2019-04

Analysis of plasma biomarkers and their association with treatment response in patients with tuberculosis

Mvungi, Happiness Cornel

NM-AIST

https://doi.org/10.58694/20.500.12479/238

ANALYSIS OF PLASMA BIOMARKERS AND THEIR ASSOCIATION WITH TREATMENT RESPONSE IN PATIENTS WITH TUBERCULOSIS

Happiness Cornel Mvungi

A Dissertation submitted in partial fulfillment of the requirements for the Degree of Master's in Life Sciences of the Nelson Mandela African Institution of Science and Technology

Arusha, Tanzania

April, 2019

ABSTRACT

Human tuberculosis is a chronic inflammatory disease caused by *mycobacterium tuberculosis*. Pulmonary tuberculosis is the result of the failure of host immune system to control *mycobacterium tuberculosis*. The aim of the study was to analyze and associate plasma biomarkers that can be used in monitoring treatment in active pulmonary tuberculosis patients before and after the use of anti-TB therapy. Multiple cytokine responses in active tuberculosis (TB) patients were investigated in this study following anti-TB drug therapy after 2 months. Ninety-six participants with pulmonary TB were engaged in the study between May 2018 and October 2018. Samples of blood were taken early before treatment at 0 and 2 months after using anti-TB therapy. The levels of interferon-gamma (IFN)-γ, interleukin-4 (IL-4), IL-6, IL-10, and tumor necrosis factor (TNF)-α in whole blood plasma collected from the QuantiFERON-TB Gold Plus were measured. Compared with baseline levels, TNF-α, IL6 and IL10 were significantly lower following treatment whereas the IFN-γ and IL-4 increased significantly after treatment. The responses of five cytokines varied significantly after treatment ($P < 0.0001$) where IFN-γ was highest compared to other cytokines with 123.6%, AUC=0.757and $P < 0001$, TNF-α AUC = 0.529 and $P = 0.743$, IL-4 AUC = 0.557 and $P = 0.514$, IL-6 AUC = 0.629 and $P = 0.047$, IL-10 AUC = 0.549 and $P = 0.581$. It was concluded that changes of cytokines that observed during the treatment of TB patients play a very important role in monitoring pulmonary TB and can be suitable biomarkers to assess the effectiveness of anti-TB therapy in patients with TB.

.

DECLARATION

I, Happiness Cornel Mvungi, do hereby declare to the Senate of Nelson Mandela African Institution of Science and Technology that this dissertation is my own original work and that it has neither been submitted nor being concurrently submitted for degree award in any other institution.

Happiness Cornel Mvungi	**Date**
Name and signature of candidate	

The above declaration is confirmed by;

Dr. Elingarami S. Nkya	**Date**
Name and signature of Supervisor 1	

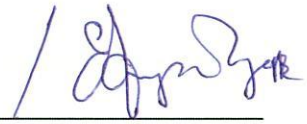
Dr. Stellah Mpagama	**Date**
Name and signature of supervisor 2	

COPYRIGHT

CERTIFICATION

The undersigned certify that has read and hereby recommend for acceptance by Nelson Mandela African Institution of Science and Technology a dissertation entitled: "Analysis of Plasma Biomarkers and their Association with Treatment Response in Patients with Tuberculosis". The dissertation is submitted by Happiness C. Mvungi in partial fulfillment of the requirements for degree of Masters in Life Sciences of Nelson Mandela African Institution of Science and Technology Arusha, Tanzania.

Approval of the Dissertation:



9th April 2019

Dr. Elingarami S. Nkya **Date**

Name and signature of Supervisor 1

8/4/2019

Dr. Stellah Mpagama **Date**

Name and signature of supervisor 2

ACKNOWLEDGMENT

First and foremost, I am very grateful to my supervisors for their great dedication, contribution, commitment and tireless guidance in providing invaluable professional guidance in this work which was made possible through great support provided by the following supervisors:

Dr Elingarami Nkya of Nelson Mandela African Institution of Science and Technology who tirelessly supervised this work from research proposal development, data collection and report writing.

Dr. Stellah Mpagama of Kibong'oto Infectious Diseases Hospital who was a close mentor and supervisor during this research work from data collection and report writing.

Miss Margareta Sariko a laboratory scientist at Kilimanjaro Clinical Research Institute who was a mentor during laboratory activities.

Special thanks also goes to Elifuraha Mngumi for his advice on statistical analysis.

I am greatly indebted to all members of Kibong'oto Infectious Diseases Hospital who contributed directly or indirectly to my capability to perform this work. Furthermore, I would like to express my sincere gratitude to CREATES for supporting me financially, including research funds, as they really played a big role in completion of this work. I also extend my special thanks to my respondents; the patients who willingly gave their time to answer the questions and provided their specimens for laboratory investigation. I have no words to thank my parents, my family, for showing me the value of education.

Above all, I thank the Almighty God for his blessings and for giving me strength, guidance and capacity to accomplish this work.

DEDICATION

This work is dedicated to the Almighty God, lecturers, my family, my husband Eng. Gerald Shayo for his immeasurable support, assistance and encouragement, and to my child Paris for bearing with my absence at the time I was needed most to make this work possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

AFB	Acid Fast Bacilli
ANOVA	Analysis of Variance
BCG	Bacille Calmette Guerin
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CFP-10	Culture Filtrate Protein 10
CI	Confidence Interval
CO	Carbon Mono-oxide
DNA	Deoxyribonucleic acid
DOTS	Directly Observed Therapy
EPTB	Extra pulmonary Tuberculosis
ESAT-6	Early Secreted Antigenic Target 6
HIV/AIDS	Human Immune Virus/Acquired Immune deficiency Syndrome
IFN-γ	Interferon Gamma
IGRA	Interferon Gamma Release Assays
IL-	Interleukin
LITB	Latently Infected TB
MDR	Multi-drug resistant
MHC	Major Histocompatibility Complex
Mtb	Mycobacterium tuberculosis
NO	Nitric Oxide
PCR	Polymerase chain reaction
PPD	Purified Protein Derivative
QFT	QuantiFERON Gold in Tube Test
Rcf	Relative centrifuge force
RPF	Resuscitation promoting factor
rRNA	ribosomal ribonucleic acid
RT	Room temperature
TB	Tuberculosis
Th-	T Helper cell
TNFα	Tumor Necrosis Factor alpha
WHO	World Health Organization

XDR	Extensively/Extremely Drug-Resistant
ZN	Ziehl -Neelsen

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Tuberculosis is a chronic inhalational infectious bacterial disease caused by Mycobacteria tuberculosis complex (MTBC). TB is the ninth leading cause of death worldwide, and the leading cause from a single infectious agent, ranking above HIV/AIDS. In 2016, there were an estimated 1.3 million TB deaths among HIV negative cases and an additional 374 000 deaths among HIV-positive cases (WHO, 2017).

Tanzania is among 30 countries in the world with highest burdens of TB having approximately 200 to 299 TB cases per 100 000 adult (WHO, 2017). Furthermore, about 5 percent of adults in Tanzania are living with HIV or AIDS, which according to the World Health Organization, makes them 26 or 31 times more likely to become infected with *Mycobacterium tuberculosis* due to their impaired immune system.

The most common method for diagnosing TB worldwide remains to be sputum smear microscopy developed more than 100 years ago, in which bacteria are observed in sputum samples examined under a microscope. However, developments in TB diagnostics in the last few years mean that the use of rapid molecular tests to diagnose TB and drug-resistant TB is increasing, and some countries are phasing out use of smear microscopy for diagnostic purposes. In countries with more developed laboratory capacity, cases of TB are also diagnosed by using culture methods. Treatment using drug sensitive Tuberculosis requires combination of four drugs namely; Rifampicin, Isonizid, Pyrazinamide and Ethambutol. These must be administered for six to nine months consecutively. During treatment, patients must be monitored after two and five months for anti-TB. In some cases, Tuberculosis has shown poorer therapeutic outcome with elevated mortality rate.

Biomarkers are objective characteristics that indicate a normal or pathogenic biological process or pharmacological response to a therapeutic intervention or vaccination (Atkinson *et al*., 2001). Sputum acid fast bacilli (AFB), and sputum culture are the most common TB biomarkers currently used to monitor drug sensitive TB patients. However, sputum acid fast bacilli (AFB) does not distinguish live and dead organisms and is not predictive of outcome or relapse. Meanwhile, culture takes 6 to 8 weeks to get the results. Thus, there is an urgent need to identify early biomarkers of treatment efficacy that could be used for patient

stratification and management. A number of immunological markers measured in blood plasma at the start of treatment have shown promising results as prognostic markers for clinical severity and/or predictors of microbiological outcome in TB patients. The inflammation process observed in patients with Tb is mediated by activation of the immune system, with excessive production of immune markers (cytokines/chemokines), such as IL-1, IL-2, IFN-γ, and TNF-α. The following cytokines/chemokines were therefore analyzed and associated with anti-TB treatment in this study; Interleukins (IL-6) and (IL-10), IL-4, gamma interferon (IFN-γ) and Tumor necrosis factor alpha (TNF-α).

Cytokines are proteins that can alter the behavior or properties of the cell itself or of another cell. These proteins are involved in the immunopathology of different diseases. Study of the cytokines in *Mycobacterium tuberculosis* infection is very important, as they participate in establishment, persistence and evolution of the infection. The intricate complexity of these regulating proteins stimulates the investigation in searching for more effective treatments that permit eradication of tuberculosis, which is one of the leading causes of mortality and morbidity worldwide despite efforts made by the scientific community.

1.2 Problem Statement and Justification

Tuberculosis (TB) has existed for millennia and remains a major global health problem. It causes ill-health for approximately 10 million people each year and is one of the top ten causes of death worldwide (ref). For the past 5 years, TB has been the leading cause of deaths from a single infectious agent, ranking above is HIV/AIDS (WHO, 2017). Tanzania is among the 30 countries having the highest TB burden in the world attributed to challenges in diagnosis, treatment and monitoring. During treatment, patients should be monitored after two and five months.

The most common TB biomarkers currently used to monitor Drug sensitive TB patients in Tanzania and Worldwide in general are Sputum AFB and Sputum culture. However, sputum AFB does not distinguish live and dead organisms and is not predictive of outcome or relapse. It is also relatively insensitive, as at least 5000 bacilli per milliliter of sputum are required for direct microscopy to be positive. On the other hand, sputum culture takes about 2 months to get the results, and its sensitivity is limited as it requires about 100 bacilli per milliliter of sputum to yield a positive culture. Response to drug sensitive TB treatment has mostly based on the decrease of acid-fast bacilli in sputum during anti-TB chemotherapy. TB patients on treatment are thus required to produce sputum for AFB examination at second and

fifth month of therapy. As for drug resistant tuberculosis sputum, culture is evaluated every month during treatment.

Most TB patients cannot produce sputum after two months of anti-TB therapy, primarily because coughing ceases or is reduced dramatically, making expectorating difficult. Children as well cannot produce sputum, and therefore prediction of their therapeutic response becomes difficult. It is also difficult to use sputum for smear or culture in extra pulmonary TB as a marker that can predict its treatment response, because it is not applicable in the context of extra-pulmonary tuberculosis. Hence, a blood-based biomarker would be ideal, as it is easier to obtain blood and assessment of immunological parameters can be done at second and fifth month of anti-TB treatment.

Despite the ongoing global TB problem and extensive research into protective immunity against this intracellular pathogen, mechanisms for protective immunity against *Mtb* in humans have not been fully clarified.

This proposed study therefore aimed to identify potential plasma biomarkers that can be used in monitoring patient's prognosis 2 months after the start of anti- TB treatment, in a bid to stratify patient risk and optimize TB patient care.

1.3 Objective of the Study

1.3.1 General Objective

The general objective of this study was to analyse and associate plasma biomarkers (IL-6, IL-10, IL-4, IFN-γ and TNF-α) with treatment response in patients with tuberculosis.

1.3.2 Specific Objectives

(i) To compare plasma markers (IL-6, IL-10, IL-4, IFN-γ, and TNF-α) at baseline and 2 months of anti-TB treatment.

(ii) To determine plasma biomarkers (IL-6, IL-10, IL-4, IFN-γ, TNF-α) that can predict favorable treatment outcome.

(iii) To compare clinical presentation/symptoms at baseline and two months after anti-TB treatment.

1.4 Research Question

The study addressed the following question: “Do plasma biomarkers have a role when monitoring treatment response in patients with tuberculosis?”

1.5 Significance of the Study

Results from this study will provide baseline information on improving management of tuberculosis patients, by avoiding diagnosis delays and failure of anti-tuberculosis treatment, hence reduction of infection transmission and death. Furthermore, studied blood plasma biomarkers will serve as treatment biomarkers for patients who cannot expectorate during follow up, and those patients with Extra pulmonary tuberculosis (EPTB) to know their prognosis.

1.6 Expected Outcome

The expected outcomes of this study are:

(i) Improved management of tuberculosis patients, by avoiding diagnosis delays and failure of anti-tuberculosis treatment, hence reduction of transmission of infection to others and death.

(ii) Improved knowledge of studied blood plasma biomarkers will serve patients who cannot expectorate during follow up, and those patients with EPTB to know their prognosis.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

TB is a chronic infectious disease caused by the bacillus *Mycobacterium tuberculosis*. It typically affects the lungs (pulmonary TB) but can also affect other sites (extra pulmonary TB). TB causes ill-health in millions of people each year and was one of the top 10 causes of death in 2017 worldwide, ranking above HIV/AIDS as one of the leading causes of death from infectious diseases. The best estimate is that there were 1.3 million TB deaths among HIV negative people (down from 1.7 million in 2000) and an additional 374 000 deaths among HIV-positive people (WHO, 2017). An estimated 10.4 million people (90% adults; 65% male; 10% people living with HIV) fell ill with TB in 2016 (WHO, 2017). Treatment of drug sensitive Tuberculosis requires combination of four drugs Rifampicin, Isonizid, Pyrazinamide, Ethambutol and must be administered for six to nine months. Patients must be monitored after two and five months of anti-TB drugs during treatment because of we want to see the prognosis of the patient and the bacteriological status.

WHO has recommended sputum-smear examination at the end of second and five months of treatment in patients diagnosed with pulmonary tuberculosis, as a predictive marker for treatment outcome, this being the only marker that is available. Both sputum-smear microscopy and mycobacterial culture during tuberculosis treatment have low sensitivity and modest specificity for predicting failure and relapse (Horne *et al*., 2010). In addition, Sputum culture is the Gold standard for TB diagnosis and monitoring of multidrug resistant tuberculosis patients. However, its sensitivity is limited as it requires about 100 bacilli per ml of sputum to yield a positive culture (Van Deun *et al*., 2004). A negative result can be obtained because of low bacterial numbers resulting from paucibacillary sputum or decreased bacilli load resulting from the decontamination process. Insufficient follow-up of TB treatment poses a major threat for individual patients due to variable treatment outcomes and therefore, call for new approaches such as using plasma biomarkers to monitor tuberculosis treatment outcome responses. Measurement of *Mycobacterium tuberculosis* specific antigen induced responses in blood has been proposed as a better option, hence characterization of cellular response to *Mycobacterium tuberculosis* antigens during treatment was the major focus of this study.

2.2 Human immune response to tuberculosis infection

Recent understanding of the roles played by leukocytes and the cytokines has revealed delicacy underlying balance between the strategies used by *M. tuberculosis* to survive within a host and concomitant efforts of the host to kill it.

The main route of entry for the bacillus into the body is via the respiratory tract through inhalation of infectious droplet nuclei. Only small sized droplet nuclei are able to gain entry into the lower respiratory tract while larger ones are excluded by physical barriers of the nasopharynx and upper respiratory tract (Paper, 1995). Alveoli is the first point of entry for the bacilli where they interact with phagocytic cells, such as macrophages and dendritic cells through different receptors (Ernst, 2012).

Once the bacilli lodges into the lungs may assume either of the four potential fates (Dannenberg, 1994). These includes; initial host response that clears the bacilli with no chance of developing tuberculosis at any time in the future or organisms can begin to multiply and immediately resulting into a clinical disease (primary tuberculosis). Furthermore, the bacilli may become dormant, contained in the lung granuloma and never cause the disease with patient referred to have latent infection. This is manifested only by a positive tuberculin skin test. The last fate is the latent organisms that eventually begin to multiply resulting into clinical disease.

Studies have shown that there was a 5 to 10% chance over a lifetime of developing active disease (Of *et al*., 2014). However, latent infection in severely compromised patients with HIV/AIDS have 7% chance for developing clinical tuberculosis annually (Selwyn *et al*., 1989).

Reactivation is where the latent bacilli exit the dormancy mode through resuscitation to establish active infection as reported by Schluger *et al*. (2010). However, recent advances in studying the immune response to TB suggest a paradigm shift from this old model of well-defined outcomes towards a view of Mtb infection outcome as a continuous spectrum generated by a range of lesions providing multiple microenvironments that support bacterial replication, persistence or killing as reported by Barry *et al*. (2009). This continuous spectrum extends from sterilizing immunity, to subclinical active disease, to fulminate active disease, with conventional designations of latent infection and active disease corresponding to partially overlapping regions of biological heterogeneity (Young *et al*., 2009).

2.3 Innate immune response to tuberculosis

In humans, upon entry of mycobacterium into the body, the *Mtb* components are recognized by multiple pattern recognition receptors (PRR) of the host, including toll like receptors (TLR), specific members of the C-type lectin receptor (CLR) family, including DC-SIGN, dectin 1, the mannose receptor and Mincle-monocyte-inducible C-type lectin (Ernst, 2012). The stimulation of these receptors either individually or collectively induces the expression of pro-inflammatory cytokines, selected chemokines and cell adhesion receptors that contribute to local and systemic immune cell mobilization and activation.

Phagocytic cells engulf the invading microbes in a membrane-bound tight vacuole created when the pseudopods surround the bacterium and fuse distally. Engulfment inside the phagosome leads to killing of pathogenic bacteria via several pathways; including fusion of the phagosome with lysosome to form the phagolysosome, resulting in release of cytotoxic granules, generation of reactive oxygen intermediaries (ROIs) and reactive nitrogen intermediaries (RNIs) as reported by Schluger *et al*. (2009).

However *Mycobacterium tuberculosis*, accomplishes intracellular survival through several evasion strategies, including neutralization of the phagosomal pH and interference with autophagy, which serves as a cell autonomous defense mechanism by Gutierrez *et al*. (2004).

In contrast to other infectious diseases, where the recruitment of phagocytic cells restricts and even eliminates invading pathogens, the recruitment of phagocytes to sites of mycobacterial infection actually benefits the pathogen during the early stages of infection, by providing additional cellular niches for bacterial population expansion by Davis *et al*. (2009).

2.4 Adaptive immune response to tuberculosis

The onset of the adaptive immune response to *Mycobacterium tuberculosis* is delayed compared with that of other infections or immunization, that allows the bacterial population in the lungs to expand markedly during the pre- immune phase of infection by Wolf *et al*. (2008).

Subsequently, most human become asymptomatic, do not shed bacteria and are considered to have latent TB infection (Ernst, 2012) as defined by detectable memory *Mtb*-specific T cell response, signifying the important role of lymphocytes as co-effectors in mycobacterial host defense.

Adaptive immune responses are mediated by B and T lymphocytes. Adaptive immune responses mediated by T cells play a vital role in the elimination of *M. tuberculosis* (Joanne *et al*., 2001). Cytotoxicity and cytokine production are the two major effector mechanisms utilized by T cells against intracellular pathogens. Among various cytokines, the role of IFN-g in *Mtb* infection was demonstrated in various experiments as reported by (Flynn, 1993).

The T cells are involved in protective anti–TB immunity evidenced from studies on TB in mice, in which the expression of immunity can be measured in terms of control of infection in major organs in the absence of selected T cell subpopulations (Cooper *et al*., 1997). On the other hand, our knowledge on the T cells involved in immunity to TB in humans is based on correlative evidence that comes from experiments designed to identify T cells that respond to appropriately presented *Mtb* antigens in vitro as reported by Mogues *et al*. (2001).

2.5 Biomarkers for TB

Biomarkers are objective characteristics that indicate a normal or pathogenic biological process or pharmacological response to therapeutic intervention or vaccination as defined by Atkinson *et al*. (2001). They can provide information about disease status, risk of progression, likelihood of response to treatment or of drug toxicity and protective immunity after vaccination. Biomarkers are useful as surrogate endpoints in clinical trials, replacing typical clinical endpoints that describe how a patient feels, functions or survives (Wallis *et al*., 2009). The requirement for biomarkers in TB stems from two critical features of human *Mtb* infection: its long and varied natural history, and essential role played by minority bacillary sub-populations. Non-replicating persisters are thought to be the main impediment to shortening therapy, because they are relatively unaffected by most TB drugs (Rao *et al*., 2008).

Both human and *Mtb* biomarker studies have focused on three specific areas of research over the past decade: biomarkers predicting treatment efficacy and cure of active TB, the reactivation of latent tuberculosis infection and induction of protective immune responses by vaccination (Walzl *et al*., 2011). This study was set up to provide new information that will be useful for studies aimed at looking for immunological biomarkers predicting early treatment response and cure of tuberculosis.

2.6 Biomarkers predicting treatment response and cure

Biomarkers for TB treatment response and cure are urgently required for proper management of patients, as well as for clinical trials of novel drugs and vaccines. For the TB patients, 6 to 8 months of anti-TB therapy makes adherence very difficult and this long duration also puts pressure on health care systems in developing countries as reported by Wallis *et al*. (2009). Tools such as plasma biomarkers that provides an early indication of treatment efficacy during chemotherapy or markers that stratify patients into risk groups requiring different durations of treatment even prior to the start of therapy would improve therapeutic strategies and possibly reduce drug resistance due to non-adherence. Multi-drug resistant (MDR)-TB poses a threat to global control of TB this was reported by Lange *et al*. (2014) and has reinforced the need to find biomarkers that indicate relapse-free treatment success. It will also make it easier to focus more attention on patients who have a high risk of poor treatment outcomes and ease pressure on healthcare systems, especially in developing countries this was reported by Wallis *et al*. (2009).

Chemokines and cytokines are key molecules that regulate immunological responses, and have been extensively studied in relation with their potential as diagnostic and prognostic biomarkers for tuberculosis (Markos and Abebe, 2013).

Several studies have reported that the level of cytokines and chemokine in unstimulated plasma or after stimulation with mycobacterium antigens could be used as an additional or alternative to the existing tests in development of a rapid, sensitive and user-friendly test for monitoring effective anti-tuberculosis therapy. Currently, polyfunctional CD4 and CD8 T cells expressing multiple cytokines (IL-2, TNF-α and IFN-γ) are being increasingly studied as these cells may be involved in mediating protection and curative host responses in TB (Caccamo *et al*., 2010)

In vitro levels of IL-10 and ratio of IFN- γ/IL-10 in response to a recombinant 32-kilodalton antigen of *M. bovis* BCG has been reported as a good marker in monitoring treatment of TB, where the level of IL-10 decreases and the ratio of IFN-γ/IL-10 increases after the treatment (Sai *et al*., 2010). Another study reported utility of serial quantitative T-cell responses as treatment monitoring tools as they showed a significant decline in IFN-γ in the quantitative result for both QFT-IT and T-SPOT.TB® after treatment (Chee *et al*., 2010). Other study has also showed that the IP-10 response to RD1 selected peptides (similar to IFN-gamma) might

be a useful biomarker for monitoring therapeutic efficacy in patients with active TB this was reported by Syed *et al*. (2011).

Several research studies have looked on the role of IFN-γ levels as markers for monitoring anti- TB therapy. However, these studies have reported varied results, mainly because a complex network of other cytokines are involved and therefore, studies involving multiple cytokines are needed.

Gamma interferon and IL-10 are considered among the main cytokines responsible for protection and pathogenesis of TB, respectively. For example; High IFN-γ/IL-10 ratios have been shown to strongly correlate with protection and TB cure, whereas low ratios correlate with disease severity. Interleukin -10 has been identified as an important clinical biomarker for TB disease progression, as high levels at the end of treatment may function as a risk factor for TB recurrence. Moreover, another association study by Xiong *et al*. (2016) demonstrated that IL-6 was closely associated with TB patients, suggesting that IL-6 can serve as a valuable biomarker for distinguishing TB patients from healthy individuals.

Tumor necrosis factor alpha has also been investigated in many studies on its role in immune response during TB infection. It has been found that serum TNF-α levels are raised as treatment progresses. However, TNF-α levels have been found to increase in severe TB, due primarily to the fact that the initiation of therapy in individuals with severe TB often begins with clinical deterioration before occurrence of clinical improvement.

2.6.1 Interferon gamma (IFN-γ)

The cytokine interferon- gamma plays a pivotal role in protective immunity against intracellular pathogens (Lalvani *et al*., 2008). Specifically, IFN-γ is an important mediator of macrophage activation (Flynn, 1993) due to its critical role in inducing macrophage synthesis of enzyme inducible nitric oxide synthase (NOS2). Upon secretion by activated CD4 T cells, IFN-γ activates macrophages to generate nitric oxide and other reactive nitrogen intermediates (RNIs), the best characterized anti-tuberculous effector molecules in humans (Nicholson *et al*., 1996).

Since the strength of host immune response against *Mtb* infection is directly proportional to the level of cellular CD4+ production of IFN-γ (Feng *et al.,* 1999). IFN-γ level has been widely used for diagnosis of TB infection following stimulation with *Mtb* specific antigens (Goldsack *et al*., 2007) .

Due to its pivotal role in TB pathology, several studies have looked into the role of IFN-γ levels in monitoring reaction to anti- TB therapy. The increased level of IFN-γ during anti-TB treatment has also been demonstrated in other studies carried out in patients with TB (Torres 1998; Turner and Dockrell, 2000). These studies however, reported varied results mainly because it is known that IFN-γ plays an important role against *Mtb* infection, a complex network of other cytokines involvement (Lalvani and Millington, 2008) with multiple cytokines that may need further studies.

2.6.2 Tumor necrosis factor alpha (TNF-α)

Tumor necrosis factor alpha (TNF-α) is one of the most important pro-inflammatory cytokines, critical to the control of tuberculosis infection prior to initiation of the adaptive immune response. It is produced mainly by macrophages in response to stimuli activating toll-like receptors, but can as well be expressed by activating T cells, B cells, and NK cells (Old, 1988). In concert with IFN-γ, it increases the phagocytic ability of macrophages and enhances the killing of mycobacteria, and may also induce apoptosis of permissive macrophages (Bekker *et al*., 2000). Baseline levels of TNF-α are thus thought to be low in peripheral blood and high at the sites of infection during the early phase of active tuberculosis infection. The importance of TNF-α especially in the early levels of TB infection had long been shown in mouse experiments proving that they play a vital part in the establishment of early granuloma (Bean *et al*., 2018). This was also reported by Biology (1999) but the cell was unstimulated. However, the observation that there was an increased incidence of TB in persons given anti-TNF-α treatment for autoimmune diseases was easily reinforced the protective function of TNF-α in TB in human (Stenger *et al*., 2005). TNF-α has been investigated in many studies exploring the immune response during TB infection and it has been found that during the early stages of the disease, serum TNF-α levels are high and decrease as treatment progresses (Kim *et al*., 2014). However, TNF-α levels have been found to increase transiently in severe TB, due primarily to the fact that the initiation of therapy in individuals with severe TB often begins with clinical deterioration (even death) before improvement occurs (Bekker *et al*., 1998).

Most studies on TB-antigen stimulated TNF-α responses reported a reduction in TNF-α concentrations during treatment. This was consistent with study that used flow cytometry to measure T-cell TNF-α responses (Kim *et al.,* 2014).

2.6.3 Interleukin 10 (IL-10)

Interleukin-10 is a potent immunomodulatory cytokine that has been shown in vitro to directly or indirectly affect multiple cell types, including macrophages, monocytes, dendritic cells, CD4 T cells, and CD8 T cells (Moore *et al*., 2001). Its main biological function seems to be limitation and termination of inflammatory responses and regulation of differentiation and proliferation of several immune cells, such as T cells, B cells, natural killer cells, antigen-presenting cells, mast cells, and granulocytes (Asadullah *et al*., 2003). Produced by macrophages and T lymphocytes during infection with *Mtb,* IL-10 reduces the secretion of interferon-gamma by T-cells through the negative regulation of IL-12 production and co-stimulatory molecule expression (Kaphingst *et al*., 2010). It also has a TNF-α opposite effect by protecting against tissue damage and regulating inflammation and apoptosis (Rojas *et al*., 1999). The interplay between IFN-γ and IL-10 is so critical in that the IFNγ/IL10 ratio provides a useful objective marker for disease activity in TB and can be important in disease management (Jamil *et al*., 2007). High IFN-γ/IL-10 ratios strongly correlate with protection and TB cure, whereas low ratios correlate with disease severity.

Recently, the secretion of *Mycobacterium tuberculosis* (MTB) enhanced intracellular survival protein from MTB cells was reported, which possibly increased IL-10 expression (Eum *et al*., 2010).

2.6.4 Interleukin 6 (IL-6)

Interleukin-6 is an important cytokine whose serum levels are commonly high in active pulmonary tuberculosis. IL-6 screening in patients with TB may be useful in monitoring the progress of infectious process and inferring the risk of progression to active disease (Henrique *et al*., 2013). Interleukin-6 has been noted for having a relevant role in the immunopathogenesis of tuberculosis. For instance, it is known for stimulating the secretion of IFN-gamma, a crucial cytokine in the activation of macrophages infected with *Mtb*, although the precise mechanism for such interaction still requires further clarification (Saunders *et al*., 2000).

It is also secreted by macrophages infected with *Mycobacterium tuberculosis*, and is able to inhibit the response to INF-gamma by non-infected macrophages adjacent to infected ones (Nagabhushanam *et al*., 2018). These results revealed that IL-6 may be involved in inability of the cellular immune response to eradicate the infection. This cytokine can also be harmful

to mycobacterial infections, in so far as it is able to inhibit the production of TNF-α and interleukin-1 beta (Schindler *et al*., 1990). However, some study that predominantly included HIV-positive patients found no changes in IL-6 levels in tuberculosis patients (Silva *et al*., 2013).

2.6.5 Interleukin 4 (IL-4)

In tuberculosis, cell-mediated immunity is responsible for eradication of mycobacteria. The major mechanism for cell-mediated immunity is thought to activate the infected macrophages by Th1-type cytokines, particularly interferon gamma. The protective effects of Th1-type cytokines may be antagonized by Th2-type cytokines, primarily interleukin -4 (Lucey *et al*., 1996).

The deleterious role of IL-4 in various infectious diseases, including leprosy and leishmaniasis, has been ascribed to its suppression of the protective inflammatory response of Th1-type cytokines (Lucey *et al*., 1996).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

The study was conducted at Kibong'oto Infectious Diseases Hospital (KIDH) located at Siha district in Kilimanjaro region. This is a National Tuberculosis hospital that receives patients from all over the country especially complicated cases who are referred to this facility.

3.2 Study Design

This was a prospective cohort study that involved new patients diagnosed with pulmonary tuberculosis. These patients were followed for two months, with measurements of their plasma immune markers (IL-6, IL-10, IFN-γ, TNF-α and IL-4) before and after two months of anti-TB treatment.

3.3 Study Population

The study population comprised of new smear positive pulmonary TB patients aged 18 years and above who were diagnosed at Kibong'oto Infectious Disease hospital during the study period.

3.4 Inclusion Criteria

TB patients 18 years of age or older and newly diagnosed with sputum smear positive pulmonary TB, including those co-infected with HIV and had ability to give informed consent.

3.5 Exclusion Criteria

All Patients with extra pulmonary TB and who are very sick i.e. unconscious were excluded.

3.6 Administration of Informed Consent

Details of the study were discussed with potential study participants. These participants were then given the informed consent form to read or the contents were translated into their local dialect for them to read and consent. Participants were assured of the confidentiality of the information and laboratory investigation results and that their participation in the study was voluntary and they were free to withdrawal without any negative impact. This consent form

was approved by the National Institute for Medical Research, NIMR. Those who were satisfied with the explanation of the purpose of the research study and agreed to enroll in the study were asked to sign or thumb-print the consent form. Details of sex, age, contact details, HIV status, and previous history of tuberculosis were taken using a semi-structured questionnaire.

3.7 HIV Testing

All study participants were offered voluntary counseling for HIV testing by trained health personnel in accordance with the National TB control programme guidelines at the health facilities. Appropriate post- test counseling and further treatment advice was offered to those with positive results.

3.8 Sample Size

The desired sample size was 96 patients with total of 192 samples. Before starting antitb's total of 96 samples were collected and after two months of treatment another 96 samples were also collected. The number of sample size was according to the budget was given.

3.9 Sample Collection

3.9.1 Blood collection, in vitro stimulation and Plasma preparation

Blood samples were taken at two-point intervals; before treatment (baseline), and at 2 months post TB treatment. From each participant, 4mls of venous blood was collected using a syringe and immediately 1ml of blood was dispensed in each 4 QFT-plus blood collection tubes (Nil, TB1, TB2 and Mitogen) labeled with participant unique identification number. A 4-tube system of QFT was used, including the negative control tube (Nil tube), positive control tube (Mitogen tube), and TB-antigen tube (TB 1 and TB 2 tubes). Immediately after filling the tubes, they were shaken ten times firmly enough to ensure the entire inner surface of the tube was coated with blood, to dissolve antigens on tube walls at 25°C. Tubes were transferred to a 37°C incubator for 16 to 24 hours. After incubation, the tubes were centrifuged for 15 minutes at 3000 rcf and lastly plasma samples were harvested by using a pipette and they were transferred into cryotubes in which they were stored at -20°C until analysis.

3.9.2 Simultaneous analysis of cytokine/chemokine biomarkers using Luminex Immunology Multiplex Assay

The Multiplex Map Kit was used to quantify 5 different human cytokines and chemokines, in accordance with the manufacturer's instructions. The 5-plex consisted of IFN-y, IL-10, IL-4, IL-6, and TNFα. The assay sensitivities for the 5-plex markers ranged from 0.1 – 0.4 pg./ml, and 5 pg./ml for IL-2Rα and Granzyme B.

3.10 Reagent Preparation

3.10.1 Wash Buffer

In preparation of the wash buffer, 20 mls of wash buffer concentrates were added into 480 mls of distilled water to prepare 500 mls of wash buffer.

3.10.2 Diluted Microparticle cocktail Preparation

The microparticle cocktail vial was centrifuged for 30 seconds at 1000 g prior to cap removal, followed by gentle vial vortexing to suspend the microparticles. The microparticles cocktail of 500 microliter was diluted by using Diluent RD2-15ml in the mixing bottle provided.

3.10.3 Diluted Biotin Antibody cocktail preparation

The Biotin Antibody cocktail vial was centrifuged for 30 seconds at 1000 g prior to remove of the cap, followed by gentle vial vortexing with precaution not to invert the vial. The Biotin Antibody cocktail 500 microliter was then diluted by using Diluent RD2 5 mls, followed by gentle mixing.

3.10.4 Streptavidin- PE Preparation

The polypropylene test tube was wrapped with aluminum foil to protect the streptavidin-PE from light during handling and storage. The Streptavidin-PE vial was centrifuged for 30 seconds at 1000 g prior to remove of the cap, followed by gentle vial vortexing and 220 microliters of Streptavidin-PE concentrate was then diluted by using 5.35 mls wash buffer (Fig. 3).

Figure 1: Streptavidin- PE Preparations

3.11 Measurements of Biomarkers

Measurement of biomarkers was done after preparing all the reagents. The following steps were involved in assay procedure:

(i) 50 microliters of sample were added to each well, and a plate layout was provided to record standards and samples assayed.

(ii) The diluted microparticle cocktail was resuspended by vortexing, and then 50 microliters of microparticle cocktail was added into each well of the microplate. The microplate was securely covered with a foil plate sealer, incubated for 2 hours at room temperature on a microplate shaker set at 800 rpm. After that, it was washed by applying the magnet to the bottom of the microplate, to allow 1 minute before

removing the liquid from each well filled with 100 microliter wash buffer and removing the liquid again. The washing procedure was done three times according to manufacturer's instructions.

(iii) After washing, 50 microliters of diluted Biotin-Antibody were added to each well, and the microplate was securely covered with foil plate sealer and incubated for 1 hour at room temperature on the shaker set at 800 rpm. After that, it was washed again by applying the magnet to the bottom of the microplate, to allow 1 minute before removing the liquid from each well filled with 100 microliter wash buffer and removing the liquid again. The wash procedure was done three times again.

(iv) Fifty microliters of diluted Streptavidin-PE were added to each well securely covered with a foil plate sealer and incubated for 30 minutes at room temperature on the shaker set at 800 rpm.

(v) Microplate was washed again by applying the magnet to the bottom of the microplate for1 minute before removing the liquid from each well filled with 100 microliters of wash buffer and removing the liquid again. The washing procedure was again done three times.

(vi) 100 microliters of wash buffer were then added to each well, incubated for 2 minutes at room temperature on a shaker at 800 rpm. Plates were finally read with a Luminex instrument (Luminex 200) within 90 minutes.

3.12 Statistical Analysis

Data analysis consisted of computing the descriptive statistics for each study variable, and then comparing them. To describe the demographic and clinical characteristics, we used proportions or means. Analysis of descriptive and inferential statistics was done by using GraphPad Prism version 3.02. The baseline data were compared with final data using *Wilcoxon Matched Pair test*. Furthermore, *Mann Whitney test* was used to compare male and female cytokine levels. The responses of five cytokines were compared using *Friedman test* – which is a repetitive non-parametric One-way ANOVA alternative. In each case, a significant level was set at 5% with 95% CI. The discriminative power of each biomarker for 2-months smear status was analyzed using the receiver-operating characteristic (ROC) curve and area under the ROC curve. The optimal cut-off value was set according to Youden's index, which depended on the maximized value of sensitivity plus specificity minus one.

The power of cytokines to discriminate infection was tested by doing Receiver Operating Characteristic (ROC) curve analysis, where the AUC of 0.5 was considered. Receiver Operating Characteristic curve analysis was done using MedCalc version 18.10 − © 1993-2018 MedCalc Software bvba. Logistic regression analysis was used to identify cytokines associated with persistent smear positivity after 2 months of anti-TB treatment. Cytokine levels were transformed into binary variables (according to Youden's index) before being entered into the logistic regression analysis.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Participants' characteristics

All ninety-six (96) participants were sputum smear positive, diagnosed by GeneXpert. During monitoring at month two, sputum for microscopy AFB was used where ninety-five patients were smear negative and one was smear positive (Table 1).

Table 1: Participant's characteristics

Participant	Age	Gender	GeneXpert results at diagnosis	Smear result at Month 2
1	43	M	Mtb detected medium	Negative
2	23	M	mtb detected high	Negative
3	30	F	mtb dwtected high	Negative
4	25	M	mtb detected medium	Negative
5	20	M	mtb detected medium	Negative
6	23	M	mtb detected low	Negative
7	48	M	mtb detected medium	Negative
8	26	F	mtb detected medium	Negative
9	33	M	mtb detected high	Negative
10	37	F	mtb detected low	Negative
11	19	M	mtb detected medium	Negative
12	40	M	mtb detected very low	Negative
13	22	M	mtb detected medium	Negative
14	25	M	mtb detected very low	Negative
15	23	M	mtb detected medium	Negative
16	28	M	mtb detected high	Negative
17	24	M	mtb detected low	Negative
18	20	F	mtb detected medium	Negative
19	27	F	mtb detected low	Negative
20	42	M	mtb detected high	Scant (Positive)
21	35	F	mtb detected high	Negative
22	21	M	Mtb detected medium	Negative
23	29	M	mtb detected very low	Negative
24	26	M	mtb detected high	Negative
25	38	M	mtb detected high	Negative
26	35	M	mtb detected low	Negative
27	29	M	mtb detected high	Negative
28	33	M	mtb detected very low	Negative
29	30	M	Mtb detected medium	Negative
30	28	F	mtb detected high	Negative
31	45	M	mtb detected low	Negative
32	32	M	mtb detected high	Negative
33	27	M	mtb detected high	Negative
34	28	M	mtb detected very low	Negative
35	39	M	Mtb detected medium	Negative

Participant	Age	Gender	GeneXpert results at diagnosis	Smear result at Month 2
36	22	M	Mtb detected medium	Negative
37	31	M	mtb detected high	Negative
38	36	M	mtb detected very low	Negative
39	24	M	mtb detected low	Negative
40	26	M	mtb detected low	Negative
41	40	M	Mtb detected medium	Negative
42	37	F	mtb detected low	Negative
43	33	M	mtb detected very low	Negative
44	39	M	Mtb detected medium	Negative
45	22	F	mtb detected very low	Negative
46	35	M	Mtb detected medium	Negative
47	41	M	Mtb detected medium	Negative
48	26	M	mtb detected high	Negative
49	29	M	mtb detected low	Negative
50	25	M	mtb detected high	Negative
51	26	M	Mtb detected medium	Negative
52	31	M	mtb detected low	Negative
53	34	M	mtb detected high	Negative
54	25	M	mtb detected very low	Negative
55	38	F	Mtb detected medium	Negative
56	30	M	mtb detected low	Negative
57	27	M	Mtb detected medium	Negative
58	29	M	Mtb detected medium	Negative
59	20	M	mtb detected high	Negative
60	37	M	Mtb detected medium	Negative
61	22	M	mtb detected low	Negative
62	29	M	Mtb detected medium	Negative
63	31	F	mtb detected very low	Negative
64	46	M	mtb detected high	Negative
65	23	M	Mtb detected medium	Negative
66	28	M	mtb detected high	Negative
67	47	M	Mtb detected medium	Negative
68	26	M	mtb detected very low	Negative
69	25	M	mtb detected high	Negative
70	24	M	mtb detected high	Negative
71	29	M	Mtb detected medium	Negative
72	31	M	mtb detected very low	Negative
73	23	F	Mtb detected medium	Negative
74	27	M	Mtb detected medium	Negative
75	33	F	Mtb detected medium	Negative
76	43	M	Mtb detected medium	Negative
77	37	M	mtb detected very low	Negative
78	33	F	mtb detected high	Negative
79	27	M	mtb detected low	Negative
80	26	M	mtb detected high	Negative
81	29	M	Mtb detected medium	Negative
82	36	F	mtb detected very low	Negative

Participant	Age	Gender	GeneXpert results at diagnosis	Smear result at Month 2
83	42	M	mtb detected very low	Negative
84	34	M	Mtb detected medium	Negative
85	26	F	Mtb detected medium	Negative
86	48	M	mtb detected high	Negative
87	37	M	Mtb detected medium	Negative
88	44	M	mtb detected high	Negative
89	22	M	mtb detected low	Negative
90	49	M	Mtb detected medium	Negative
91	28	F	mtb detected high	Negative
92	33	M	mtb detected high	Negative
93	38	M	Mtb detected medium	Negative
94	35	F	Mtb detected medium	Negative
95	32	M	mtb detected low	Negative
96	30	F	Mtb detected medium	Negative

4.2 Comparing plasma biomarkers at baseline and two months of anti TB treatment

Plasma biomarkers were investigated before starting treatment and after two months of treatment to see if there were any changes or not.

The plasma biomarkers considered under the current study includes, IL-6, IL-10, IL-4, IFN-γ and TNF-α. The levels of different cytokines at the beginning and at 2 months after starting treatment have shown significant changes at each level (Table 2). The treatment response of tuberculosis patients resulted in increased IFNg and IL4 with decreased TNF, IL6 and IL10 (Fig. 4).

Table 2: Descriptive summary of the cytokine's levels before and after medication

Statistics	*Case Scores*		*IFNg*		*TNF*		*IL4*		*IL6*		*IL10*	
	Baseline	***Final***	***Baseline***	***Final***	***Baseline***	***Final***	***Baseline***	***Final***	***Baseline***	***Final***	***Baseline***	***Final***
Sample size	96	96	96	96	96	96	96	96	96	96	96	96
Symptomatic patients	96	15										
Minimum	3	0	0.5	3.5	87	34.5	0.2	0.5	40	30	44	36
25% Percentile	4	0	3	10.25	105	63.5	1.6	1.7	75.75	46.5	75	50.75
Median	4	0	6.75	16.25	122	76	2.5	2.8	88.25	56.5	89.75	61
75% Percentile	4	0	38.25	50	192.5	85.5	5.2	5.2	105.5	69.75	101	70
Maximum	5	2	63	80	12265	10988	55	58	162	90	411	275
Mean	3.99	0.18	16.65	28.49	409.5	289.2	4.87	5.13	91.61	57.92	92.26	63.09
Std. Deviation	0.55	0.41	18.21	22.75	1654	1487	7.9	8.17	24.29	15	37.8	24.99
Std. Error	0.06	0.04	1.86	2.32	168.8	151.8	0.81	0.83	2.48	1.53	3.86	2.55
Lower 95% CI	3.878	0.09396	12.96	23.88	74.38	-12.06	3.27	3.475	86.69	54.88	84.6	58.03
Upper 95% CI	4.102	0.2602	20.34	33.1	744.5	590.6	6.472	6.784	96.54	60.95	99.92	68.15
P value	*P*<0.0001		*P*<0.0001		*P*<0.0001		*P*<0.0001		*P*<0.0001		*P*<0.0001	

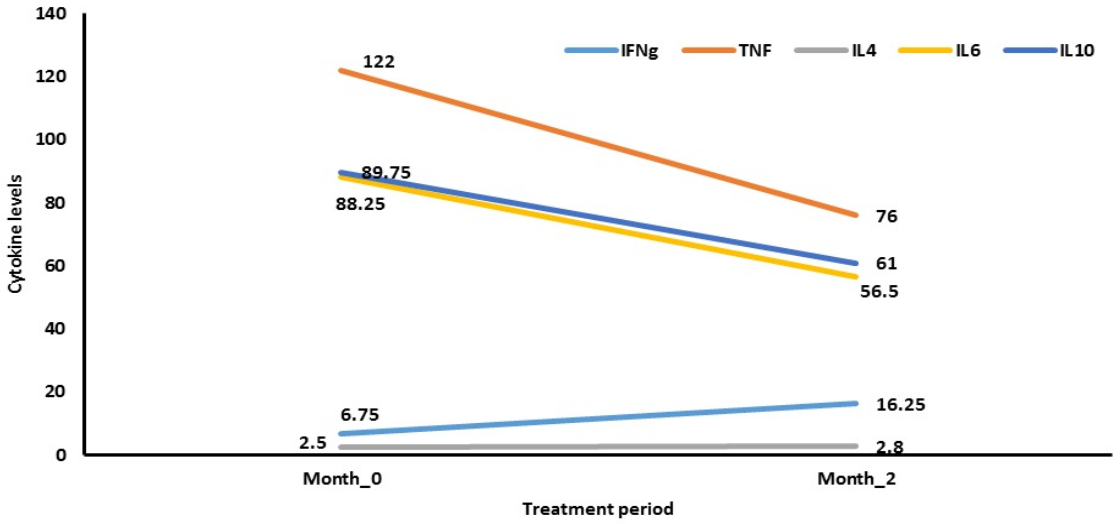


Figure 2: Changes of cytokines levels after medication

4.3 To determine plasma biomarkers that can predict favourable treatment outcome.

After investigating what happened before and after treatment cytokines with favourable treatment outcome was analysed so that it will be used as a monitoring tool during treatment of Tuberculosis.

The responses of five cytokines varied significantly after treatment (P<0.0001), where IFN-γ was highest (123.6%) followed by TNF (41.5%) and IL4 (6.9%) the least (Table 6). This is not enough to predict plasma biomarkers with favorable outcome so sensitivity and specificity test was conducted.

Table 3: Friedman test (non-parametric Repeated One-way ANOVA) results to compare median percentage responses across the five cytokines

Descriptive statistics of % changes	***IFN-γ***	***TNF***	***IL4***	***IL6***	***IL10***
Number of values	96	96	96	96	96
Minimum	11.29	10.37	-13.04	10	7.143
Median	123.6	41.49	6.905	33.69	30.68
Maximum	3100	94.68	166.7	65.91	56.12
Mean	215.2	44.92	14.77	35.58	30.8
Std. Deviation	355.7	18.52	27.23	11.99	8.509
Std. Error	36.3	1.89	2.779	1.224	0.8685
Lower 95% CI	143.1	41.17	9.251	33.15	29.08
Upper 95% CI	287.3	48.67	20.29	38.01	32.53

Friedman test

P value	*P*<0.0001
Friedman statistic	204.9

Dunn's Multiple Comparison Test	Difference in rank sum	P value	Summary
IFNg_%Change vs TNF_%Change	113	P < 0.001	***
IFNg_%Change vs IL4_%Change	305	P < 0.001	***
IFNg_%Change vs IL6_%Change	163.5	P < 0.001	***
IFNg_%Change vs IL10_%Change	183.5	P < 0.001	***
TNF_%Change vs IL4_%Change	192	P < 0.001	***
TNF_%Change vs IL6_%Change	50.5	P > 0.05	ns
TNF_%Change vs IL10_%Change	70.5	P < 0.05	*
IL4_%Change vs IL6_%Change	-141.5	P < 0.001	***
IL4_%Change vs IL10_%Change	-121.5	P < 0.001	***
IL6_%Change vs IL10_%Change	20	P > 0.05	ns

ns: Not significant *** *highly significant* * *Significant*

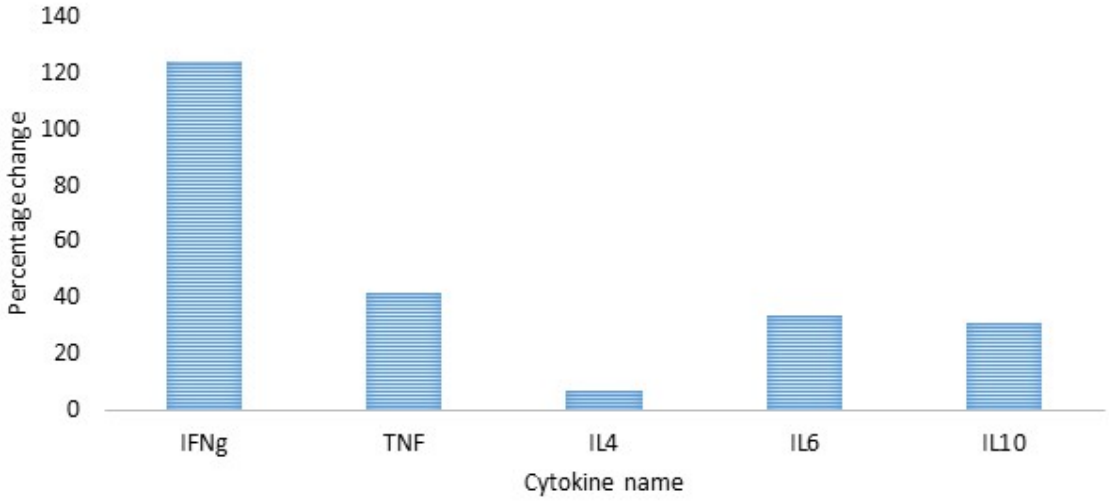


Figure 3: Comparative percentage changes of levels of cytokines recorded after 2 months of treatment of tuberculosis

The ROC curves were used to analyze the discriminating power of the cytokines against the health status of study patients after medication. We saw that IFN-γ (AUC=0.757, *P*<0001) and IL6 (AUC=0.629, *P*=0.047) had the ability to distinguish sick and healthy individuals. The corresponding sensitivity and specificity for each cytokine is shown in Table 3 and Fig. 4-7.

Table 4: Sensitivity and Specificity of each Cytokine

	IFNg	*TNF*	*IL4*	*IL6*	*IL10*
Sample size	96	96	96	96	96
Positive group [a]	16 (16.67%)	16 (16.67%)	16 (16.67%)	16 (16.67%)	16 (16.67%)
Negative group [b]	80 (83.33%)	80 (83.33%)	80 (83.33%)	80 (83.33%)	80 (83.33%)
Area under the ROC curve (AUC)	0.757	0.529	0.557	0.629	0.549
Standard Error [a]	0.0594	0.087	0.0879	0.0649	0.0884
95% Confidence interval [b]	0.659 -0.839	0.424 - 0.631	0.452 - 0.659	0.524 - 0.725	0.444- 0.651
z statistic	4.336	0.328	0.653	1.986	0.552
Significance level P (Area=0.5)	<0.0001	0.7431	0.5137	0.047	0.5807
Youden index J	0.4375	0.1375	0.2625	0.35	0.175
Associated criterion	≤8.5	≤58	>0.3	>27.5	≤16
Sensitivity	75	68.75	43.75	87.5	31.25
Specificity	68.75	45	82.5	47.5	86.25

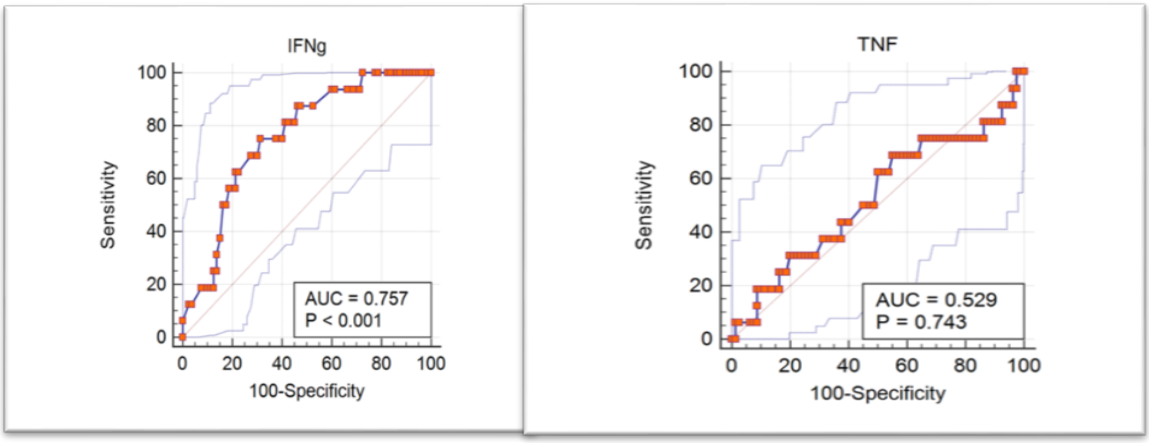


Figure 4: Sensitivity and specificity for FN-γ and TNF respectively

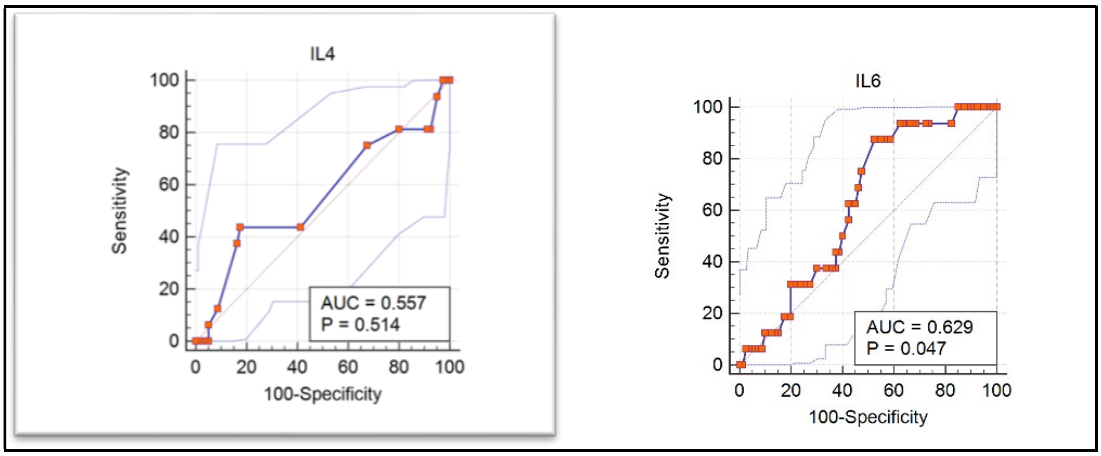


Figure 5: Sensitivity and specificity for IL4 and IL6 respectively

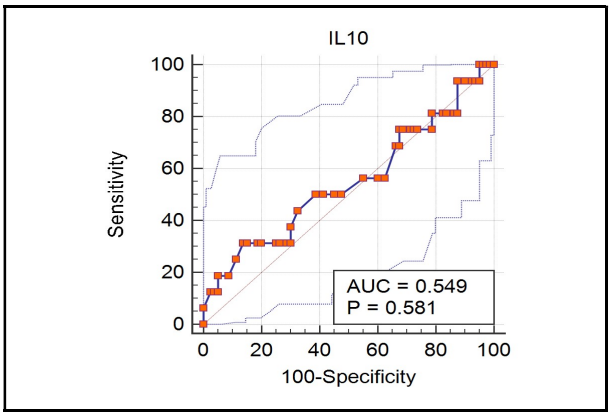


Figure 6: Sensitivity and specificity for IL10

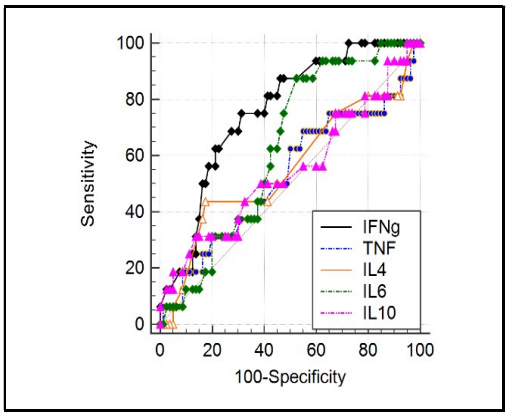


Figure 7: Combined for all Cytokine's

4.4 To compare clinical presentation/symptoms at baseline and two months after anti-TB treatment

To compare clinical characteristics/symptoms with smear results at baseline and after two months of treatment. The study involved 96 people with clinical tuberculosis. A tuberculosis case was defined by scores calculated by combination of symptoms and smear results.

The clinical symptoms/signs are as follows:

Cough, chest pain, fever, excessive night sweat and weight loss.

These are the cardinal signs of Tuberculosis that were used in scoring the patients so during diagnosis all 96 patients had these symptoms and signs but after two months of treatment they were reduced and most of the patients had mild cough only.

(i) The results from the study showed that all patients (equivalent to 100%) had symptoms of tuberculosis before medication was initiated. After two months of medication, patients with tuberculosis symptoms dropped significantly to 15.6%. Similarly, median case scores decreased from 3.99 to 0.18 (P<0.0001) Table 5.

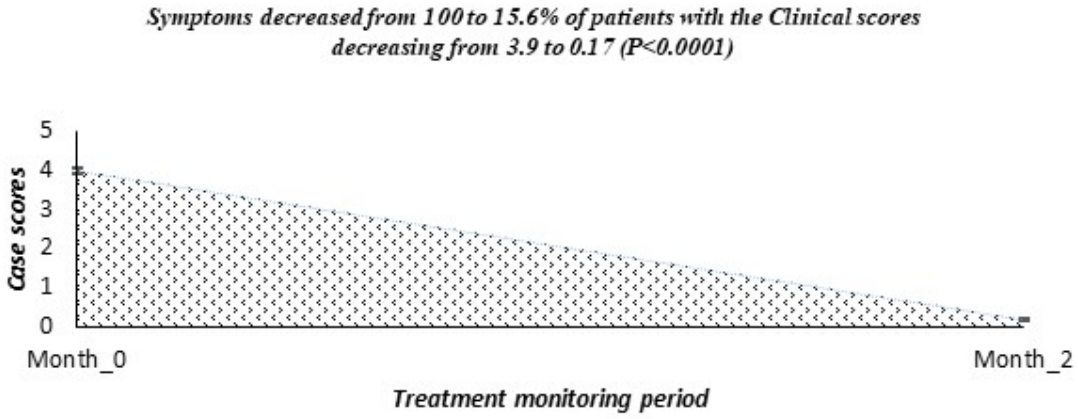


Figure 8: Graphical presentation of decreased tuberculosis case scores before and after treatment within two months

4.5 Discussion

This study enrolled 96 confirmed pulmonary TB patients and measured cytokine biomarkers at diagnosis and 2-months after the start of anti-TB treatment. Several exploratory studies have evaluated the potential of cytokine biomarkers for monitoring anti-TB therapy. TNF-α has been one of the most studied cytokines in previous studies. The largest studies found that TNF-α levels were increased during anti-TB treatment (Eum *et al.,* 2010). However, other study on TB-Ag-stimulated TNF-α responses to anti-TB treatment have consistently reported a reduction in TNF-α levels in whole blood or peripheral blood mononuclear cells during treatment (Petruccioli *et al*., 2013). In the current study, we found TNF-α responses to TB specific antigens showed significant decreased tendency following anti-TB therapy. IL-10 is an important anti-inflammatory cytokine reported to affect multiple cell types such as macrophages, monocytes, dendritic cells, CD4+ T cells, and CD8+ T cells (Moore *et al*., 2001). It inhibits CD4+ T cell responses by inhibiting the antigen-presenting cell function of TB-infected cells (Mauricio *et al*., 1999). Recently, the secretion of *Mycobacterium tuberculosis* enhanced intracellular survival protein from MTB cells was reported, which possibly increases IL-10 expression (Duan *et al*., 2016). The studies that have evaluated the role of IL-10 in TB infection, the findings are not consistent (Torres *et al*., 1998). There is also a study reported that IL-10 levels increased with treatment (Eum *et al*., 2010). However, another large longitudinal study found a reduction in IL-10 levels in response to treatment (Priya *et al*., 2009). In the current study, we found decreased IL-10 responses to TB specific antigens following anti-TB therapy. This decrease may be due to modified cytokine expression in infected individuals after treatment.

The current study was consistent with other findings where IL-6 decreased following anti-TB treatment. IL-6 appears to be the only major cytokine elaborated by mycobacteria-infected peritoneal macrophages *in vitro*, and thus may function as a potent biomarker for mycobacterial infection, either as stand-alone or along with other cytokines (Singh *et al*., 2013). There is a study that investigated the responses of IL-6 during anti-TB treatment with decreased IL-6 in the first two months of treatment (Mattos *et al*., 2010). Other studies two of which predominantly included HIV-positive patients found no change (Silva *et al*., 2013; Su *et al.*, 2010).

The increased levels of TNF-α, IL-10, IL-6 in active TB patients and their decrease following therapy may suggest the transient induction of regulatory immune mechanisms leading to

subsequent restoration of immune homeostasis. Significant decrease of these cytokines after just two months of anti-TB treatment may thus be used as an indicator of successful treatment, and hence bigger cohort studies with larger sample size are thus warranted, to assess immune mechanisms behind their respective responses to treatment with longer periods.

In this study, the average level of IFN-γ obtained from the patients with pulmonary TB increased significantly after two months of anti-tb treatment. The pleural fluid of TB patients contained high number of IFN-γ producer cells as reported by Raju *et al*. (2001) and suggested that these cells may migrate to the lung and pleural tissue during the active disease, and therefore they may be reduced, temporarily, in the peripheral blood. This fact might explain the lower levels of IFN-γ found before the use of anti-TB drugs in this study. However, in order to confirm this hypothesis, more techniques may be used to collect and study pulmonary cells in infected individuals.

The increase in the productive capacity of IFN-γ after the anti-TB treatment was also demonstrated in other studies carried out in patients with TB (Mensah *et al*., 2014; Mustafa *et al*., 2003). It is still unclear if the increased production of IFN-γ after treatment is related to cell stimulation by antigens released after the death of mycobacteria caused by chemotherapy. The increased levels of IFN-γ after the treatment in this study could be related to the use of anti-TB treatment and that voids the *Mycobacterium* after healing, which suggests an important role of the IFN-γ in the immunological response against *M. tuberculosis.*

We also found significant increase in the serum levels of IL-4. Some previous studies have shown increased production of IL-4 in human TB patients, especially those with cavitary disease (Surcel *et al*., 1994; Crevel *et al*., 2010; Ameglio *et al.*, 2005). According to other researcher, it still remains to be determined whether IL-4 causes or merely reflects disease activity in human TB (Lin *et al*., 1996). Some studies showed no detectable IL-4 in any TB patient and no significant difference in IL-4 level between TB patients and controls (Deveci *et al*., 2005; Zhang *et al.*, 1995). This finding also calls for a bigger study to show the specific role of IL-4 in TB treatment.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

From the results in this research work, the following recommendations and conclusions were made; Cytokines plays important role in the pathogenesis of active pulmonary tuberculosis. Effective chemotherapy improved cellular responses of TB patients after two months of anti-TB therapy, as characterized by general increase and decrease in secretion of various cytokines. Increase of IFN-γ and IL-4, and decrease of TNF-α, IL-6 and IL-10. IFN-γ can predict favorable treatment outcome hence can be used in monitoring.

5.2 Recommendations

Future confirmatory studies should include a sufficiently large number of patients with active TB infection, with cytokine concentrations measured over the entire course of treatment. Cytokines can be measured as early as two weeks after starting anti-TB.

REFERENCES

Ameglio, F., Casarini, M., Capoluongo, E., Mattia, P., Puglisi, G. and Giosuè, S (2005). Post-treatment changes of six cytokines in active pulmonary tuberculosis: Differences between patients with stable or increased fibrosis. *International Journal of Tuberculosis and Lung Disease*. ***9***(1): 98–104.

Asadullah, K., Sterry, W. and Volk, H. D. (2003). Interleukin-10 Therapy — Review of a New Approach. *Medical Immunology*. ***55***(2): 241–269.

Atkinson, A. J., Colburn, W. A., DeGruttola, V. G., DeMets, D. L., Downing, G. J., Hoth, D. F. and Zeger, S. L. (2001). Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework. *Clinical Pharmacology and Therapeutics*. ***69***(3): 89–95.

Barnes, P. F., Lu, S., Abrams, J. S., Wang, E., Yamamura, M. and Modlin, R. L. (1993). Cytokine Production at the Site of Disease in Human Tuberculosis. *Infection and Immunity.* ***61***(8): 3482–3489.

Barry, C. E., Boshoff, H. I., Dartois, V., Dick, T., Ehrt, S., Flynn, J. and Young, D. (2009). The spectrum of latent tuberculosis: Rethinking the biology and intervention strategies. *Nature Reviews Microbiology*. *7*(12): 845–855.

Bean, A. G. D., Roach, D. R., Briscoe, H., France, M. P., Korner, H. and Sedgwick, J. D. (2018). How the body respond to infection. *Journal of Immunology*. **8**(2):58-79

Bekker, L. G., Maartens, G., Steyn, L. and Kaplan, G. (1998). Selective increase in plasma tumor necrosis factor-alpha and concomitant clinical deterioration after initiating therapy in patients with severe tuberculosis. *Journal of Infectious Diseases*. ***178***(2): 580–584.

Bekker, L. G., Moreira, L., Bergtold, A, Freeman, S., Ryffel, B. and Kaplan, G. (2000). Immunopathologic effects of tumor necrosis factor alpha in murine mycobacterial infection are dose dependent. *Infection and Immunity*. ***68***(12): 6954–6961.

Biology, M. I. (1999). Different types of tuberculosis granuloma necrosis in immunocompetent. *Journal of Infection*. ***137***(3): 127–137.

Caccamo, N., Guggino, G., Joosten, S. A., Gelsomino, G., Carlo, P. Di., Titone, L. and Afid, D. (2010). Multifunctional CD4 cells correlate with active Mycobacterium tuberculosis infection. *The Lancet of Infectious Disease*. **61**(10): 2211–2220.

Central, L. (1994). Immune Responsiveness and Lymphokine Production in Patients with Tuberculosis and Healthy Controls. *Journal of Immunology*. ***62***(12): 5673–5678.

Chee, C. B. E., KhinMar, K. W., Gan, S. H., Barkham, T. M., Koh, C. K., Shen, L. and Wang, Y. T. (2010). Tuberculosis treatment effect on T-cell interferon-γ responses to Mycobacterium tuberculosis-specific antigens. *Journal of Infectious*. ***36***(2): 355–361.

Chowdhury, I. H., Ahmed, A. M., Choudhuri, S., Sen, A., Hazra, A., Pal, N. and Bahar, B. (2014). Alteration of serum inflammatory cytokines in active pulmonary tuberculosis following anti-tuberculosis drug therapy. *Molecular Immunology*. ***62***(1): 159–168.

Condos, R., Rom, W. N., Liu, Y. U. K. M. and Schluger, N. (2010). Local Immune Responses Correlate with Presentation. *International Journal in Immunology.* ***78***(9): 36-47.

Cooper, A. M., Callahan, J. E., Keen, M., Belisle, J. T. and Orme, I. M. (1997). Expression of memory immunity in the lung following re exposure to Mycobacterium tuberculosis. *Journal of Mycobacteriology*. ***78***(2): 67–73.

Crevel, R. Van, Karyadi, E., Preyers, F., Leenders, M., Kullberg, B., Nelwan, R. H. H. and Meer, J. W. M. Van Der. (2015.). Increased Production of Interleukin 4 by CD4 + and CD8 + T Cells from Patients with Tuberculosis Is Related to the Presence of Pulmonary Cavities. *European Respiratory Journal*. **30**(2): 1194–1197.

Dannenberg, A. M. (1994). Roles of Cytotoxic Delayed-Type Hypersensitivity and Macrophage-Activating Cell-Mediated Immunity in the Pathogenesis of Tuberculosis. *Journal of Immunobiology*. ***191***(4–5): 461–473.

Davis, J. M. and Ramakrishnan, L. (2009). The Role of the Granuloma in Expansion and Dissemination of Early Tuberculous Infection. *Journal of Respiratory*. ***136*****(**1): 37–49.

Deveci, F., Akbulut, H. H., Turgut, T. and Muz, M. H. (2005). Changes in Serum Cytokine Levels in Active Tuberculosis With Treatment. *Journal of Immunology.* ***5***(1): 256–262.

Duan, L., Yi, M., Chen, J., Li, S. and Chen, W. (2016). Mycobacterium tuberculosis EIS gene inhibits macrophage autophagy through up-regulation of IL-10 by increasing the acetylation of histone H3. *Biochemical and Biophysical Research Communications*. ***473***(4): 1229–1234.

Ernst, J. D. (2012). The immunological life cycle of tuberculosis. *Nature Reviews Immunology*. ***12***(8): 581–591.

Eum, S. Y., Lee, Y. J., Min, J. H., Kwak, H. K., Hong, M. S., Kong, J. H. and Cho, S. N. (2010). Association of antigen-stimulated release of tumor necrosis factor-alpha in whole blood with response to chemotherapy in patients with pulmonary multidrug-resistant tuberculosis. *Immunobiology*. ***80***(4): 275–284.

Falk, A. and Fuchs, G. F. (1978). Prophylaxis with isoniazid in inactive tuberculosis. A Veterans Administration cooperative study XII. *Clinical Trial*. ***73***(1): 44–48.

Feng, C. G., Bean, A. G. D., Hooi, H., Briscoe, H. and Britton, W. J. (1999). Increase in gamma interferon-secreting CD8+, as well as CD4+, T cells in lungs following aerosol infection with Mycobacterium tuberculosis. *Infection and Immunity*. ***67***(7): 3242–3247.

Flynn, J. L. (1993). An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. *Journal of Experimental Medicine*. ***178***(6): 2249–2254.

Flynn, J. L. and Chan, J. (2001). Importance of IL-10 during Tuberculosis infection. *Journal of Immunology.* **73**(3): 93–129.

Flynn, J. L., Goldstein, M. M., Triebold, K. J., Koller, B. and Bloom, B. R. (1992). Major histocompatibility complex class I-restricted T cells are required for resistance to Mycobacterium tuberculosis infection. *Proceedings of the National Academy of Sciences of the United States of America*. ***89(***24): 12013–12017.

Goldsack, L. and Kirman, J. R. (2007). Half-truths and selective memory: Interferon gamma, CD4+ T cells and protective memory against tuberculosis. *Tuberculosis*. ***87***(6): 465–473.

Gutierrez, M. G., Master, S. S., Singh, S. B., Taylor, G. A., Colombo, M. I. and Deretic, V. (2004). Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell*. ***119***(6): 753–766.

Henrique, F., Lopes, A., Botelho, K. P., Frota, C. C., Victor, M. and Freitas, C. (2013). Serum levels of interleukin-6 in contacts of active pulmonary tuberculosis. *Infectious Diseases.* **66**(8): 410–414.

Horne, D. J., Royce, S. E., Gooze, L., Narita, M., Hopewell, P. C., Nahid, P. and Steingart, K. R. (2010). Sputum monitoring during tuberculosis treatment for predicting outcome : systematic review and meta-analysis. *The Lancet Infectious Diseases*. ***10***(6): 387–394.

Jamil, B., Shahid, F., Hasan, Z., Nasir, N., Razzaki, T., Dawood, G. and Hussain, R. (2007). Interferonγ/IL10 ratio defines the disease severity in pulmonary and extra pulmonary tuberculosis. *Tuberculosis*. ***87***(4): 279–287.

Kaphingst, K. A., Persky, S. and Lachance, C. (2010). Roles of cytokines. *Journal of Immunology*. ***14***(4): 384–399.

Kim, C. H., Choi, K. J., Yoo, S. S., Lee, S. Y., Won, D. Il, Lim, J. O. and Lee, J. (2014). Comparative analysis of whole-blood interferon-γ and flow cytometry assays for detecting post-treatment Immune responses in patients with active tuberculosis. *Clinical Cytometry*. ***86***(4): 236–243.

Kim, C. H., Yoo, S. S., Lee, S. Y., Cha, S. I., Park, J. Y. and Lee, J. (2015). Post-treatment change in Mycobacterium tuberculosis antigen- stimulated tumor necrosis factor-alpha release in patients with active tuberculosis. *Journal of Mycobacteriology*. ***7***(5): 903–907.

Lai, C. K. W., Ho, S., Chan, C. H. S. and Chan, J. (1997). Cytokine Gene Expression Profile of Circulating CD4 + T Cells in Active Pulmonary Tuberculosis. *Mycobacteriology*. ***111***(3), 606–611.

Lalvani, A. and Millington, K. A. (2008). T-cell interferon-γ release assays: Can we do better. *European Respiratory Journal*. ***32***(6): 1428–1430.

Lalvani, A. and Millington, K. A. (2018). T Cells and Tuberculosis : Beyond Interferon. *Journal of Immunology*. ***197***(8): 941–943.

Lange, C., Abubakar, I., Alffenaar, J. C., Bothamley, G., Caminero, J. A., Carvalho, A. and Williams, B. (2014). Roles of cytokines in infection consensus statement. *Respiratory Journal*. **6**(4): 1–41.

Lee, M., Yang, C., Shu, C., Lin, C., Weng, Y., Lee, S. and Yu, C. (2014). Biomarkers of Tuberculosis. *Clinical Microbiology and Infection*. **26**(2): 97-110

Lin, Y., Zhang, M., Hofman, F. M., Gong, J., Barnes, P. and Caminero, J. (1996). Absence of a Prominent Th2 Cytokine Response in Human Tuberculosis. *International Immunology.* ***64***(4): 1351–1356.

Lucey, D. R., Clerici, M. and Shearer, G. M. (1996). Type 1 and Type 2 Cytokine Dysregulation in Human Infectious, Neoplastic, and Inflammatory Diseases. *Clinical Microbiology and Infection*. ***9***(4): 532–562.

Markos Abebe, A. M. (2013). Cytokines and Chemokines as Biomarkers of Tuberculosis. *Mycobacterial Diseases*. ***03***(02): 1–7.

Mattos, A. M. M., de Almeida, C. S., Franken, K. L. M. C., de Alves, C. C. S., Abramo, C., de Souza, M. A. and Teixeira, H. C. (2010). Increased IgG1, IFN-γ, TNF-α and IL-6 responses to Mycobacterium tuberculosis antigens in patients with tuberculosis are lower after chemotherapy. *International Immunology*. ***22***(9): 775–782.

Mensah, G. I., Addo, K. K., Tetteh, J. A., Sowah, S., Loescher, T., Geldmacher, C. and Jackson-sillah, D. (2014). Cytokine response to selected MTB antigens in Ghanaian TB patients , before and at 2 weeks of anti-TB therapy is characterized by high expression of IFN- γ and Granzyme B and inter- individual variation. *Journal of Immunology*. **58**(9) 1–11.

Mogues, T., Goodrich, M. E., Ryan, L., LaCourse, R. and North, R. J. (2001). The relative importance of T cell subsets in immunity and immunopathology of airborne Mycobacterium tuberculosis infection in mice. *The Journal of Experimental Medicine*. ***193***(3): 271–280.

Moore, K. W., de Waal Malefyt, R., Coffman, R. L. and O'Garra, A. (2001). Interleukin -10 and the I Nterleukin -10 R Eceptor. *Annual Review of Immunology*. ***19***(1): 683–765.

Mustafa, A. S., Abal, A. T., Madi, N. M. and Andersen, P. (2003). Restoration of mycobacterial antigen-induced proliferation and interferon- Q responses in peripheral blood mononuclear cells of tuberculosis patients upon active chemotherapy. *Journal of Infectious Diseases*. ***38***(1): 249–256.

Nagabhushanam, V., Solache, A., Ting, L., Escaron, C. J., Zhang, J. Y. and Ernst, J. D. (2018). Innate Inhibition of Adaptive Immunity: Mycobacterium tuberculosis -Induced IL-6 Inhibits Macrophage Responses to IFN-γ. *International Journal of Mycobacteriology*. **22**(6): 550-692.

Nicholson, B. S., Bonecini-almeida, M. G., Roberto, J., Nathan, I. I. C., Xie, Q., Mumford, R. and Ho, J. L. (1996). Inducible Nitric Oxide Synthase in Pulmonary Alveolar. *Clinical Immunology*. ***183***(5): 312-425.

Of, S., Among, T. and Nursing, O. F. (2014). Large General Hospital. *Journal of Infectious Diseases.* **62**: 9–14.

Old, J. (1988). Tumor necrosis factor remarkable complex response to injury and infection. *Immunology and Infection.* ***258***: 59–75.

Orozcoe, H., Sampieri, A., Pavon, L. and Velasquillo, C. (1996). Correlation between the kinetics of Thl / Th2 cells and pathology in a murine model of experimental pulmonary tuberculosis. *Tuberculosis*. **5**:26–33.

Paper, H. (1995). Aerial dissemination op pulmonary tuberculosis. *American Journal of Epidemiology*. ***142***: 185–196.

Petruccioli, E., Petrone, L., Vanini, V., Sampaolesi, A., Gualano, G., Girardi, E. and Goletti, D. (2013). IFNγ/TNFα specific-cells and effector memory phenotype associate with active tuberculosis. *Journal of Infection*. ***66***(6): 475–486.

Priya, V. H. S., Anuradha, B., Gaddam, S. L., Hasnain, S. E., Murthy, K. J. R. and Valluri, V. L. (2009). In vitro levels of interleukin 10 (IL-10) and IL-12 in response to a recombinant 32-kilodalton antigen of Mycobacterium bovis BCG after treatment for tuberculosis. *Clinical and Vaccine Immunology*. ***16***(1): 111–115.

Raju, B., Tung, C. F., Cheng, D., Yousefzadeh, N., Condos, R., Rom, W. N. and Tse, D. B. (2001). In Situ Activation of Helper T Cells in the Lung. *Journal of Infection*. ***69***(8): 4790–4798.

Rao, S. P. S., Alonso, S., Rand, L., Dick, T. and Pethe, K. (2008). The protonmotive force is required for maintaining ATP homeostasis and viability of hypoxic, nonreplicating Mycobacterium tuberculosis. *Proceedings of the National Academy of Sciences*. ***105***(33): 11945–11950.

Rojas, M., Olivier, M., Gros, P., Barrera, L. F. and García, L. F. (1999). TNF-alpha and IL-10 modulate the induction of apoptosis by virulent Mycobacterium tuberculosis in murine macrophages. *Journal of Immunology.* **162**(10): 6122–6131.

Sai, V. H., Latha, G. S., Hasnain, S. E., Murthy, K. J. and Lakshmi, V. (2010). Cytokine Enhanced T cell responsiveness to Mycobacterium bovis BCG r32-kDa Ag correlates with successful anti-tuberculosis treatment in humans. *Cytokine*. **52**(3): 190–193.

Saunders, B. M., Frank, A. A., Orme, I. A. N. M. and Cooper, A. M. (2000). Interleukin-6 Induces Early Gamma Interferon Production in the Infected Lung but Is Not Required for Generation of Specific Immunity to Mycobacterium tuberculosis Infection. *Journal of Immunology*. **68**(6): 3322–3326.

Schindler, B. R., Mancilla, J., Endres, S., Ghorbani, R., Clark, S. C. and Dinarello, C. A. (1990). Cytokines for diagnosis of Tuberculosis. *Cytokines*. **75**(1): 4–11.

Schisterman, E. F., Perkins, N. J., Liu, A. and Bondell, H. (2005). Optimal Cut-point and Its Corresponding Youden Index to Discriminate Individuals Using Pooled Blood Samples. *Epidemiology.* **16**(1): 73–81.

Schluger, N. W. and Rom, W. N. (2010). State of the Art The Host Immune Response to Tuberculosis. *Journal of Infection.* **5**(3): 21-30.

Selwyn, P., Hartel, D., Lewis, V., Schoenbaum, E., Vermund, S., Klein, R. and Walker, A. (1989). A prospective study of the risk of tuberculosis among intravenous drug users with human immunodeficiency virus infection. *Journal of Biomedicine.* **320**(21): 545–550.

Silva, T. P., Giacoia-gripp, C. B. W., Schmaltz, C. A., Sant, F. M., Rolla, V. and Morgado, M. G. (2013). T Cell Activation and Cytokine Profile of Tuberculosis and HIV-Positive Individuals during Antituberculous Treatment and Efavirenz-Based Regimens. *Journal of Infectious.* **8**(6): 4–11.

Singh, A., Dey, A. B., Mohan, A., Sharma, P. K. and Mitra, D. K. (2012). Foxp3+ Regulatory T Cells among Tuberculosis Patients: Impact on Prognosis and Restoration of Antigen Specific IFN-γ Producing T Cells. *PLoS ONE*. **7**(9): 1–10.

Singh, P. P. and Goyal, A. (2013). Interleukin-6 : a potent biomarker of mycobacterial infection. *Cytokines and Chemokines.* **2**(1): 9-15.

Stenger, S. (2005). Immunological control of tuberculosis: Role of tumour necrosis factor and more. *Annals of the Rheumatic Diseases*. **64**(4): 24–28.

Su, W., Perng, W., Huang, C., Yang, C., Wu, C. and Chen, J. (2010). Association of Reduced Tumor Necrosis Factor Alpha , Gamma Interferon , and Interleukin-1 but Increased IL-10 Expression with Improved Chest Radiography in Patients with Pulmonary Tuberculosis. *Cytokines*. **17**(2): 223–231.

Surcel, H., Andersson, S. P. G., Moreno, C. and Pasvol, G. (1994). Thl / Th2 profiles in tuberculosis , based on the proliferation and cytokine response of blood lymphocytes to mycobacterial antigens. *Journal of Infectious Diseases.* **18**(1): 4–9.

Syed, B., Kabeer, A., Raja, A., Raman, B., Thangaraj, S., Leportier, M. and Goletti, D. (2011). IP-10 response to RD1 antigens might be a useful biomarker for monitoring tuberculosis therapy. *Immunobiology.* **8**(2): 1–9.

Torres, M., Herrera, T., Villareal, H., Rich, E. A. and Sada, E. (1998). Cytokine profiles for peripheral blood lymphocytes from patients with active pulmonary tuberculosis and healthy household contacts in response to the 30-kilodalton antigen of Mycobacterium tuberculosis. *Infection and Immunity*. **66**(1): 176–180.

Turner, J., Corrah, T., Sabbally, S., Whittle, H. and Dockrell, H. M. (2000). A longitudinal study of in vitro IFN production and cytotoxic T cell responses of tuberculosis patients in The Gambia. *Journal of Biomedicine*. **80**(5): 161–169.

Van der Wel, N., Hava, D., Houben, D., Fluitsma, D., van Zon, M., Pierson, J. and Peters, P. J. (2007). M. tuberculosis and M. leprae Translocate from the Phagolysosome to the Cytosol in Myeloid Cells. *Cell.* **129**(7): 1287–1298.

Van Deun, A., Hamid Salim, M. A., Kumar Das, A. P., Bastian, I. and Portaels, F. (2004). Results of a standardised regimen for multidrug-resistant tuberculosis in Bangladesh. *International Journal of Tuberculosis and Lung Disease*. **8**(5): 560–567.

Verbon, A., Juffermans, N. and Speelman, P. (1999). Serum concentrations of cytokines in patients with active tuberculosis (TB) and after treatment. *Journal of Immunology* **9**(1):113–116.

Wallis, R. S., Doherty, T. M., Onyebujoh, P., Vahedi, M., Laang, H., Olesen, O. and Zumla, A. (2009). Biomarkers for tuberculosis disease activity, cure, and relapse. *The Lancet Infectious Diseases*. ***9***(3): 162–172.

Walzl, G., Ronacher, K., Hanekom, W. and Scriba, T. J. (2011). Immunological biomarkers of tuberculosis. *Nature Publishing Group*. ***11***(5): 343–354.

Wang, J., Chang, H., Liu, J. and Shu, C. (2012). Expression of toll-like receptor 2 and plasma level of interleukin-10 are associated with outcome in tuberculosis. *Journal of Immunology*. **9**(3): 2327–2333.

WHO. (2017). *Global Tuberculosis Report 2017*.

Wolf, A. J., Desvignes, L., Linas, B., Banaiee, N., Tamura, T., Takatsu, K. and Ernst, J. D. (2008). Initiation of the adaptive immune response to *Mycobacterium tuberculosis* depends on antigen production in the local lymph node, not the lungs. *The Journal of Experimental Medicine*. ***205***(1): 105–115.

Xiong, W., Dong, H., Wang, J., Zou, X. and Wen, Q. (2016). Analysis of Plasma Cytokine and Chemokine Profiles in Patients with and without Tuberculosis by Liquid Array-Based Multiplexed Immunoassays. *Cytokines*. **3**(1): 1–14.

Young, D. B., Gideon, H. P. and Wilkinson, R. J. (2009). Eliminating latent tuberculosis. *Trends in Microbiology*. ***17***(5), 183–188.

Young, J. M., Adetifa, I. M. O., Ota, M. O. C. and Sutherland, J. S. (2010). Expanded polyfunctional T cell response to mycobacterial antigens in TB disease and contraction post-treatment. *PLoS ONE*. **5**(6): 18-36

Zhang, M., Lin, Y., Iyer, D. V, Gong, J., Abrams, J. S. and Barnes, P. F. (1995). T-Cell Cytokine Responses in Human Infection with Mycobacterium tuberculosis. *Journal of Infectious.* ***63***(8): 3231–3234.

APPENDICES

Appendix 1: Ethical Clearance

THE UNITED REPUBLIC OF TANZANIA

National Institute for Medical Research
3 Barack Obama Drive
P.O. Box 9653
11101 Dar es Salaam
Tel: 255 22 2121400
Fax: 255 22 2121360
E-mail: ethics@nimr.or.tz

Ministry of Health, Community Development, Gender, Elderly & Children
University of Dodoma, Faculty of Arts and Social Sciences
Building No. 11
P.O. Box 743
40478 Dodoma

NIMR/HQ/R.8a/Vol. IX/2763

09th May 2018

Dr. Happiness Mvungi
C/o Dr. Elingarami Nkya
Nelson Mandela African Institute of Science and Technology
P.O. Box 447
Arusha

RE: ETHICAL CLEARANCE CERTIFICATE FOR CONDUCTING MEDICAL RESEARCH IN TANZANIA

This is to certify that the research entitled: Analysis of plasma biomarkers and their association with treatment response in patients with tuberculosis (Mvungi H. *et al.*) whose supervisor is Dr. Elingarami Nkya of Nelson Mandela African Institute of Science and Technology has been granted ethical clearance to be conducted in Tanzania.

The Principal Investigator of the study must ensure that the following conditions are fulfilled:

1. Progress report is submitted to the Ministry of Health, Community Development, Gender, Elderly & Children and the National Institute for Medical Research, Regional and District Medical Officers after every six months.
2. Permission to publish the results is obtained from National Institute for Medical Research.
3. Copies of final publications are made available to the Ministry of Health, Community Development, Gender, Elderly & Children and the National Institute for Medical Research.
4. Any researcher, who contravenes or fails to comply with these conditions, shall be guilty of an offence and shall be liable on conviction to a fine as per NIMR Act No. 23 of 1979, PART III Section 10(2).
5. Site: Kilimanjaro

Approval is valid for one year: 09th May 2018 to 08th May 2019.

Name: Prof. Yunus Daud Mgaya

Signature
CHAIRPERSON
MEDICAL RESEARCH
COORDINATING COMMITTEE

Name: Prof. Muhammad Bakari Kambi

Signature
CHIEF MEDICAL OFFICER
MINISTRY OF HEALTH, COMMUNITY DEVELOPMENT, GENDER, ELDERLY & CHILDREN

CC: RMO of Dar es Salaam.
DMO/DED of Selected districts.

Appendix 2: Consent form

THE NELSON MANDELA AFRICAN

INSTITUTION OF SCIENCE AND TECHNOLOGY

Consent form

TITLE: ANALYSIS OF PLASMA BIOMARKERS AND THEIR ASSOCIATION WITH TREATMENT RESPONSE IN PATIENTS WITH TUBERCULOSIS

You are being asked to take part in a research study on "Analysis of plasma biomarkers and their association with treatment response in patients with tuberculosis". We are asking you to take part in this study because you fit in the set criteria. Please listen carefully and ask any questions you may have before agreeing to take part in the study.

What the study is about: The purpose of this study is to establish association between plasma biomarkers (IL-6, IL-10, IFN-γ, TNF alpha with treatment response in patients with tuberculosis, and if there will be association it means these biomarkers will be also used as a monitoring tools.

What we will ask you to do: If you agree to participate in this study, we will collect 5ml of your blood for analysis of plasma biomarkers (IL-6, IL-10, IFN-γ, TNF-α and IL-4) before you start treatment and after 2 months of treatment and all the results will be given to you.

Benefits: After participating in our study you will get to know your blood test results and in a longer-term this is beneficial in improving monitoring investigation to patients with tuberculosis, to plan and design an appropriate intervention for prevention and control of tuberculosis to the whole community.

Compensation: There is no compensation for taking part in this study

Confidentiality: Your answers will be confidential. The records of this study will be kept private. In any sort of report we make public we will not include any information that will make it possible to identify you. Research records will be kept in a locked file; only the researchers will have access to the records.

Taking part is voluntary: Taking part in this study is completely voluntary. If you refuse it will not affect your health care at Kibong'oto hospital, current or future relationship with the researcher or Nelson Mandela African Institute of Science and Technology, the District or regional Council. If you decide to take part, you are free to withdraw at any time.

If you have questions: The researchers conducting this study: Dr Happiness Mvungi email: mvungih@nm-aist.ac.tz Phone: +255 755 900 764

Statement of Consent: I have read the above information, and I consent to take part in the study

Participant Name: ……. Date: ……. Signature: ……

Name (Researcher): ……Date: …….. Signature: …….

Thank you very much for your participation in this study.

For any inquiries, please contact:
National Health Research Ethics Sub-Committee (NatHREC)
The National Institute for Medical Research (NIMR)
3 Barack Obama Drive, P. O. Box 9653,
11101 Dar es Salaam, Tanzania.
Tel: +255222121400
E-mail: hq@nimr.or.tz**;** info@nimr.or.tz**.**

RESEARCH OUTPUTS

Output 1: Paper Presentation

Original Article

Blood Cytokine Responses to Early Secreted Protein Antigen-6/Culture Filtrate Protein-10 Tuberculosis Antigens 2 Months after Antituberculosis Treatment among Patients with Drug-Susceptible Pulmonary Tuberculosis

Happiness Cornel Mvungi[1,2], Peter Masunga Mbelele[2], Joram Josephat Buza[1], Stellah George Mpagama[2], Elingarami Sauli[1]

[1]Department of Global Health and Biomedical Sciences, School of Life Science and Bioengineering, Nelson Mandela-African Institution for Science and Technology, Arusha, [2]Kibong'oto Infectious Diseases Hospital, Kilimanjaro Clinical Research Institute, Kilimanjaro, Tanzania

Abstract

Background: Human tuberculosis is a chronic inflammatory disease caused by *mycobacterium tuberculosis*. Pulmonary tuberculosis is the result of the failure of host immune system to control *mycobacterium tuberculosis*. The aim of the study was to asses the changes of the cytokines in active pulmonary tuberculosis patients before and after the use of anti-TB therapy. **Methods:** Multiple cytokine responses in active tuberculosis (TB) patients were investigated in this study following anti-TB drug therapy after 2 months. Ninety-six participants with pulmonary TB were engaged in the study between May 2018 and October 2018. Samples of blood were taken early before treatment at 0 and 2 months after using anti-TB therapy. The levels of interferon-gamma (IFN)-γ, interleukin-4 (IL-4), IL-6, IL-10, and tumor necrosis factor (TNF)-α in whole blood plasma collected from the QuantiFERON-TB Gold Plus were measured. **Results:** Compared with baseline levels, TNF-α, IL6 and IL10 were significantly lower following treatment whereas the IFN-γ and IL-4 increased significantly after treatment. The responses of five cytokines varied significantly after treatment ($P < 0.0001$) where IFN-γ was highest compared to other cytokines with 123.6%, AUC=0.757 and $P < 0001$, TNF-α AUC: 0.529 and $P = 0.743$, IL-4 AUC:0.557 and $P = 0.514$, IL-6 AUC:0.629 and $P = 0.047$, IL-10 AUC:0.549 and $P = 0.581$. **Conclusion:** It is concluded that changes of cytokines that observed during the treatment of TB patients play a very important role in monitoring pulmonary TB and can be suitable biomarkers to assess the effectiveness of anti-TB therapy in patients with TB.

Keywords: Antituberculosis therapy, biomarkers, cytokines, pulmonary tuberculosis, QuantiFERON-TB Gold Plus

Introduction

Tuberculosis (TB) infection remains a major challenge globally.[1] It is one of the leading infectious causes of death worldwide[1,2] responsible for an estimated 1.7 million deaths.[3,4] In 2017, TB caused an estimated 1.3 million deaths among HIV-negative people, and there were an additional 300,000 deaths from TB among HIV-positive people.[4] Tanzania is one of the highest burden countries with TB (among 30 countries) in the world.[5]

Mechanisms underlying host defense to *Mycobacterium* infection is poorly understood.[6,7] In response to infection, host immune cells secrete a number of cytokines and chemokine signals[7,8] which play active roles in initiation and regulation of the immune response at various stages of disease development.[7,9] Following anti-TB therapy, research has shown that the cytokine-mediated cell signaling is altered, which ultimately leads to recovery of the TB infection.[7] The knowledge of understanding different cytokines at different stages of TB infection is necessary in knowing the host response after treatment. TB is an old disease, but still, there is an urgent need of rapid and precise monitoring tools to assess

Address for correspondence: Dr. Happiness Cornel Mvungi, Kibong'oto Infectious Diseases Hospital, P. O. Box 12 Siha, Kilimanjaro, Tanzania.
E-mail: happinesscornel@gmail.com

ORCID:
https://orcid.org/0000-0001-6527-4887

Access this article online

Quick Response Code:

Website: www.ijmyco.org

DOI: 10.4103/ijmy.ijmy_30_19

How to cite this article: Mvungi HC, Mbelele PM, Buza JJ, Mpagama SG, Sauli E. Blood cytokine responses to early secreted protein antigen-6/culture filtrate protein-10 tuberculosis antigens 2 months after antituberculosis treatment among patients with drug-susceptible pulmonary tuberculosis. Int J Mycobacteriol 2019;8:53-9.

the early response of anti-TB treatment. Sputum culture status is the only acceptable biomarker for TB treatment response,[10] but in resource-poor settings, the culture is unavailable. In additional waiting for the culture results for 6- 8 weeks to monitor the response of anti-TB is a challenge. The Zichl–Neelsen sputum for smear is the available tool for diagnosis and the same time monitoring in resource-poor setting even though its limitations are well published.[11] Sputum for smear is not useful for monitoring anti-TB treatment response as it is unable to distinguish live from dead bacilli.[12] Using sputum has its limitation as for the patients with extrapulmonary TB and in children. Furthermore after 2 months of anti-TB medication to get quality sputum sample is difficult due to cough cessation or reduction, patients end up giving inadequate volume of sputum or excessive saliva. Given the limitations of using sputum for monitoring treatment, blood has been suggested as alternative.

Cell-mediated immunity plays an important role in human host defenses against TB.[13,14] The essential role of T cells in protection against TB infection has been well-documented.[13,14] In this research, we studied changes of different cytokines in pulmonary TB patients following anti-Tb treatment after 2 months.

Methods

Ethical approval

The study was approved by the National Institute of Medical Research in Tanzania. All participants were given written informed consent.

Study design and population

This was a prospective cohort study involving two sites. Participants with pulmonary TB were enrolled between May 2018 and October 2018. Patients were considered eligible for inclusion if they were >18 years of age and had sputum smear-positive pulmonary TB detected by GeneXpert. Exclusion criteria includes participants who refused to provide informed consent, participants with extra pulmonary TB, and participants who were very sick i.e unconscious. The QuantiFERON-TB Gold Plus (QFT-Plus) was performed in each patient from the baseline (before starting treatment) and at 2 months of anti-TB medication. The levels of interferon-gamma (IFN-γ), interleukin-6 (IL-6), IL-10, IL-4, and tumor necrosis factor-α (TNF-α) were measured consecutively in the plasma harvested from the QFT-Plus tubes. All methods were carried out in accordance with the manufacturer's instructions.

Diagnosis and pulmonary tuberculosis treatment

The diagnosis of pulmonary TB was made based on clinical and microbiological status. Active pulmonary TB was confirmed using GeneXpert as derived from respiratory specimen (sputum). The standard regimen of fixed-dose combination of rifampicin, isoniazid, ethambutol, and pyrazinamide was used, and the dosage was given according to body weight of the patients. The duration of treatment was 6 months.

Plasma preparation using QuantiFERON-Tuberculosis Gold Plus

The preparation of plasma samples was performed using the QFT-TB Gold Plus tubes according to the manufacturer's instructions. For each patient, 1 ml of blood by venipuncture directly into each QFT-Plus blood collection (nil, TB1, TB2, and mitogen) where TB1 and TB2 tubes had specific TB antigen early secreted protein antigen-6 (ESAT-6), culture filtrate protein-10 (CFP-10), and the blood was incubated within 16 h at 37°C of blood collection. The plasma samples were harvested after centrifugation and stored below −20°C until use.

Human 5-Plex Magnetic Luminex Assay

The plasma which was stored below −20°C was removed from the freezer and kept to room temperature until all ice were completely melted. The assay of all cytokines, such as IFN-γ, IL-6, IL-4, IL-10, and TNF-α, were performed using Human Premixed Multi-Analyte Kit with the catalog number LXSAHM, and multiplexing analysis was done using Luminex™ 200 system. All the samples and reagents were prepared according to the manufacturer's protocol.

Statistical analysis

The analysis of data consisted of computing descriptive statistics for each study variable and then comparing them. Analysis of descriptive and inferential statistics was done using GraphPad Prism version 3.02. The baseline data were compared with final data using Wilcoxon matched-pair test. The responses of five cytokines were compared using Friedman test – a repetitive nonparametric one-way ANOVA alternative. In each case, a significant level was set at 5% with 95% confidence interval. The power of cytokines to discriminate infection was tested by doing receiver operating characteristic (ROC) curve, where area under the curve (AUC) of 0.5 was considered. ROC curve analysis was done using MedCalc version 18.10−© 1993–2018 MedCalc Software bvba.

Results

The study involved 96 people with pulmonary TB. A TB case was defined by scores calculated by combination of symptoms and smear results. The results from the study showed that all patients (equivalent to 100%) had symptoms of TB before medication was initiated. After 2 months of medication, patients with TB symptoms dropped significantly to 15.6%. Similarly, median case scores decreased from 3.99 to 0.18 ($P < 0.0001$) [Table 1 and Figure 1].

As indicated in Table 1 and Figure 2, the result showed significant changes of initial levels of each cytokine ($P < 0.0001$). Treatment of TB resulted into increased IFN-γ from 6.75 to 16.25 pg/ml and IL-4 from 2.5 to 2.8 pg/ml and the decreased TNF-α from 122 to 76 pg/ml, IL-6 from 88.25 to 56.5 pg/ml, and IL-10 from 89.7 to 61 pg/ml [Figure 2]. The percentage

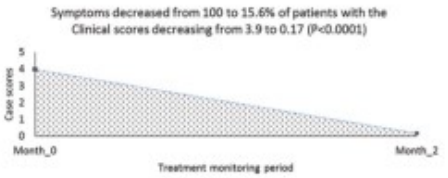


Figure 1: Graphical presentation of decrease of tuberculosis case scores before and after treatment within 2 months

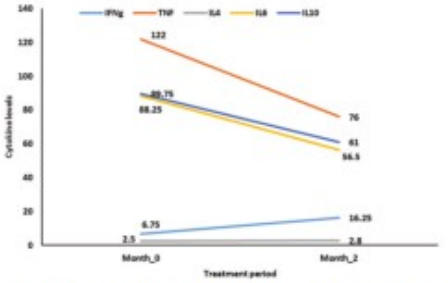


Figure 2: Changes of cytokine levels after medication (from month 0 to month 2). IFN-γ: Interferon-gamma, TNF: Tumor necrosis factor, IL-4: Interleukin-4, IL-6: Interleukin-6, IL-10: Interleukin-10

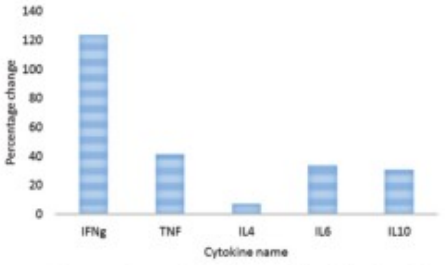


Figure 3: Comparative percentage changes of the levels of cytokines recorded after 2 months of tuberculosis treatment. IFN-γ: Interferon-gamma, TNF: Tumor necrosis factor, IL-4: Interleukin-4, IL-6: Interleukin-6, IL-10: Interleukin-10

response for each cytokine is presented in Table 2 and Figure 3. As indicated in Table 2, the responses for five cytokines varied significantly after treatment ($P < 0.0001$), where IFN-γ was highest compared to other cytokines. The percentage responses are summarized in Figure 3, indicating that IFN-γ had the highest response of 123.6%, followed by TNF-α (41.5%), IL-6 (33.7%), IL-10 (30.68%), and IL-4 (6.9%). The ROC curves were used to analyze the discriminating power of the cytokines against the health status of study patients after medication. We saw that IFN-γ (AUC = 0.757, $P < 0001$) and IL-6 (AUC = 0.629, $P = 0.047$) had the ability to distinguish sick and healthy individuals. The corresponding sensitivity and specificity of each cytokine is shown in Table 3 and Figures 4-9.

Discussion

The profile of cytokine secretion in response to mycobacteria infection has been studied to identify immunological changes in active TB. Changes in cytokines could be designed as important biomarkers for monitoring progress of the patients with TB.

There have been several studies that explained about the importance of cytokines as a biomarker for monitoring the progress of the patients using anti-TB. Previously, the most studied cytokine was TNF-α. One of the largest studies found that TNF-α increased after the use of anti-TB treatment.[15] On Other studies where Tumor necrosis factor alpha was stimulated with TB- specific antigens reported to decrease after the use of anti-TB medication.[16-19] In our study, we found that after the stimulation of the cells with TB-specific antigens ESAT-6/CFP-10, TNF-α decreased following anti-TB therapy.

IL-10 is an important anti-inflammatory cytokine reported to affect multiple cell types, such as macrophages, monocytes, dendritic cells, CD4+ T-cells, and CD8+ T-cells.[13,20] It inhibits CD4+ T-cell responses by inhibiting the antigen-presenting cell function of TB-infected cells.[13,21] Recently, the secretion of *Mycobacterium tuberculosis* (MTB)-enhanced intracellular survival protein from MTB cells was reported, which possibly increases IL-10 expression.[13,22] Findings from different studies that have reported the role of IL-10 in TB infection are inconsistent.[15,23-25] One study reported that IL-10 increased with treatment.[15] On the other hand, another longitudinal study reported the reduction in IL-10 levels after being in treatment.[23] In this study, we found that IL-10 after being stimulated with TB-specific antigens decreased after anti-TB treatment. This decrease may be due to modified cytokine expression in infected individuals after treatment.[13]

IL-6 appears to be the major cytokine elaborated by mycobacteria-infected peritoneal macrophages(PMs) *in vitro* and thus may function as a potential biomarker of mycobacterial infection, either stand-alone or along with other cytokines.[26] Several studies have investigated the responses of IL-6 during anti-TB therapy.[13] Some studies reported decreased IL-6 during the intensive phase of anti-TB therapy.[1,18,27] Other studies (two of which predominantly included HIV-positive patients) found no change of IL-6.[1,28,29] In this study, IL-6 tends to decrease following anti-TB treatment. The increased levels of these three cytokines, such as IL-6, IL-10, and TNF-α, in active TB patients and their decrease following anti-TB treatment might be due to temporary stimulation of regulatory immune mechanisms that can lead to subsequent repair of immune homeostasis.

Table 1: Descriptive summary of cytokine's levels before and after medication

Statistics	Case scores		IFN-λ		TNF-α		IL4		IL6		IL10	
	Baseline	Final	Baseline	Final	Baseline	Final	Baseline	Final	Baseline	Final	Baseline	Final
Sample size	96	96	96	96	96	96	96	96				
Symptomatic patients	96	15										
Minimum	3	0	0.5	3.5	87	34.5	0.2	0.5	40	30	44	36
25% percentile	4	0	3	10.25	105	63.5	1.6	1.7	75.75	46.5	75	50.75
Median	4	0	6.75	16.25	122	76	2.5	2.8	88.25	56.5	89.75	61
75% percentile	4	0	38.25	50	192.5	85.5	5.2	5.2	105.5	69.75	101	70
Maximum	5	2	63	80	12265	10988	55	58	162	90	411	275
Mean	3.99	0.18	16.65	28.49	409.5	289.2	4.87	5.13	91.61	57.92	92.26	63.09
Std. Deviation	0.55	0.41	18.21	22.75	1654	1487	7.9	8.17	24.29	15	37.8	24.99
Std. Error	0.06	0.04	1.86	2.32	168.8	151.8	0.81	0.83	2.48	1.53	3.86	2.55
Lower 95% ci	3.878	0.09396	12.96	23.88	74.38	12.06	3.27	3.475	86.69	54.88	84.6	58.03
Upper 95% ci	4.102	0.2602	20.34	33.1	744.5	590.6	6.472	6.784	96.54	60.95	99.92	68.15
P	*P*<0.0001		*P*<0.0001		*P*<0.0001		*P*<0.0001		*P*<0.0001		*P*<0.0001	

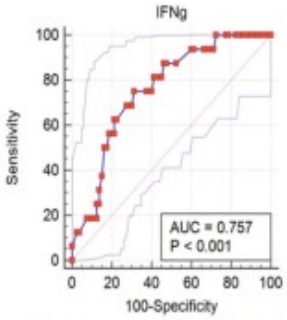


Figure 4: Sensitivity and specificity for interferon gamma which also show area under the curve in receiver operating characteristics with *P* value. IFNg: Interferon gamma, AUC: Area under the curve, *P*: *P* value

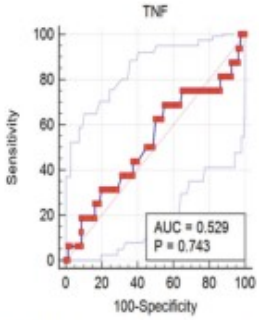


Figure 5: Sensitivity and specificity for tumor necrosis factor which also show area under the curve in receiver operating characteristics with *P* value. TNF: tumor necrosis factor alfa, AUC: Area under the curve, *P*: *P* value

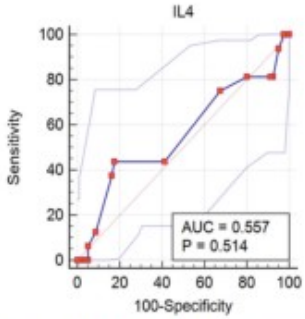


Figure 6: Sensitivity and specificity for IL-4 which also show area under the curve in receiver operating characteristics with *P* value. IL-4: Interleukin 4, AUC: Area under the curve, *P*: *P* value

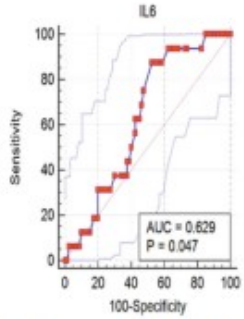


Figure 7: Sensitivity and specificity for IL-6 which also show area under the curve in receiver operating characteristics with *P* value. IL-6: Interleukin 6, AUC: Area under the curve, *P*: *P* value

Table 2: Friedman test (non-parametric Repeated One-way ANOVA) results to compare median percentage responses across the five cytokines

Descriptive statistics of % changes	IFN-λ	TNF-α	IL4	IL6	IL10
Number of values	96	96	96	96	96
Minimum	11.29	10.37	-13.04	10	7.143
Median	123.6	41.49	6.905	33.69	30.68
Maximum	3100	94.68	166.7	65.91	56.12
Mean	215.2	44.92	14.77	35.58	30.8
Std. Deviation	355.7	18.52	27.23	11.99	8.509
Std. Error	36.3	1.89	2.779	1.224	0.8685
Lower 95% CI	143.1	41.17	9.251	33.15	29.08
Upper 95% CI	287.3	48.67	20.29	38.01	32.53
Friedman test					
P	*P*<0.0001				
Friedman statistic	204.9				

Dunn's Multiple Comparison Test	Difference in rank sum	*P*	Summary
IFNg %Change vs TNF% Change	113	*P*<0.001	***

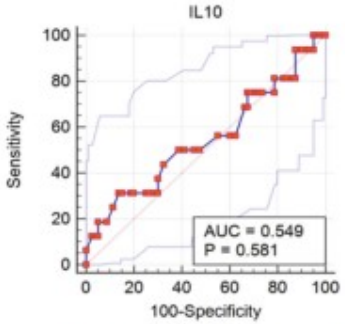


Figure 8: Sensitivity and specificity for IL-10 which show area under the curve in receiver operating characteristics with *P* value. IL-10: Interleukin 10, AUC: Area under the curve, *P*: *P* value

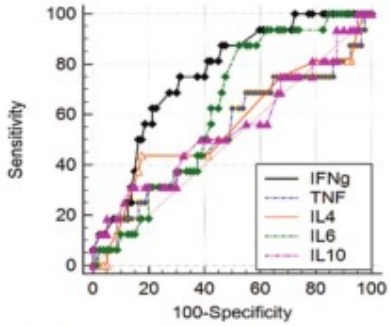


Figure 9: Combined for all cytokines. Sensitivity and specificity of 5 cytokines which also show area under the curve in receiver operating characteristics with *P* value of each cytokine. IFN-γ: Interferon-gamma, TNF: Tumor necrosis factor, IL-4: Interleukin-4, IL-6: Interleukin 6, IL-10: Interleukin 10, AUC: Area under the curve, *P*: *P* value

In our study, the level of IFN-γ found from the participants with active pulmonary TB increased significantly after 2 months of anti-TB therapy. Several studies reported that the pleural fluids of patients with TB have a high number of IFN-γ-induced cells;[30,31] this suggest these cells move to the pleural tissue and lungs during the active phase of the disease, and hence, they may be low temporarily in the blood. This explains the reason of lower level of IFN-γ obtained before the use of medication (anti-TB) in our study. To support our results there are other studies reported the increase of Interferon gamma after the anti-TB therapy.[10,25,32,33]

In this study, the increase of IFN-γ after 2 months could be due to the use of anti-TB therapy that cleared out the *Mycobacterium*, and this suggests the important role of IFN-γ in the response of immunity against MTB.

We found a slight increase of IL-4 in our study. Some studies reported an increased level of IL-4 in patients with pulmonary TB, especially those who had cavities in their lungs.[9,34,35] Still, it is not well explainable if IL-4 mirrors disease activity in human.[36,37] Many studies reported that no IL-4 level was detected in patients with TB, and also, there is no significant difference in IL-4 levels between patients with TB and healthy individuals.[7,38-42]

Our study had some limitation such as small sample size (only 96 participants) and this is due to limited cases that met inclusion criteria and also few cytokines tested (5 cytokines). We could not continue with the study further up to 5 months of anti-TB where another blood samples could be taken which would allow us to observe the changes in cytokines up to the end of anti-TB therapy may be it would help to describe the better immunological changes up to the end of treatment to our participants.

Table 3: Sensitivity and Specificity of each Cytokine

	IFN-λ	TNF-α	IL4	IL6	IL10
Sample size	96	96	96	96	96
Positive group[a]	16 (16.67%)	16 (16.67%)	16 (16.67%)	16 (16.67%)	16 (16.67%)
Negative group[b]	80 (83.33%)	80 (83.33%)	80 (83.33%)	80 (83.33%)	80 (83.33%)
Area under the ROC curve (AUC)	0.757	0.529	0.557	0.629	0.549
Standard Error[c]	0.0594	0.087	0.0879	0.0649	0.0884
95% Confidence interval[d]	0.659 to 0.839	0.424 to 0.631	0.452 to 0.659	0.524 to 0.725	0.444 to 0.651
Z statistic	4.336	0.328	0.653	1.986	0.552
Significance level *P* (Area=0.5)	<0.0001	0.7431	0.5137	0.047	0.5807
Youden index J	0.4375	0.1375	0.2625	0.35	0.175
Associated criterion	≤8.5	≤58	>0.3	>27.5	≤16
Sensitivity	75	68.75	43.75	87.5	31.25
Specificity	68.75	45	82.5	47.5	86.25

Conclusion

From the results of this research work, cytokine has a major role in immunological response during the pathogenesis of pulmonary TB. The use of anti-TB therapy effectively can cause immunological response in TB patients after 2 months of treatment which can be categorized by increase and decrease in secretion of cytokines these are IFN-γ, IL-4, and TNF-α, IL-6, IL-10 respectively. The studied markers herein can still be used to predict/determine early anti-TB response, which is crucial in determining whether a given treatment is effective or not, which may then help reduce unnecessary treatment cost that may be incurred in nonresponsive/resistant treatments.

Future studies should include a large number of participants, and if possible, all forms of TB (pulmonary and extrapulmonary TB) and cytokine levels measured the entire course of anti-TB treatment. Finally, the cytokine concentration can be measured as early as 2 weeks after starting anti-TB.

Acknowledgment

The authors acknowledge KIDH and KCRI authorities for giving permission to conduct this study. In addition, we acknowledge The Centre for Research, Agricultural Advancement, Teaching Excellence and Sustainability in Food and Nutritional Security (CREATES), for sponsorship in undertaking this research. We also acknowledge patients for voluntary participating in this study.

Financial support and sponsorship

The study was funded by the Centre for Research, Agricultural Advancement, Teaching Excellence and Sustainability in Food and Nutritional Security (CREATES), which also supported Happiness Mvungi's MSc study program at the Nelson Mandela-African Institution of Science and Technology in Tanzania.

Conflicts of interest

There are no conflicts of interest.

References

1. Clifford V, Zufferey C, Street A, Denholm J, Tebruegge M, Curtis N, *et al.* Cytokines for monitoring anti-tuberculous therapy: A systematic review. Tuberculosis (Edinb) 2015;95:217-28.
2. Perez-Velez CM. Pediatric tuberculosis: New guidelines and recommendations. Curr Opin Pediatr 2012;24:319-28.
3. Lawn SD, Zumla AI. Tuberculosis. Lancet 2011;378:57-72.
4. Global tuberculosis report 2018. Geneva: World Health Organization; 2018. Licence: CC BY-NC-SA 3.0 IGO.
5. World Health Organization. Global tuberculosis report 2017 (WHO/HTM/TB/2017.23). Geneva: WHO; 2017 Available from: http://apps.who.int/iris/bitstream/handle/10665/259366/9789241565516-eng.pdf. [Last accessed on 2018 Jun 21].
6. Grange JM. The mystery of the mycobacterial 'persistor'. Tuber Lung Dis 1992;73:249-51.
7. Chowdhury IH, Ahmed AM, Choudhuri S, Sen A, Hazra A, Pal NK, *et al.* Alteration of serum inflammatory cytokines in active pulmonary tuberculosis following anti-tuberculosis drug therapy. Mol Immunol 2014;62:159-68.
8. Cooper AM. The role of cytokines in the initiation, expansion, and control of cellular immunity to tuberculosis. Immunological Reviews 2008; 226:191-204.
9. Ameglio F, Casarini M, Capoluongo E, Mattia P, Puglisi G, Giosuè S, *et al.* Post-treatment changes of six cytokines in active pulmonary tuberculosis: Differences between patients with stable or increased fibrosis. Int J Tuberc Lung Dis 2005;9:98-104.
10. Mensah GI, Addo KK, Tetteh JA, Sowah S, Loescher T, Geldmacher C, *et al.* Cytokine response to selected MTB antigens in Ghanaian TB patients, before and at 2 weeks of anti-TB therapy is characterized by high expression of IFN-γ and granzyme B and inter- individual variation. BMC Infect Dis 2014;14:495.
11. Desikan P. Sputum smear microscopy in tuberculosis: Is it still relevant? Indian J Med Res 2013;137:442-4.
12. Horne DJ, Royce SE, Gooze L, Narita M, Hopewell PC, Nahid P, Steingart KR. Sputum monitoring during tuberculosis treatment for predicting outcome: Systematic review and meta-analysis. The Lancet Infectious Diseases 2010;10:387-94.
13. Leem AY, Song JH, Lee EH, Lee H, Sim B, Kim SY, *et al.* Changes in cytokine responses to TB antigens ESAT-6, CFP-10 and TB 7.7 and inflammatory markers in peripheral blood during therapy. Sci Rep 2018;8:1159.
14. Cooper AM. Cell-mediated immune responses in tuberculosis. Annual Review of Immunology 2009;27:393-422.
15. Eum SY, Lee YJ, Min JH, Kwak HK, Hong MS, Kong JH, *et al.* Association of antigen-stimulated release of tumor necrosis factor-alpha in whole blood with response to chemotherapy in patients with pulmonary multidrug-resistant tuberculosis. Respiration 2010;80:275-84.
16. Petruccioli E, Petrone L, Vanini V, Sampaolesi A, Gualano G, Girardi E, *et al.* IFNγ/TNFα specific-cells and effector memory phenotype associate with active tuberculosis. J Infect 2013;66:475-86.
17. Kim CH, Choi KJ, Yoo SS, Lee SY, Won DI, Lim JO, Lee J. Comparative analysis of whole-blood interferon-γ and flow cytometry assays for detecting post-treatment Immune responses in patients with active

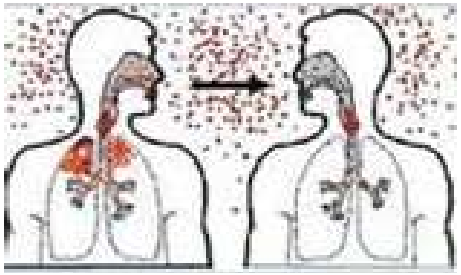
tuberculosis. Cytometry Part B: Clinical Cytometry 2013;86:236-43.
18. Mattos AM, Almeida Cde S, Franken KL, Alves CC, Abramo C, de Souza MA, *et al.* Increased igG1, IFN-gamma, TNF-alpha and IL-6 responses to *Mycobacterium tuberculosis* antigens in patients with tuberculosis are lower after chemotherapy. Int Immunol 2010;22:775-82.
19. Young JM, Adetifa IM, Ota MO, Sutherland JS. Expanded polyfunctional T cell response to mycobacterial antigens in TB disease and contraction post-treatment. PLoS One 2010;5:e11237.
20. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin -10 and the I nterleukin -10 R eceptor. Annu Rev Immunol 2001;19:683-765.
21. Rojas M, Olivier M, Gros P, Barrera LF, Garcia LF. TNF-alpha and IL-10 modulate the induction of apoptosis by virulent *Mycobacterium tuberculosis* in murine macrophages. J Immunol 1999;162:6122-31.
22. Duan L, Yi M, Chen J, Li S, Chen W. *Mycobacterium tuberculosis* EIS gene inhibits macrophage autophagy through up-regulation of IL-10 by increasing the acetylation of histone H3. Biochem Biophys Res Commun 2016;473:1229-34.
23. Sai Priya VH, Anuradha B, Latha Gaddam S, Hasnain SE, Murthy KJ, Valluri VL, *et al. In vitro* levels of interleukin 10 (IL-10) and IL-12 in response to a recombinant 32-kilodalton antigen of *Mycobacterium bovis* BCG after treatment for tuberculosis. Clin Vaccine Immunol 2009;16:111-5.
24. Singh A, Dey AB, Mohan A, Sharma PK, Mitra DK. Foxp3+ regulatory T cells among tuberculosis patients: Impact on prognosis and restoration of antigen specific IFN-γ producing T cells. PLoS One 2012;7:e44728.
25. Torres M, Herrera T, Villareal H, Rich EA, Sada E. Cytokine profiles for peripheral blood lymphocytes from patients with active pulmonary tuberculosis and healthy household contacts in response to the 30-kilodalton antigen of *Mycobacterium tuberculosis*. Infect Immun 1998;66:176-80.
26. Singh PP, Goyal A. Interleukin-6: A potent biomarker of mycobacterial infection. Springerplus 2013;2:686.
27. Wang JY, Chang HC, Liu JL, Shu CC, Lee CH, Wang JT, *et al.* Expression of toll-like receptor 2 and plasma level of interleukin-10 are associated with outcome in tuberculosis. Eur J Clin Microbiol Infect Dis 2012;31:2327-33.
28. da Silva TP, Giacoia-Gripp CB, Schmaltz CA, Sant Anna FM, Rolla V, Morgado MG, *et al.* T cell activation and cytokine profile of tuberculosis and HIV-positive individuals during antituberculous treatment and efavirenz-based regimens. PLoS One 2013;8:e66095.
29. Su WL, Perng WC, Huang CH, Yang CY, Wu CP, Chen JH, *et al.* Association of reduced tumor necrosis factor alpha, gamma interferon, and interleukin-1beta (IL-1beta) but increased IL-10 expression with improved chest radiography in patients with pulmonary tuberculosis. Clin Vaccine Immunol 2010;17:223-31.
30. Barnes PF, Lu S, Abrams JS, Wang E, Yamamura M, Modlin RL, *et al.* Cytokine production at the site of disease in human tuberculosis. Infect Immun 1993;61:3482-9.
31. Raju B, Tung CF, Cheng D, Yousefzadeh N, Condos R, Rom WN, *et al. In situ* activation of helper T cells in the lung. Infect Immun 2001;69:4790-8.
32. Turner J, Corrah T, Sabbally S, Whittle H, Dockrell HM. A longitudinal study of in vitro IFNγ production and cytotoxic T cell responses of tuberculosis patients in The Gambia. Tubercle and Lung Disease 2000; 80:161-9.
33. Al-Attiyah R, Mustafa AS, Abul AT, Madi NM, Andersen P. Restoration of mycobacterial antigen-induced proliferation and interferon-gamma responses in peripheral blood mononuclear cells of tuberculosis patients upon effective chemotherapy. FEMS Immunol Med Microbiol 2003;38:249-56.
34. Surcel HM, Troye-Blomberg M, Paulie S, Andersson G, Moreno C, Pasvol G, *et al.* Th1/Th2 profiles in tuberculosis, based on the proliferation and cytokine response of blood lymphocytes to mycobacterial antigens. Immunology 1994;81:171-6.
35. van Crevel R, Karyadi E, Preyers F, Leenders M, Kullberg BJ, Nelwan RH, *et al.* Increased production of interleukin 4 by CD4+ and CD8+T cells from patients with tuberculosis is related to the presence of pulmonary cavities. J Infect Dis 2000;181:1194-7.
36. Lai CK, Ho S, Chan CH, Chan J, Choy D, Leung R, *et al.* Cytokine gene expression profile of circulating CD4+T cells in active pulmonary tuberculosis. Chest 1997;111:606-11.
37. Lin Y, Zhang M, Hofman FM, Gong J, Barnes PF. Absence of a prominent th2 cytokine response in human tuberculosis. Infect Immun 1996;64:1351-6.
38. Deveci F, Akbulut HH, Turgut T, Muz MH. Changes in serum cytokine levels in active tuberculosis with treatment. Mediators Inflamm 2005;2005:256-62.
39. Zhang M, Lin Y, Iyer DV, Gong J, Abrams JS, Barnes PF, *et al.* T-cell cytokine responses in human infection with *Mycobacterium tuberculosis*. Infect Immun 1995;63:3231-4.
40. Verbon A, Juffermans N, Van Deventer SJ, Speelman P, Van Deutekom H, Van Der Poll T, *et al.* Serum concentrations of cytokines in patients with active tuberculosis (TB) and after treatment. Clin Exp Immunol 1999;115:110-3.
41. Condos R, Rom WN, Liu YM, Schluger NW. Local immune responses correlate with presentation and outcome in tuberculosis. Am J Respir Crit Care Med 1998;157:729-35.
42. Sánchez FO, Rodríguez JI, Agudelo G, García LF. Immune responsiveness and lymphokine production in patients with tuberculosis and healthy controls. Infect Immun 1994;62:5673-8.

Output 2: Poster Presentation

Happiness Cornel Mvungi(Reg.M337/T16)
School of life science
The Nelson Mandela African Institute of science and Technology

Introduction

- Tuberculosis is a chronic inhalational infectious bacterial disease caused by Mycobacteria tuberculosis complex .
- TB is the ninth leading cause of death worldwide, and the leading cause from a single infectious agent, ranking above HIV/AIDS
- Tanzania is one of the highest burdens of TB among 30 countries in the world(WHO ,2017)



- Sputum acid fast bacilli and Sputum culture are the most common TB biomarkers currently used to monitor pulmonary TB patients.
- These methods have disadvantages and they have been over 100 years.
- There is urgent need to identify blood biomarker for monitoring TB patients.

- Cytokines are proteins that can alter the behavior or properties of the cell itself or of another cell.
- Inflammation process in patients with Tb is mediated by activation of the immune system, with excessive production of cytokines.
- cytokines play important role in the pathogenesis of active pulmonary tuberculosis and can be useful biomarkers .

- The increased level of IFN-γ in the supernatant after treatment may therefore be useful biomarker for monitoring therapy in active TB patients.
- Also increase of IL-6 level can be used as the diagnosis in TB patients and its decrease can be a monitor tool to TB patients.

References

WHO. (2017). *Global Tuberculosis Report 2017.*