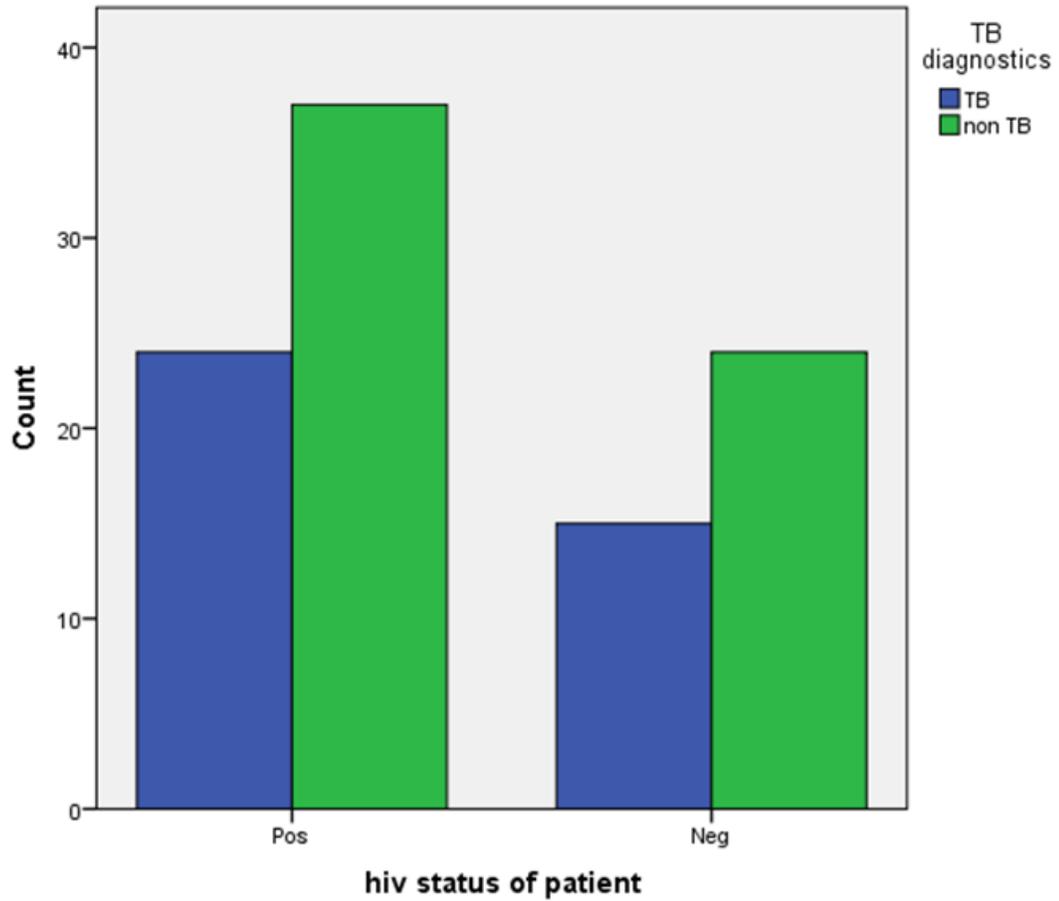
Figure 7: TB classification compared to HIV status

4.5 IFN-ɣ in QFT supernatants at different time points to monitor treatment

The longitudinal study expected 39 TB cases that were classified by the clinician to be on treatment and followed up. Only 19/39 (48.7%) were followed-up at two month while on treatment and at six month after initiation of TB treatment.

The last objective was to find if there was a change in the IFN-ɣ levels at different time points, it was hypothesised that IFN-ɣ levels will change in TB patients during treatment and at the end of treatment as compared to pre-treatment, implying that QFT test can be used to measure if the patients are responding to TB treatment. Figures 8-12 and 13 show the vertical bars (denoting 0.95 confidence interval) indicating the means of IFN-gammas in Nil, TB antigen, mitogen, TB antigen minus Nil and mitogen minus nil, and the body mass index at different time points. The graphs were plotted after performing repeated measures of ANOVA.
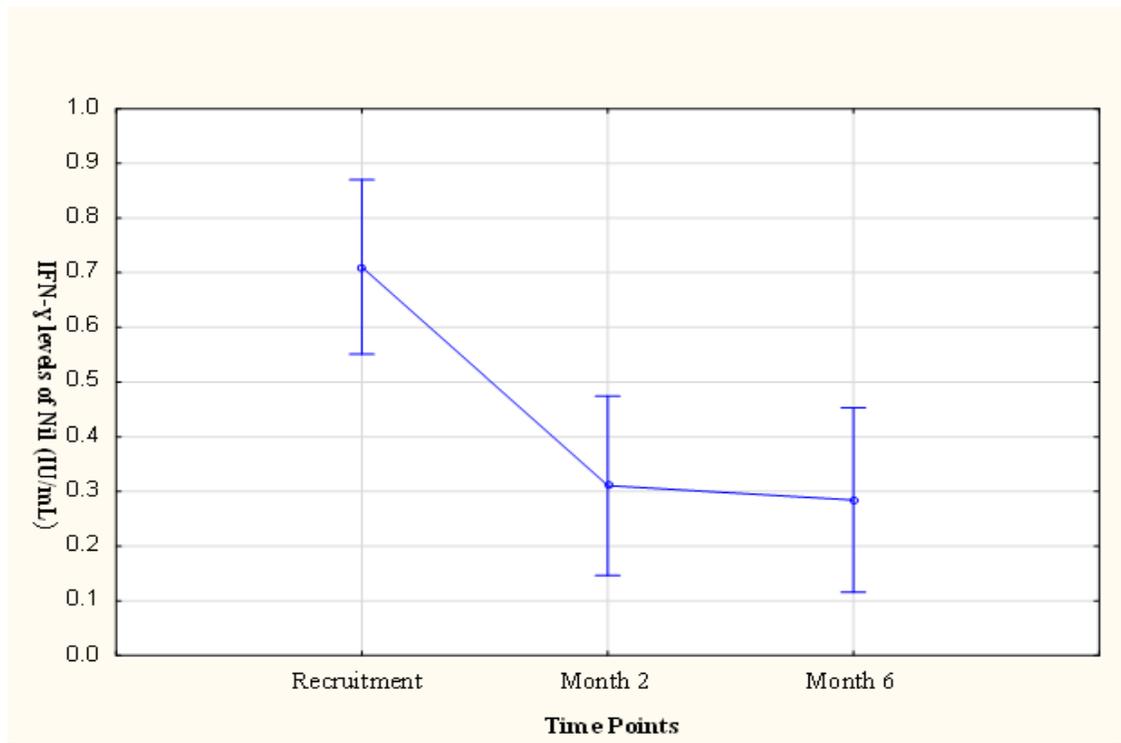
Figure 8: IFN-ɣ levels within the Nil control from quantiferon supernatants at different time points
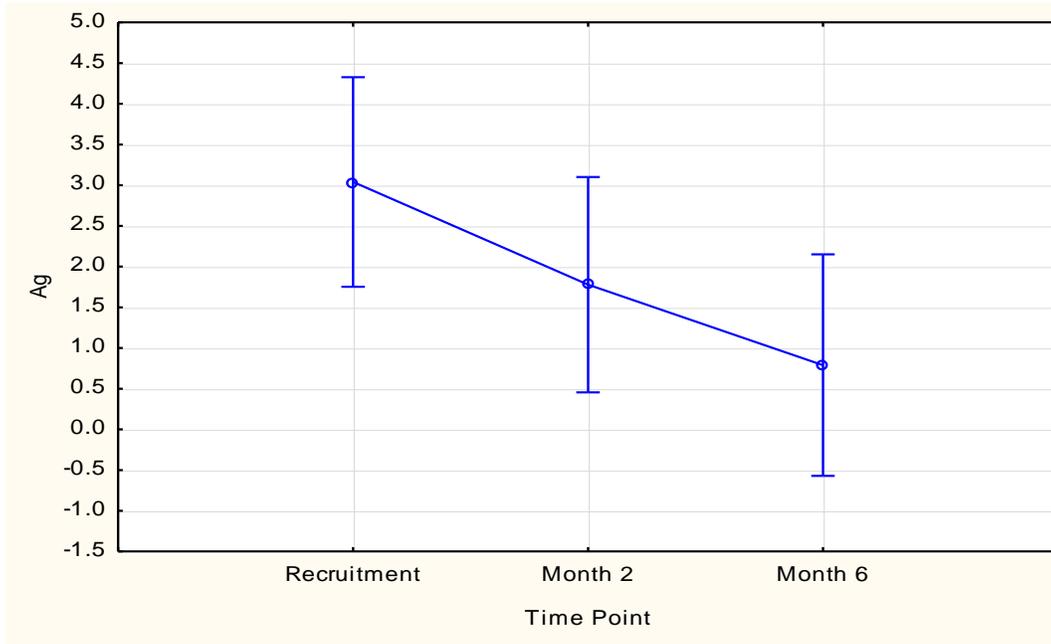
Figure 9: IFN- ɣ levels of the TB antigen from quantiferon supernatants
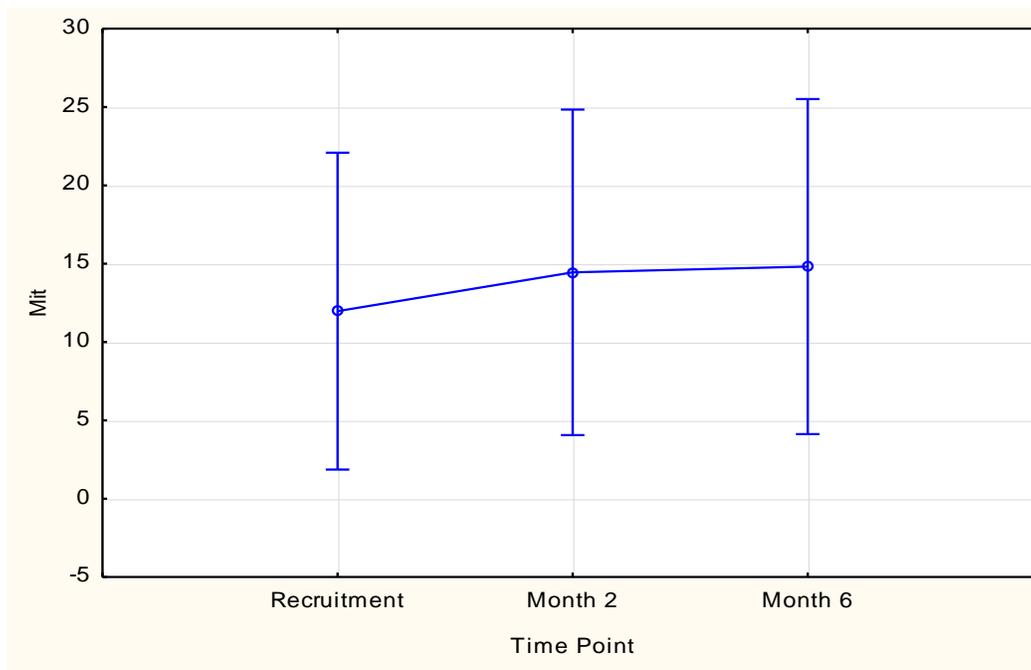
Figure 10: IFN-ɣ levels for the Mitogen from quantiferon supernatants
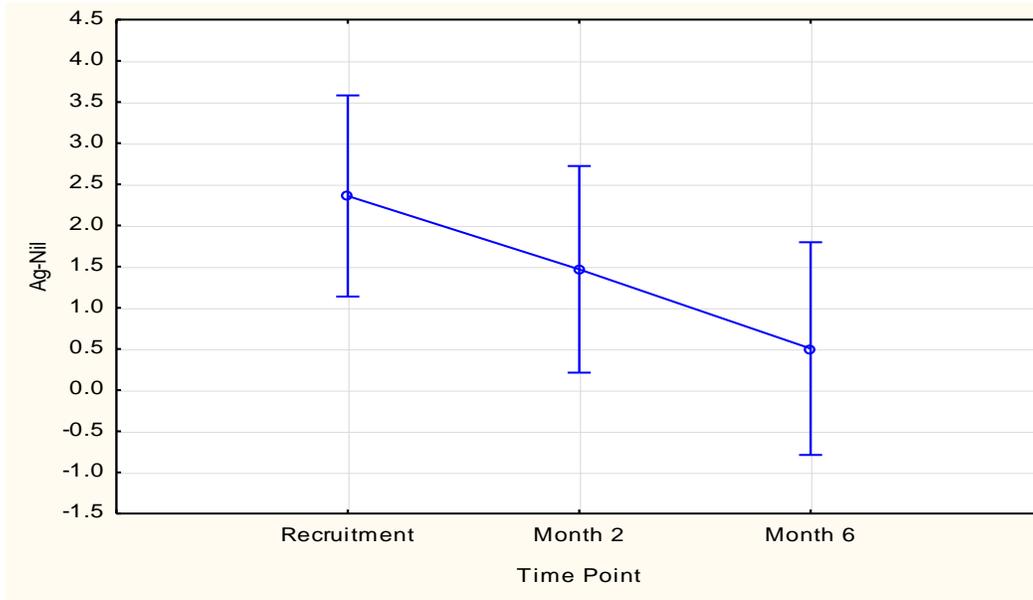
Figure 11: IFN-ɣ levels for TB antigen minus Nil form quantiferon supernatants

Figure 12: IFN-ɣ levels for the Mitogen minus Nil from quantiferon supernatants
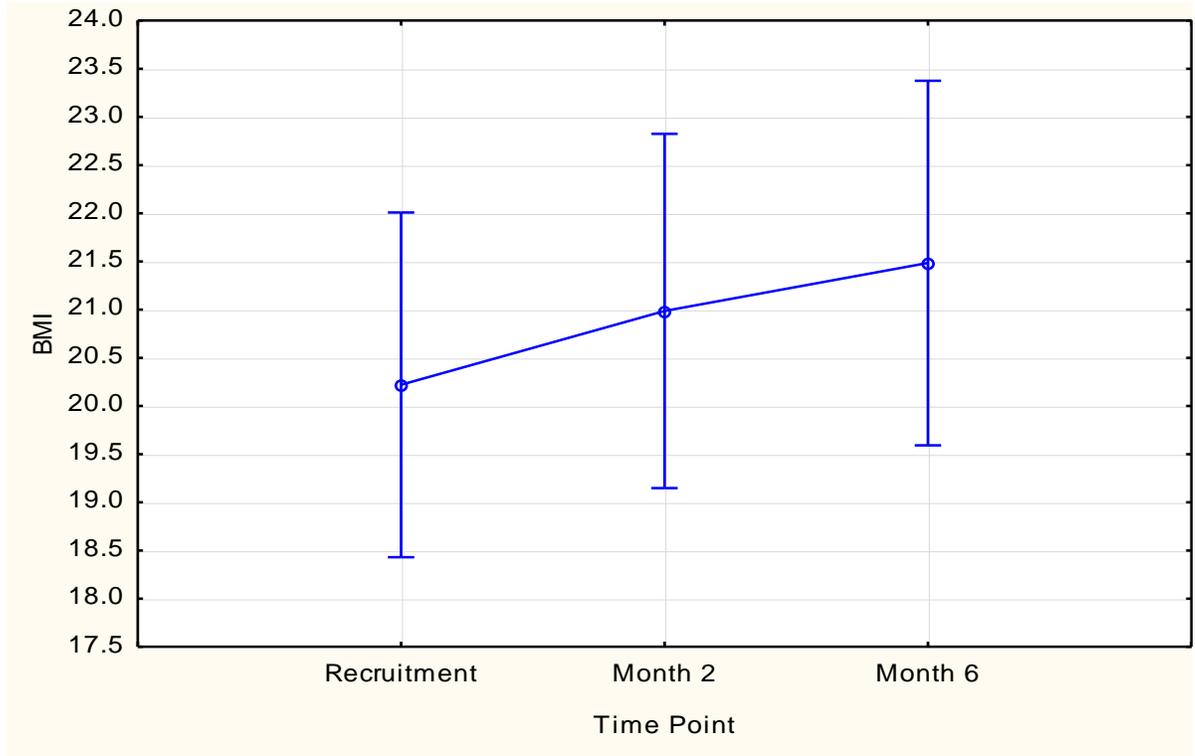
Figure 13: Body mass index (BMI) in TB patients before treatment and at other different time points

Table 9: The *p*-values of IFN-ɣ levels and BMI at different time points

	Recruitment and Month 2	Recruitment and Month 6	Month 2 and Month 6
Nil	0.00	0.00	0.82
TB Antigen	0.18	0.02	0.30
Mitogen	0.73	0.70	0.96
TB Antigen – Nil	0.31	0.04	0.29
Mitogen – Nil	0.02	0.01	0.92
BMI	0.55	0.33	0.71

The ANOVA test showed statistical significant difference, $p < 0.05$ within the IFN-ɣ levels of Nil ($p = 0.00$), TB antigen ($p = 0.02$), TB antigen minus Nil ($p = 0.04$) and mitogen minus nil ($p = 0.01$) when compared to recruitment (before initiation of treatment) and at completion of treatment (month 6). There was no statistical significant difference within the IFN-ɣ levels between recruitment and month 2 as well as between month 2 and month 6 (Table 9). In addition, there was no statistical significant difference at different time points on the body mass index, $p > 0.05$.

4.6 Conclusion

The participation of patients in the study was voluntary; there were a higher proportion of males compared to females; and the majority of the patients were HIV positive. Although the study targeted the TB suspects; not every patient who had suggestive symptoms was a TB case.

The sensitivity of QFT-IT test as compared to MGIT culture in diagnosing pulmonary TB caused by *M. tuberculosis* infection was 90.5% and a specificity of 57.8%, with a low PPV (41%) and high NPV (95%). A statistically significant difference was observed between QFT-IT and MGIT culture test.

There was a statistical significant difference between QFT-IT test and HIV status of an individual.

There was a statistical significant difference within the IFN-ɣ levels of quantiferon supernatants between recruitment and at completion of treatment.

CHAPTER 5: DISCUSSION

5.1 Introduction

This chapter interprets the results that were presented in the previous chapter and compares to the existing findings and elaborates more on the meaning of the results.

5.2 Usefulness of quantiferon TB Gold In-Tube test for the diagnosis of pulmonary TB

The sputum and blood were the specimens collected from the participants. Sputum is a true specimen representative of infections in the lungs; therefore its collection is very important in diagnosis for direct microscopy of the smear to detect AFB and incubating it in the MGIT culture machine for the growth of the mycobacteria. Blood was collected directly into a set of three quantiferon tubes that were incubated at 37°C and the QFT ELISA was performed.

The ability of a diagnostic test or procedure to correctly classify individuals into two categories such as positive and negative is assessed by two parameters; sensitivity and specificity (Kirkwood & Sterne, 2003). An individual may be classified as diseased or non-diseased, exposed or non-exposed, positive or negative or at high risk or not. To

estimate sensitivity and specificity of a test; each individual needs to be classified definitely (using a 'gold standard' assessment) as true positive or true negative and, in addition, to be classified according to the test being assessed. In this study, the TB 'gold standard' test was microbiological MGIT culture which could diagnose pulmonary TB caused by *M. tuberculosis* from sputum culture and it signifies active TB. The assessed test was Quantiferon TB Gold In-Tube for the detection of *M. tuberculosis* infection from blood samples and it indicates latent and active TB. Due to lack of a 'gold standard' test for latent TB infections, the sensitivity of QFT was evaluated on the results of participants who were confirmed to have active TB (TB disease), and these tests employ different immunological conditions. The approach is similar to many other analyses including that of Diel, Loddenkemper & Nienhaus, 2010. Microbiological culture of *M. tuberculosis* is a suitable surrogate to evaluate sensitivity of Quantiferon TB Gold In-Tube since patients with disease (active TB) are by definition infected (Quantiferon TB Gold (In-Tube method), Package Insert, Cellestis).

Sensitivity is the ability of a test to correctly classify an individual as diseased. Specificity is the ability of a test to correctly classify an individual as disease-free (Parikh, Mathai, Parikh, Sekhar & Thomas, 2008). Predictive values are also used to interpret the results of a diagnostic test and it indicates a probability (Kirkwood & Sterne, 2003). Positive predictive value (PPV) is the probability that a patient is truly

positive if the test result is positive and negative predictive value (NPV) is the probability that the patient is truly negative if the test result is negative.

Evaluation of Quantiferon TB Gold In-Tube test compared to MGIT culture test yielded the sensitivity of 90.5%, [95% confidence interval (CI) 69.6 – 98.55%) and a specificity of 57.8%, [95% CI 44.8 – 70.1%]. The Quantiferon TB Gold In-Tube test showed the ability to correctly classify individuals with pulmonary TB infections and disease at 90.5% and it is correctly classifying individuals as free from pulmonary TB infections and disease at 57.8%.

The sensitivity of 90.5% obtained in this study on the quantiferon TB Gold In-Tube test is high compared to those of the reviewed studies by Fan et al. (2012); Pai & Menzies, (2007) and Sester et al. (2011), but it is in the range given in QFT package insert. However, it has shown a low specificity (57.8%) which is similar to the specificity range of the low/middle-income countries at [95% CI 48-89%], and comparing it to the specificity given in the QFT package insert, it is too low. However, the PPV is only 41%; exhibiting that the probability that the 90.5% positive by quantiferon is only 41% true positive, so the test is not doing well enough in identifying pulmonary TB positive patients who have TB disease compared to the 'gold standard' MIGT TB culture. This result is not surprising because according to literature, quantiferon test cannot distinguish between active and latent TB. The PPV indicates the likeliness of the

patients to have both the infection and disease when the test result is positive. Although specificity was 57.8%, the NPV value is 95%; suggesting that quantiferon method is doing very well in identifying participants as negative. In this study the PPV of 41% and NPV of 95% indicate that the patients are likely not to have pulmonary TB infections when the test result is negative. The QFT test could be positive when patients have both latent and active TB (TB infection and disease). In some settings, QFT test is being used to screen for *M. tuberculosis* infection, which is a good attempt rather than waiting for the infection to develop into a disease. The specificity of QFT obtained in this study is low compared to the specificity reported in the QFT package insert. This is due to that QFT was evaluated against MGIT culture test instead of the TST test.

The sensitivity and specificity of QFT using direct microscopy of the sputum smear as the gold standard test were 75% and 52% respectively. This sensitivity is indeed in an agreement with the sensitivities reviewed by Pai & Menzies, 2007 with an exception of their sensitivity in low income countries. There was no statistical significant difference between QFT and direct microscopy of the stained smear in diagnosing pulmonary TB caused by *M. tuberculosis*; the two tests cannot differentiate between latent and active TB. QFT has specific antigens to *M. tuberculosis* infection while direct microscopy could pick up all acid fast bacilli.

On the consideration of quantiferon test results against TB classifications as non-TB or TB case by the clinicians; the sensitivity was 65.7% [95% CI, 47.8-80.9%)] and the specificity was 53.3% [95% CI, 40-66.3%]. Although, the isolation of *M. tuberculosis* from a sputum culture in the laboratory is the evidence of a causative agent for pulmonary TB, not all the patients that were classified as TB cases had sputum culture confirmed as *M. tuberculosis*. This could have contributed to the significant difference observed between TB classification and MGIT culture. The classification of TB by the clinician considered other factors such as symptoms, X-ray and direct microscopy and in many instances the culture results comes out very late. The identification of participants as TB cases or non-TB cases was not only to obtain data for the research, but it was also to help them recover through treatment; therefore the hospital sputum samples that were taken before the participants gave written informed consents to the study were also used.

The sensitivities of quantiferon as compared with MGIT culture, direct microscopy and clinical TB classification were 90.5%, 75% and 65.7% respectively. The latter is low compared to the others, and this could be because of the subjectivity by the clinician. The specificities of QFT as compared with MGIT culture, direct microscopy test results and clinical TB classification which were 57.8%, 52% and 53.3% are of interest in this study. They are closer to each other and low because QFT is a test for diagnosing latent and active TB and it was evaluated against a test that can diagnose active TB disease. The low specificity may indicate limited value of IGRAs to distinguish between patients

with specific immunity due to latent *M. tuberculosis* infection from patients with active disease (Sester et al., 2011).

Specificity for QFT analysed according to immune status subgroups; had a pooled specificity of 80% in studies without immune-compromised patients, almost similar to a value in studies with immune-compromised patients of 87% (Fan et al., 2012). The two values are not too far from each other, indicating that the specificity of QFT is less or not affected by the severity of immunity. This is an indication that the immune status of patients does not greatly affect the diagnostic accuracy of IGRAs. Although QFT test and HIV status will be addressed next in this chapter, it is worthy to mention that this study did not do a test on CD4 count to assess the immunity of patients and therefore made it difficult to reason based on the immunity. Since the study in Namibia was formed by a large number (61%) of HIV positive participants; it could be assumed that some of them could have been immune-compromised although immune-compromise is not only limited to HIV status. The specificity of QFT obtained in this study was 57.8%, which is not similar to the pooled specificities in studies with and without immune-compromised patients.

The QFT showed no agreement with MGIT culture; however, it indicated its usefulness as a test to rule-out pulmonary TB caused by *M. tuberculosis* in patients because of the NPV which is 95%. Since QFT can pick up both latent and active TB, the test is not

specifically suited for ruling-in active pulmonary TB in patients which is a case with MGIT culture test because of the low PPV which is 41%.

5.3 Quantiferon test results and HIV status

Analysis of the quantiferon test results with HIV status to answer the question whether HIV status influences the QFT test results indicated no influence with the chi-square test. In this study, although a large number 54% of HIV positive participants were QFT negative as compared to 28% of HIV negative participants that tested QFT negative; this cannot be attributed to HIV status. This finding differs to the study done by Cheallaigh et al. (2013) which was done in low TB prevalence setting, and it confirmed the association between low CD4+ T-cell and negative QFT-IT results which concluded that HIV positive individuals with advance immunosuppression are likely to have negative QFT-IT results.

The indeterminate results for any IGRAs are attributed to factors such as: a high level of immunosuppression and/or technical error in blood collection and handling or essay performance (Diel et al., 2010). In this study, the 5% indeterminate are both from the HIV infected participants; therefore their immune system could have been suppressed making it difficult for the QFT test to detect any immune activity and quantify the IFN-ɣ. The other factors are less likely to be contributing factors because precautions were

taken into consideration during the process from blood collection to assay performance. Since 2 of the 5 with indeterminate were followed up; the 2 indeterminate results became QFT negative at both time points (month 2 and 6), and the smears as well as culture were negative at all three time points. The study of Ferrara et al. (2005) reported a significant rate of indeterminate results in most vulnerable population of immunosuppressed hosts. Cheallaigh et al. (2013) stated that negative QFT-IT results in HIV positive patients need to be interpreted with caution.

In the literature review, on the advantages of quantiferon over TST; a positive internal control in QFT eliminates the false negative results due to T-cell anergy or immune-suppression (Van Rooyen & Brink, 2007). While on the disadvantages, it indicated that the quantiferon sensitivity decreases in severely immunocompromised patients. This study focused on the TB suspects and not necessarily known TB patients with the aim to avoid very sick people that could be immune-compromised, although participation on the study was voluntary, a large group was HIV positive whose immunity was not compromised. Therefore, the findings of this study suggested that QFT test was not influenced by HIV status.

5.4 IFN-ɣ levels for monitoring TB treatment response

The final TB classification in this study was based on the results of clinical, radiologic examination, direct microscopy of the sputum smear and/or isolation of *M. tuberculosis* through MGIT culture on the study sputum specimen or routine hospital sputum specimen. The 19 participants that were started on anti-TB medication and followed up and had their samples taken at two months while on treatment and (at month 6) on completion of treatment. Some participants (n=20) that were classified as TB cases in the study either revoked the consent due to discordant results between study and routine hospital results, some got transferred out of the region and few died (exact number not recorded). Therefore, only 19/39 participants were followed up and successfully completed the study.

The final specific objective was to find out if IFN-ɣ levels can be used as a tool to monitor TB treatment response. When the treatment was initiated and the IFN-ɣ levels were analysed; a statistical significant difference ($p < 0.05$) was observed between recruitment and at end of 6 months within IFN-ɣ levels for nil, TB antigen, TB antigen minus nil and mitogen minus nil. However, there was no statistical significant difference in the IFN-ɣ values at recruitment and month 2 of treatment and between month 2 of treatment and at the end of therapy (month 6). Denkinger, Pai, Patel & Menzies, 2013 reported a significant difference in the IFN-ɣ values at the time of diagnosis compared to the measurement after 2 months of treatment and at the end of treatment; median IFN-ɣ

of 6.94 IU/mL at diagnosis, compared to 3.7 IU/mL after 2 months of treatment and 2.19 IU/mL at the end of therapy. It further reported that a large proportion of patients remained QFT positive even at the completion of treatment. The current study showed that IFN-ɣ levels declined when compared between recruitment and at completion of treatment.

In this study; considering the number (19) of patients that were followed-up; 9 patients had positive QFT results at recruitment but 4/9 (44.4%) remained positive and 5/9 (55.6%) were QFT negative at completion of anti-TB treatment. Pai et al. (2007) pointed out that even if the IFN-ɣ levels dropped sharply, it is unlikely that QFT will revert to negativity because IFN-ɣ levels at baseline is mostly higher than the QFT cut-off point of 0.35 IU/mL. It is also noted that ongoing exposure and/or exogenous re-infection keep T-cells partially activated; therefore T-cell responses can remain strong even after antigen load had declined with therapy (Pai et al., 2006 cited in Pai et al., 2007). Although this study observed a statistically significant difference within the IFN-ɣ levels between before initiation of treatment and at the end of anti-TB treatment, not all the patients converted from QFT positive to QFT negative (Appendix G). This is due to that IFN-ɣ values at recruitment were mostly higher making it impossible to be lower than the cut-off point even if they decline by the end of treatment.

Levels of IFN-ɣ at different time points could inform clinical management of pulmonary TB patients by the healthcare practitioners as this gives information on the effects of anti-TB medications on the patients as expressed by the immune activity. Besides the variations in the IFN-ɣ levels which could be investigated as it might be affected by other factors, a decline in the levels between recruitment and after completion of therapy gave an idea that the anti-TB medications worked. Although the patient's IFN-ɣ values fluctuated between time points; the overall mean statistically depicted a declining pattern which indicates a possible role as a therapeutic monitoring indicator.

Body mass index is a good indicator for body mass especially in disease conditions whereby muscles are wasted. As stated in the literature, weigh loss is one of the symptoms of tuberculosis which can be gradual and/or dramatic if treatment is not started (Davies, 2008). Therefore, the analysis of BMI and seeing how it changed over time through treatment could give an idea on anti-TB treatment response. The graph on Figure 13 showed an increase in the mean BMI, however, there were no statistical significant difference at all time points on the participant's BMI. A question remains as to whether patients do take their mediations accordingly, so that treatment is effective and the body mass can recover. Some participants could have been exposed to different other factors that made a change to their body mass during the time of anti-TB treatment. So, BMI cannot exclusively be looked at as a factor to monitor pulmonary TB treatment response but it is an indication that body mass has changed.

5.5 Conclusion

The findings on the objectives as they were stated in chapter one have been interpreted. It was hypothesised that QFT test is a better test for diagnosing pulmonary TB than other methods such as sputum MGIT culture and direct microscopy. Although the sensitivity and sensitivity as well as the great findings shown by the statistical tests, the literature cautioned that QFT results of patients must be interpreted carefully especially in an HIV infected population.

Findings in this study differ to some extend from those reported in the literature; the conclusion will be included in the next chapter which presents the final conclusion and recommendations drawn in this study.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Introduction

This chapter summarises the principal conclusions of the findings reached in this study, elaborates on the limitations and advances on recommendations as to how methods for diagnosing pulmonary TB can be improved. The conclusion attempts to generate findings and possible suggestions and solutions as informed by the study.

6.2 Main findings and limitations

The effectiveness of quantiferon test for diagnosing pulmonary TB caused by *M. tuberculosis* infection was assessed, and the utility of IFN-ɣ levels to monitor TB treatment response in patients was evaluated for Katutura State Hospital in Namibia.

The quantiferon test showed no agreement with MGIT culture test; however, it indicated a better test to rule-out pulmonary TB caused by *M. tuberculosis* in patients (NPV = 95%). Since QFT test detects both latent and active TB; therefore not useful for ruling-in pulmonary TB in patients (PPV = 41%). The sensitivity and specificity of QFT when compared to MGIT culture the 'gold standard' test were 90.5% and 57.8% respectively. However, the study did not do TST test which is a standard test for latent TB infections

to evaluate the specificity of QFT-IT test. Another limitation in this study was lack of a control group which prevented the estimation of less error specificity.

The QFT test results were not influenced by the HIV status, although HIV positive patients mostly gave QFT negative results, the study had a large portion of participants who were HIV positive. Nevertheless, there was no CD4 cell count done in this study that can be used to interpret the immune activity of patients in relation to HIV status.

The IFN-ɣ levels in QFT supernatants declined when compared between the time before treatment (recruitment) and to the completion of treatment (at end of 6 months), suggesting a possible role in monitoring TB treatment. Regardless of this finding, the number of patients (n=19) assessed for treatment response in this study was too small to make an informed decision.

Interferon gamma release assays such as Quantiferon TB Gold In-Tube test must be interpreted with caution in ruling-out and ruling-in pulmonary TB infections caused by *M. tuberculosis*. In addition, specimens must be tested by agreed combination of available diagnostic tests in order to have a through definitive confirmation of the causative agent of pulmonary TB. The focus to fight pulmonary TB infection can be reached when there is proper diagnosis and proper treatment as well as prevention of

disease reactivation and spread of infection. Proper and early diagnosis can result into early treatment and finally in the decline of TB cases.

6.3 Recommendations

Studies pertaining to TB diagnostic methods involving emerging technologies must be validated to inform and improve the public health sector. The validation of the QFT-IT needs to be done nationally with the right population size. Additionally, since the study had a large portion of HIV positive participants; it is recommended that a large study that will have sufficient power to allow for stratification into HIV positive and negative groups is needed in order to find out if the sensitivity and specificity reported in this study will change. Future research must also evaluate the influence of HIV status on MGIT culture test and QFT-IT test to compare the findings.

Furthermore, there is also a need for conducting studies that could develop other TB cytokines which may be common in Namibian patients which can possibly differentiate between latent and active TB.

Finally, the Ministry of Health and Social Services of Namibia should refer to this study as a basis on the use of quantiferon in the Namibian context with specific reference to Katutura State Hospital.

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APPENDICES

Appendix A: AE-TBC case definitions used in classifying participants as TB cases or non-TB

cases

Old Category	New Category	Culture 1	Culture 2*	AFB 1	AFB 2*	CXR	Symptoms	TB Treatment response	Failed AB	New Class
1	A	pos	pos							Definite
2	B	pos				pos				Definite
9	C	pos		pos						Definite
7	D			pos	pos	pos				Probable
8	E			pos	pos		pos			Probable
17	F			pos		pos				Probable
4	G	pos					pos	pos		Probable
10	H	pos					pos			Possible
new	I			pos	pos			pos		Possible
14	J			pos			pos			Possible
11	K					pos	pos	pos	pos	Possible
new	L	pos								Questionable
new	M			pos	pos					Questionable
new	N			pos						Questionable
16	O					pos	pos			Questionable
new	P					pos				Questionable
19	Q						pos			Questionable
20	R	Neg/ND	Neg/ND	Neg/ND	Neg/ND	Neg/ND	pos	TB treatment never started		Non-TB case
21	S	Neg/ND	Neg/ND	Neg/ND	Neg/ND	Neg/ND	Neg/ND	TB treatment never started		Non-TB case

(For rows 20/R and 21/S: ≥ 1 of shaded cells **must** be negative)

Acid-fast bacilli (AFB) sputum smear positive: ≥scanty (≥1-9 acid-fast organisms per 100x oil field) **CXR**: chest X-ray compatible with active TB

Failed antibiotic (AB) treatment: No response of symptoms to a 7 day, broad spectrum oral or IV antibiotic

Culture positive: liquid or solid agar positive with confirmed speciation for M. tuberculosis complex

*Any second sputum result (culture or AFB): a separate sample must be provided by the suspect (not an aliquot of one sample), preferably, but not necessarily, on separate days

Appendix B: Permission letter from the project principal investigator (PI) in Namibia

TO: The Chairperson

Post Graduate Studies Committee

University of Namibia

FROM: The Principal Investigator African European –Tuberculosis Consortium (AE-TBC);

UNAM

University of Namibia

DATE: July 25, 2012

SUBJECT: **ASSESSMENT OF THE QUANTIFERON TB GOLD IN-TUBE TEST FOR THE DIAGNOSIS OF TUBERCULOSIS IN NAMIBIAN PATIENTS**

This letter serves to confirm that Ms J Nelongo conducts the abovementioned study for her Master in Science degree. The study is part of a bigger research project, The evaluation of *Mycobacterium tuberculosis* specific host cytokine signatures in whole blood culture supernatants as diagnostic biomarkers for active TB infection, that the Faculty of Health Science at the University of Namibia is currently involved in. Ms Nelongo is one of the UNAM team members and is the laboratory assistant for the project.

Attached please find the letter of permission from The Ministry of Health and Social Services that the study be conducted in Namibia together with the Host Institution: Stellenbosch University, Faculty of Health Sciences and collaborators in The Gambia, Armauer Hansen Research Institute Ethiopia, Max Planck Institute for the Advancement of Science, LUMC The Netherlands, London School of Hygiene & Tropical Medicine, European Research & Project Office (Eurice), Makarere University Uganda, and The Karonga Prevention study Malawi.

THANK YOU

DR JM VAN DER VYVER

Appendix C: Permission from the Ministry of Health and Social Services

REPUBLIC OF NAMIBIA

Ministry of Health and Social Services

Private Bag 13198	Ministerial Building	Tel : (061) 203 2562
Windhoek	Harvey Street	Fax: (061) 272286
Namibia	Windhoek	Email: hnangombe@mhss.gov.na
Enquiries: Mr. H Nangombe	Ref: 17/3/3	Date: 02nd September 2010

OFFICE OF THE PERMANENT SECRETARY

Dr. J. M. Van der vyver
Project Coordinator
University of Namibia
Private Bag 13301
Windhoek
Namibia

Dear Dr. Van Der Vyver

Re: The Evaluation of Mycobacterium Tuberculosis Specific Host cytokine signatures n whole blood culture supernatants as Diagnostic Biomarkers or Active TB infection.

1. Reference is made to your application to conduct the above-mentioned study.

2. The proposal has been evaluated and found to have merit.

3. Kindly be informed that approval has been granded under the following conditions

3.1 A quarterly progress report to be submitted to the Ministry's Research Unit;
3.2 Preliminary findings are to be submitted to the Ministry before the final report;
3.3 Final report to be submitted upon completion of the study;
3.4 Separate permission to be sought from the Ministry for the publication of the findings.

Wishing your success with your project

Yours sincerely

Mr. K. Kahuure
Permanent Secretary

MINISTRY OF FINANCE
Customs & Excise

2 1 -06- 2012

TECHNICAL CONTROL
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REPUBLIC OF NAMIBIA

"Health for All"

Appendix D: Information leaflet and consent form

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE AE-TBC RESEARCH PROJECT: The evaluation of *Mycobacterium tuberculosis* specific host cytokine signatures in whole blood culture supernatants as diagnostic biomarkers for active TB infection

TITLE OF THE MASTER OF SCIENCE: Assessment of the Quantiferon TB Gold In-Tube test for the diagnosis of tuberculosis in Namibian patients

PRINCIPAL INVESTIGATOR at Stellenbosch University: Prof. Gerhard Walzl

ADDRESS: Stellenbosch University Immunology Research Group, Division of Molecular Biology and Human Genetics, Department of Biomedical Sciences, Faculty of Health Sciences, PO Box 19063, Tygerberg, 7505.

CONTACT NUMBER: (University of Stellenbosch): +27 21 938 9158 or 0825923212

PRINCIPAL INVESTIGATOR at the **University of Namibia**: Dr J M van der Vyver

ADDRESS: University of Namibia, Faculty of Health Sciences, Private Bag 13301, Windhoek, Namibia

CONTACT NUMBER: (University of Namibia): +264 61 20631111 or 0812846270

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the **Health Research Ethics Committee (HREC) at Stellenbosch University** and by the **Ministry of Health and Social Services in Namibia** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

➢ *The study will recruit approximately 100 patients visiting Katutura Hospital. The project is part of a bigger international project and will also recruit patients in South Africa, Malawi, Uganda, Ethiopia and The Gambia.*

➢ *The aim of the project is to find new ways to diagnose tuberculosis (TB). The test that are now available require several clinic visits by the patient and are not very accurate in children and in people with HIV infection, We would therefore like to investigate new*

tests that are done on blood and other samples from people who have symptoms that could be due to active TB. We will investigate the samples that we collect from TB patients and those with other diagnosis with modern laboratory techniques to look for markers that can be used to accurately diagnose TB. The tests that will be conducted and the samples that will be collected include the following:

Routine clinical/diagnostic interventions: All participants will undergo routine investigations to confirm or to rule out active TB. This will include:

- a questionnaire

- a clinical examination by a nurse or doctor

- sputum for smear and culture examination (at least two separate   samples   will   be examined)

- a chest radiograph (CXR). As CXR does not form part of the routine clinical work up of TB suspects at all primary health care clinics the project will cover the cost for these tests, which will be   done at either public health care facilities (if agreed with health facility managers) or at private radiology facilities.

- HIV rapid tests after pre-test counseling with a confirmatory second rapid test if the first test is positive. If the two tests give different results we will perform a more sensitive test in a laboratory.

- Hemoglobin will be done in all participants

In all cases the results will be made available to the public sector clinic staff for further management of patients. If applicable the patients will be referred to secondary or

tertiary care hospitals for further management if serious medical conditions are diagnosed.

Research investigations:

Blood (3 ml, which is perfectly safe to donate) will be collected at the first, the second visit and the third visit (two months and six months later).

Sputum: 10ml will be collected

A subset of samples may be shipped to laboratories outside Namibia or outside Africa for analysis with advanced techniques (ultra-sensitive methods) that are not available in Namibia.

➢ *The results of the established diagnostic tests will be made available to the clinic staff who will then be able to use this information to guide your treatment decisions. No experimental medicines will be given to anyone.*

Why have you been invited to participate?

➢ *You have been invited to participate because you have symptoms that could can be caused by TB although they could also be caused by other diseases. We would like to*

test you samples for signs of TB and to compare the results of people who are ultimately diagnosed with TB with those in people without TB.

What will your responsibilities be?

➢ *We would like to ask your permission to use your clinical information, to collect the samples that we have discussed above and would like you to return for a follow-up visit in 8 weeks.*

Will you benefit from taking part in this research?

➢ *The experimental tests will not benefit you but the results may lead to new TB diagnostic tests that could benefit your community in the future. You will also undergo additional tests like X-ray examination and sputum culture that would not be part of routine care at the clinic and which may help to diagnose your condition.*

Are there in risks involved in your taking part in this research?

➢ *The only risk to you is minor discomfort at the site of the blood draw. We will do a pregnancy test in women of child bearing age to ensure that the X-rays do not harm an unborn baby.*

If you do not agree to take part, what alternatives do you have?

➢ *You can simply undergo the routine investigations that are offered by the clinic.*

Who will have access to your medical records?

➢ *All the information collected will be treated as confidential and protected. If it is used in a publication or thesis, the identity of the participant will remain anonymous. Only the clinic staff, the research nurse and research doctors and the project co-ordinator will have access to names that are linked to the information. All samples will be stored with barcodes to identify them and no names will appear on the containers.*

What will happen in the unlikely event of some form injury occurring as a direct result of your taking part in this research study?

➢ *In the very unlikely event of an injury that is the direct result of your taking part in this study University of Namibia has insurance that would cover any expenses or losses that you would suffer.*

Will you be paid to take part in this study and are there any costs involved?

No you will not be paid to take part in the study but your transport and meal costs will be covered for each study visit. There will be no costs involved for you, if you do take part.

Is there anything else that you should know or do?

➢ *You can contact Dr Gerhard Walzl at tel +27 21 825923212 if you have any further queries or encounter any problems or Dr J M van der Vyver at the University of Namibia: +264 61 2063111 or 0812846270 or Nurse Amutenya 0812482710.*

➢ *You can contact the **Ministry of Health and Social Services** at +264 61 2032562 if you have any concerns or complaints that have not been adequately addressed by your study doctor.*

➢ *You will receive a copy of this information and consent form for your own records.*

Declaration by participant

By signing below, I …………………………………..…………. agree to take part in a research study entitled '***The evaluation of Mycobacterium tuberculosis specific host cytokine signatures in whole blood culture supernatants as diagnostic biomarkers for active TB infection'. / Assessment of the Quantiferon TB Gold In-Tube test for the diagnosis of tuberculosis in Namibian patients***

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.

- I have had a chance to ask questions and all my questions have been adequately answered.

- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.

- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.

- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) ....…........………… on (*date*) …………....………………...

....
Signature of participant   **Signature of witness**

Declaration by the research nurse

I *(name)* …………………………………………….……… declare that:

- I explained the information in this document to …………………………………..

- I encouraged him/her to ask questions and took adequate time to answer them.

- I am satisfied that he/she adequately understands all aspects of the research, as discussed above

- I did/did not use a interpreter. (*If an interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) ....….......…………….. on (*date*)………....………..………

....
Signature of the research nurse   **Signature of witness**

Declaration by interpreter

I *(name)* ………………………………………..……… declare that:

- I assisted the research nurse (*name*) ……………………………………. to explain the information in this document to (*name of participant*) ……………..………………………….. using the language medium of Oshiwambo/ Herero/ Nama/ Damara/ Kavango/ Caprivi.

- We encouraged him/her to ask questions and took adequate time to answer them.

- I conveyed a factually correct version of what was related to me.

- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*)...................…........……………on (*date*) …………...………………

....
Signature of interpreter   **Signature of witness**

Appendix E: Quantiferon ELISA optical density (OD)

	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.127	0.115	2.989	1.143	1.131	1.103	0.117	0.129	OVRFL W	0.139	0.131	OVRFL W	450
B	0.142	0.277	OVRFL W	0.373	0.311	0.326	0.177	0.388	OVRFL W	0.218	0.271	2.661	450
C	0.302	0.95	2.729	0.156	0.154	0.139	0.129	0.134	OVRFL W	0.468	0.852	OVRFL W	450
D	0.618	2.005	OVRFL W	0.11	0.132	0.122	0.133	0.448	OVRFL W	0.21	0.903	OVRFL W	450
E	0.122	0.139	OVRFL W	0.103	0.141	2.91	0.12	0.145	2.913	0.142	0.212	1.728	450
F	0.264	0.899	1.855	0.107	0.121	0.896	0.126	0.288	OVRFL W	0.756	0.932	1.483	450
G	0.096	0.384	OVRFL W	0.871	3.269	3.202	0.125	0.12	OVRFL W	0.106	0.179	0.208	450
H	0.153	0.114	OVRFL W	0.136	0.512	1.199	0.131	0.141	3.631	0.125	0.137	3.302	450

Appendix F: Quantiferon ELISA analysis performed by the software.

P5 1 of 2 4 ✓ (ok.

cellestis

Version 2.62

QuantiFERON®-TB Gold In-Tube Results

Run Date:	Monday 3 September 2012
Operator:	J. Nelongo
Run Number:	5
Kit Batch Number:	51242

Valid ELISA test run.

Results (IU/mL)

Subject ID	Nil	TB Ag	Mitogen	TB Ag-Nil	Mitogen-Nil	Result
ID 1 NAM00178	0.22	0.19	> 10	-0.03	> 10	NEGATIVE
ID 2 NAM00179	0.25	0.64	> 10	0.39	> 10	POSITIVE
ID 3 NAM00180 ✓	0.72	3.47	> 10	2.75	> 10	POSITIVE
ID 4 NAM00181	1.92	9.68	> 10	7.76	> 10	POSITIVE
ID 5 NAM00183	0.21	0.25	> 10	0.04	> 10	NEGATIVE
ID 6 NAM00182	0.60	3.22	8.70	2.62	8.10	POSITIVE
ID 7 NAM00184	0.15	1.00	> 10	0.85	> 10	POSITIVE
ID 8 NAM00185	0.28	0.19	> 10	-0.09	> 10	NEGATIVE
ID 9 NAM00186	0.16	0.25	> 10	0.09	> 10	NEGATIVE
ID 10 NAM00187	0.17	0.20	3.20	0.03	3.03	NEGATIVE
ID 11 NAM00188	3.08	> 10	> 10	> 10	> 10	POSITIVE
ID 12 NAM00189	0.24	1.48	4.78	1.24	4.54	POSITIVE
ID 13 NAM00070	0.20	0.22	> 10	0.02	> 10	NEGATIVE ✓
ID 14 NAM00190	0.34	1.01	> 10	0.67	> 10	POSITIVE
ID 15 NAM00191	0.22	0.24	> 10	0.02	> 10	NEGATIVE
ID 16 NAM00133	0.23	1.23	> 10	1.00	> 10	POSITIVE
ID 17 NAM00135	0.20	0.26	> 10	0.06	> 10	NEGATIVE
ID 18 NAM00192	0.22	0.67	> 10	0.45	> 10	POSITIVE
ID 19 NAM00193	0.21	0.20	> 10	-0.01	> 10	NEGATIVE
ID 20 NAM00194	0.23	0.25	> 10	0.02	> 10	NEGATIVE
ID 21 NAM00126	0.25	0.23	> 10	-0.02	> 10	NEGATIVE
ID 22 NAM00128	0.46	0.62	> 10	0.16	> 10	NEGATIVE
ID 23 NPM00195	1.31	2.99	> 10	1.68	> 10	POSITIVE
ID 24 NAM00196	0.44	3.24	> 10	2.80	> 10	POSITIVE
ID 25 NAM00197	0.25	0.44	7.89	0.19	7.64	NEGATIVE
ID 26 NAM00198	2.53	3.38	6.40	0.85	3.87	POSITIVE
ID 27 NAM00199	0.17	0.35	0.43	0.18	0.26	INDETERMINATE
ID 28 NAM00200	0.21	0.24	> 10	0.03	> 10	NEGATIVE

Signature _Nelongo_

Date _Nelongo_ 3/09/12

Appendix G: Participant's IFN-ɣ levels (TB Ag-Nil) and QFT results at different time points

		Recruitment (before treatment)		2 Months on treatment		Completion of treatment (end of 6 month)	
Patient ID	HIV status	TB Ag-Nil (IU/mL)	QFT	TB Ag-Nil (IU/mL)	QFT	TB Ag-Nil (IU/mL)	QFT
NAM_S_00007	Neg	1.98	Pos	9.48	Pos	4.97	Pos
NAM_S_00013	Neg	1.3	Pos	1.14	Pos	0.91	Pos
NAM_S_00014	Neg	0.06	Neg	0.68	Pos	0.55	Pos
NAM_S_00010	Pos	0.02	Indet	0.06	Neg	0	Neg
NAM_S_00017	Neg	0.02	Neg	0.39	Pos	0.17	Neg
NAM_S_00021	Pos	0	Neg	0.05	Neg	0.02	Neg
NAM_S_00020	Pos	5.74	Pos	0.69	Pos	0.32	Neg
NAM_S_00030	Neg	-0.11	Neg	-0.08	Neg	-0.02	Neg
NAM_S_00031	Pos	-0.11	Pos	-0.08	Pos	-0.02	Neg
NAM_S_00036	Pos	9.11	Pos	0.07	Neg	0.06	Neg
NAM_S_00032	Neg	3.52	Pos	2.63	Pos	1	Pos
NAM_S_00054	Pos	-0.05	Indet	-0.02	Neg	0	Neg
NAM_S_00065	Pos	0.15	Neg	0.05	Neg	0.24	Neg
NAM_S_00068	Pos	-0.03	Neg	0.05	Neg	0.02	Neg
NAM_S_00072	Neg	2.62	Pos	1.91	Pos	N/A	N/A
NAM_S_00060	Pos	0.07	Neg	0.02	Neg	0.02	Neg
NAM_S_00071	Neg	7.76	Pos	1.04	Pos	0.09	Neg
NAM_S_00075	Pos	-0.09	Neg	0.01	Neg	0.07	Neg
NAM_S_00070	Pos	2.75	Pos	N/A	N/A	0.4	Pos