PHAGOCYTIC MECHANISM AND ITS PLASMA MODULATORS IN PULMONARY TUBERCULOSIS PATIENTS ON ANTI-TUBERCULOSIS CHEMOTHERAPY AND ZINC SUPPLEMENT

BY

VICTORY FABIAN EDEM
B.Sc. (Ibadan), M.Sc. (Ibadan)
MATRIC NUMBER 121259

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CERTIFICATION

This work entitled "PHAGOCYTIC MECHANISM AND ITS PLASMA MODULATORS IN PULMONARY TUBERCULOSIS PATIENTS ON ANTI-TUBERCULOSIS CHEMOTHERAPY AND ZINC SUPPLEMENT" was carried out by Victory Fabian EDEM under my direct supervision in the Department of Chemical Pathology, University of Ibadan, Ibadan.

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Supervisor

O. G. Arinola (Ph.D., Cert Immunology, Switzerland)

Professor of Immunology

Department of Chemical Pathology

University of Ibadan.

ABSTRACT

The progression of tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) from latent to drug-sensitive TB (DS-TB) or multi-drug resistant TB (MDR-TB) depends on factors including host-pathogen interactions. The nature and course of these interactions are largely determined by host zinc levels, a function of host immune responses. Clear understanding of these interactions is crucial to identify protection mechanisms, which are not completely elucidated. This study was designed to assess phagocytic mechanism and its plasma modulators in TB patients before and during anti-TB chemotherapy with or without zinc supplementation.

Ethical approval (UI/EC/13/0340) was obtained and 160 consenting participants (50 MDR-TB patients, 60 DS-TB patients and 50 controls) were enrolled. The MDR-TB and DS-TB patients were treated with anti-tuberculosis chemotherapy. Thirty DS-TB patients received zinc supplement. Blood sample was collected and plasma obtained from patients at baseline, 2, 4 and 6 months of anti-TB chemotherapy with or without zinc supplement. Phagocytic mechanism [percentage leucocyte migration (%LM) and intracellular killing (%NBT)] were determined by microscopy. Interleukin 6 (IL-6) and 8 (IL-8) were determined using ELISA; superoxide dismutase (SOD), myeloperoxidase (MPO), hydrogen peroxide (H_2O_2) and nitric oxide (NO) were determined by spectrophotometry. Iron (Fe), zinc (Zn) and copper (Cu) were determined using AAS while vitamins A, C, D and E were determined by HPLC. Data was analysed using Kruskal-Wallis, Mann-Whitney *U*, Friedman and Wilcoxon signed rank tests at $\alpha_{0.05}$.

In MDR-TB patients, baseline levels of %LM (91.5±0.9 vs 55.0±2.2), IL-6, IL-8 (186.4±29.9 vs 3.7±0.8; 1116.4±198.3 vs 18.9±2.4 pg/mL) and vitamin C (4.80±0.2 vs 3.5±0.7 mg/dL) were significantly higher while MPO (7.5±0.3 vs 8.3±0.3 U/mL), NO (9.2±0.8 vs 14.8±1.3 µmol/L), Fe, Zn and Vitamin A (92.2±2.3 vs 123.3±1.2; 62.6±1.0 vs 118.3±3.1; 49.8±2.6 vs 80.8±6.4 µg/dL) were lower compared with controls. In DS-TB patients, baseline IL-8 (162.6±56.3 vs 18.9±2.4 pg/mL) and MPO (9.3±0.4 vs 8.3±0.3 U/mL) were significantly higher while SOD (0.2±0.0 vs 0.3±0.0 U/mL), H_2O_2 (269.1±8.3 vs 313.8±7.4 µmol/L), NO (10.3±1.6 vs 14.8±1.3 µmol/L), Zn (81.3±6.3 vs 118.3±3.1 µg/dL), vitamins C and E (0.7±0.0 vs 3.5±0.7; 1.1±0.1 vs 1.7±0.3 mg/dL) were lower compared with controls. At 2 months of anti-TB chemotherapy compared with baseline, MPO (11.1±0.3 vs 9.6±0.3 U/mL) was significantly increased in DS-TB patients on anti-TB chemotherapy alone while SOD (0.2±0.0 vs 0.1±0.0 U/mL), MPO (17.5±1.0 vs 9.0±0.7 U/mL) and NO (25.7±2.4 vs 20.7±1.8 µmol/L) were increased in DS-TB patients on chemotherapy and zinc supplement. At 4 months of anti-TB chemotherapy, MPO (18.7±1.2 vs 9.0±0.7 U/mL) and NO (26.8±2.2 vs 20.7±1.8 µmol/L) were significantly increased in DS-TB patients on anti-TB chemotherapy and zinc supplement compared with baseline. At 6 months of anti-TB chemotherapy in DS-TB patients compared with baseline, H_2O_2 and NO (372.9±6.1 vs 316.7±7.9; 14.7±1.4 vs 12.0±1.3 µmol/L) were

increased in patients on anti-TB chemotherapy alone while MPO (20.2±1.3 vs 9.0±0.7 U/mL) increased in patients on anti-TB chemotherapy and zinc supplement.

Zinc supplementation with anti-tuberculosis chemotherapy improved phagocytic mechanism and its plasma modulators in TB patients from 2 months of treatment.

Keywords: *Mycobacterium tuberculosis*, Drug sensitive tuberculosis, Zinc supplementation

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DEDICATION

This thesis is dedicated to my parents and siblings for their unwavering support, patience and understanding throughout the course of the programme.

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Unto him who commanded light out of darkness, I lift my voice in praise for thou O Lord are a shield for me, my glory and the lifter of my head.

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LIST OF ABBREVIATIONS

AAS	Atomic absorption spectrophotometry
ABC	Avidin-biotin-peroxidase comple
AEC	Airway epithelial cell
AFB	Acid fast bacilli
ANOVA	Analysis of variance
ASL	Airway surface liquid
BAL	Bronchoalveolar lavage
BCG	Bacille Calmette –Guerin
BIA	Bioelectrical impedance analysis
BMI	Body mass index
CAT	Catalase
CCL	C-C chemokine ligand
CCL2	C-C motif chemokine ligand 2
CCL3L1	C-C motif chemokine ligand 3 like 1
CCR	C-C chemokine receptor
CCR5	Chemokine receptor type 5
CD	Cluster of differentiation
CGD	Chronic granulomatous disease
CXCL	C-X-C motif ligand
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DNA	Deoxyribonucleic acid
DOTS	Directly observed treatment scheme
ELISA	Enzyme linked immunosorbent assay
FFMI	Fat free mass index
FMI	Fat mass index
fMLP-R	N-formyl-methionyl-leucyl-phenylalanine receptor
HC	Hip circumference
HIV/AIDS	Human immunodeficiency virus/Acquired immune deficiency syndrome
HNE	Human neutrophil elastase
HOCl	Hypochlorous acid

HPLC	High pressure liquid chromatography
HRP	Horseradish peroxidase
IFN γ	Interferon gamma
IgA	Immunoglobulin A
IGRA	Interferon gamma release assay
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JAK	Janus kinase
LMIF	Leucocyte migration inhibitory factor
LTBI	Latent tuberculosis infection
MAIT	Mucosa-associated invariant T-cell
ManLAM	Mannosylated lipoarabinomannan
MAPK	Mitogen-activated protein kinase
MBL	Mannose binding lectin
MDDC	Monocyte derived dendritic cell
MDR-TB	Multi-drug resistant tuberculosis
MHC	Major histocompatibility complex
miRNA	microRNA
MPO	Myeloperoxidase
MR	Mannose receptor
MUAC	Mid-upper arm circumference
NBT	Nitroblue tetrazolium
NED	N-1-napthylethylenediamine dihydrochloride
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor-kappa-B
NO	Nitric oxide
NOX	NADPH oxidase
NTBLCP	National tuberculosis and leprosy control programme
NTM	Non-tuberculous *Mycobacteria*
PAF	Population attributable fraction
PAMP	Pathogen associated molecular pattern
PBF	Percentage body fat
PBS	Phosphate buffered saline
PD-L1	Programmed death ligand 1

PD1	Programmed death receptor 1
PET-CT	Positron emission tomography-computed tomography
PMN	Polymorphonuclear neutrophil
PPAR γ	Peroxisome proliferator activated receptor gamma
PPD	Purified protein derivative
PTB	Pulmonary tuberculosis
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROI	Reactive oxygen intermediate
ROS	Reactive oxygen species
RR-TB	Rifampicin resistant tuberculosis
Rt-PCR	Real time polymerase chain reaction
SOD	Superoxide dismutase
SPSS	Statistical package for the social sciences
STAT	Signal transducer and activator of transcription
TB	Tuberculosis
TLR	Toll like receptor
TMB	Tetramethyl benzidine
TNF	Tumor necrosis factor
TST	Tuberculin skin test
WC	Waist circumference
WHR	Waist circumference to hip circumference ratio
XDR-TB	Extensively-drug resistant tuberculosis
ZN	Ziehl-Neelsen

PUBLISHED ARTICLES

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CHAPTER ONE
INTRODUCTION

Pulmonary Tuberculosis (PTB) which has existed for millennia is still a prominent cause of ill-health and death globally (Pai *et al.*, 2016; WHO, 2016). The bacilli of the *Mycobacterium tuberculosis* (*M.tuberculosis*) complex are recognized as cause of PTB. In 2016, PTB was ranked ninth in regard to cause of mortality universally and foremost sole cause of mortality from an infectious disease (Sakamoto, 2012; WHO, 2016). Recent statistics indicate an increase in PTB associated deaths from 1.5 million in 2013 and 2014 (WHO, 2014; 2015) to 1.674 million in 2016 (WHO, 2017), which represents a 20% rise in PTB associated deaths within this period. This might not be surprising due to a consistent increase in annual global PTB incidence between 2013 and 2015, increasing from 9 million new cases in 2013 (WHO, 2014), to 9 million 6 hundred thousand incident cases in 2014 (WHO, 2015) and 10 million 4 hundred thousand incident cases in 2015 (WHO, 2016). Since, most PTB associated deaths are preventable, the disease death toll indicates an increased demand for more effort towards improved knowledge and available information on host pathogen interaction that could culminate in better control of the disease.

Sub-Saharan Africa and South-East Asia accounts annually for large proportions of incident TB cases, contributing about 70% of incident TB cases together, while sub-Saharan Africa alone accounts for about 25-28% of these (Villamor *et al.*, 2008; WHO 2016, 2017). In 2014, Nigeria recorded the highest number of new PTB cases in sub-Saharan Africa, overtaking South Africa, Mozambique and Zimbabwe, and maintained this position in 2015 (WHO, 2015, 2016). This indicates the need for more effort towards better understanding of host-pathogen interaction in PTB patients in Nigeria to foster better control of the disease in this region. Generally, progression to active TB disease in exposed persons involves a two-stage process after *M.tuberculosis* infection has been established. Majority of infected persons present as the latent disease form whereby growth and expansion of bacilli is repressed in caseous granulomas by host immune response (Narasimhan *et al.*, 2013). Between 5% and 15% of these individuals develop symptomatic TB disease, while others carry a sustained TB disease risk for life (Vynnycky and Fine, 1997; Andrews *et al.*, 2012; Pai *et al.*, 2016). Although host characteristics comprising gender, age, malnutrition, diabetes and immune status, as well as social and environmental determinants such as poor ventilation, crowding, smoking, alcohol use, and occupational risk have been classified as risk factors for advancement from latent to full-blown active TB disease (Narasimhan *et al.*,

2013). Nonetheless, underlying mechanisms by which risk factors, particularly the host factors, increase the likelihood of active disease development at biochemical, immunological and molecular levels are poorly understood. Also, the drawbacks of host immunity to *M.tuberculosis* as well as mechanisms adapted by the bacilli to effect them are largely speculative.

Understanding host immune mechanisms that confer protection against *M.tuberculosis* infection and host factors that determine development of primary active disease or reactivation from dormancy is a great scientific challenge that applies to a large proportion of individuals. This challenge arises due to the fact that in spite the development of obviously suitable immunological responses to *M.tuberculosis* infection, this does not reliably eliminate the bacilli in most cases. Instead, *M.tuberculosis* adopts a clinically quiescent, latent infection state, from which reactivation can subsequently ensue. Though the wide spectrum of immune protective mechanisms at early infection phase, from *M.tuberculosis* exposure to granuloma formation and entry into dormancy, have been unraveled through human and animal studies, the host and bacterial factors or the interplay of these factors that promote *M.tuberculosis* reactivation from the state of dormancy are not clearly understood. It must be noted that reactivation of TB disease from a state of dormancy accounts for majority of the incident TB cases in adults, and this is a major public health concern as estimates show that over 3 billion people harbor *M.tuberculosis* in latent infection (Pai *et al.*, 2016).

In most individuals with latent TB, combinations of immunological responses sufficiently support controlled, asymptomatic infection. Nonetheless, in some infected individuals, for yet unclear reasons, the infection can advance to clinical disease. Traditionally, *M.tuberculosis*-specific T-cells were regarded as the hallmark of anti-TB protective immunity, and hence an efficient vaccine target. But, regardless of enhanced levels of T-cell mediated response, a recent TB vaccine did not improve protection (Tameris *et al.*, 2013). Emerging reports continue to indicate that innate immunity is crucial in the host regulation of *M.tuberculosis* (Korbel *et al.*, 2008; Lerner *et al.*, 2015; Liu *et al.*, 2017). Phagocytosis, which is an aspect of innate immunity, includes migration of phagocytes, recognition and attachment of antigen to receptors on phagocytes, internalization and intracellular killing. The process of phagocytosis and the subsequent interactions in post-primary (reactivation) TB disease could therefore be explored to identify possible targets and design of more successful vaccines or immunotherapeutic intervention for better TB control and eventual eradication.

The process of phagocytosis involves movement of circulating immune cells to the locus of infection or tissue injury to eliminate infectious agent or cause of tissue injury, as well as contribute to tissue repair. Migration of phagocytes across the endothelium from the circulatory pool to the lungs is important in TB disease to sustain effectual immune response and prevent spread of the bacilli to extra-pulmonary spaces from the lung (Ernst, 2012). This process usually involves release of chemokines, which draw leucocytes to infection site and restrict recruited leucocytes within infection site. Previous studies demonstrated an association between active TB disease and polymorphisms in chemokines and chemokine receptors coding genes necessary for immune cell trafficking – including functional variants of C-C motif chemokine ligand 2 (CCL2), C-C motif chemokine ligand 3 like 1 (CCL3L1) and C-C chemokine receptor type 5 (CCR5) (Flores-Villanueva *et al.*, 2005; Mamtani *et al.*, 2011). However, it is unclear if migration of leucocytes is hampered in active PTB. Thus migration of circulating immune cells in PTB patients at diagnosis and during chemotherapy was examined in this study.

Attraction of phagocytes to the infection site leads to recognition and engulfment of pathogen, phagolysosome fusion and intracellular killing of pathogen. A cornerstone of pathogen intracellular killing is the generation and release of anti-microbial oxygen radicals (hydroxyl radical – OH^-, hydrogen peroxide – H_2O_2, superoxide - O_2^-, hypochlorite – HClO) and nitrogen radicals (nitric oxide – NO, peroxynitrite – $ONOO^-$) by phagocytes via the respiratory burst pathway and inducible nitric oxide synthase (iNOS) system respectively (Halliwell and Gutteridge, 2007; Nambi *et al.*, 2015). Several human, animal and cell lines studies have clearly demonstrated that oxidants from phagocyte respiratory burst are vital in defense against *M.tuberculosis* (Cooper *et al.*, 2000; Lee *et al.*, 2008; Yang *et al.*, 2012). Furthermore, moderate increases in superoxide are known to induce substantial enzymatic and growth defects in bacteria due to the remarkably higher rate constants ($10^6 M^{-1} s^{-1}$) for superoxide-mediated inactivation of essential metabolic enzymes (Flint *et al.*, 1993). Various animal models defective in active NADPH oxidase (NOX) components studied for their capacity to check *M.tuberculosis* growth and expansion gave conflicting reports (Adams *et al.*, 1997; MacMicking *et al.*, 1997; Scanga *et al.*, 2001; Jung *et al.*, 2002). Data on phagocyte respiratory burst in humans with active TB are scarce. Hence, it could be postulated that recruitment of immune cells that are defective in bactericidal potential could also allow advancement from latency to active PTB.

Control of *M.tuberculosis* expansion and containment of the bacilli within the lung is facilitated by interactions of immune cells and components that depend on interplay of factors including cytokines and micronutrients. Reports from animal and human studies have associated interleukin 6 (IL-6) with effectual early expression of immunity in the lung during infection (Domingo-Gonzalez *et al.*, 2016). Nonetheless, there is dearth of information on IL-6 level and its association with oxidative intracellular killing in individuals who progress from latent to active TB disease before and during anti-TB treatment. In addition, tissue monocyte and macrophage phagocytosis of *M.tuberculosis* forms a crucial stimulus for IL-8 secretion *in vitro* (Friedland *et al.*, 1992). Based on its chemotactic functions, IL-8 secretion is expected to promote recruitment of more phagocytic cells to the infection site with a resultant positive association between IL-8 and measures of phagocytosis as well as leucocyte migration in TB. However, there is dearth of information on the associations between circulating levels of IL-8 with measures of phagocytosis and leucocyte migration in PTB patients before and during anti-TB treatment.

The study of Botella *et al.* (2012) demonstrated utilization of iron, zinc and copper by phagocytic cells to enhance intracellular killing of microorganisms. While a possible significant role of copper, iron and zinc in the alteration of macrophage function has been suggested, evidences abound showing a close relationship between cellular immunity and the vitamins A, C, D and E (Erickson *et al.*, 2000). Though the mechanism by which these vitamins boost cellular immunity are still largely unclear, different population studies continue to associate deficiency of these vitamins with higher TB risk (Nnoaham and Clarke, 2008; Williams *et al*., 2008). Vilcheze *et al.* (2013) demonstrated extraordinary *M.tuberculosis* (drug- sensitive and resistant) sensitivity to killing by vitamin C, which depended on elevated ferrous ion and reactive oxygen species generation as well as pleiotropic activities affecting various biological processes in *M.tuberculosis*. Previous studies (Akinyoola *et al.*, 2012; Edem *et al.*, 2012) showed reversal of toxicant-induced modulations in nitric oxide production and respiratory burst-myeloperoxidase enzyme activity by supplementation of vitamins C and E in Wistar rats. Hence it can be posited that micronutrient supplementation in addition to standard anti-tuberculosis therapeutic regimen could therefore improve immunological containment of *M.tuberculosis* and thereby facilitate better control of the disease and improved treatment outcomes in drug-sensitive and drug-resistant PTB.

1.1 Justification for the study

Although, advancement from *M.tuberculosis* latency to active PTB has been linked to host characteristics such as gender, age, malnutrition, diabetes and immune state, as well as social and environmental determinants such as poor ventilation, crowding, smoking, alcohol use and occupational risk (Narasimhan *et al.*, 2013), the underlying mechanisms by which risk factors, particularly host factors, increase the likelihood of active disease development at biochemical, immunological and molecular levels are not clearly understood.

Understanding the mechanisms of host immunity against *M.tuberculosis* and host factors that determine development of primary active disease or reactivation from dormancy is a great scientific challenge with direct health impact on the health and lives of a large percentage of the human population habouring *M.tuberculosis* in the latent state of infection. Immunological and genetic studies support essential function of the innate immune cells as antigen presenters and effector cells in eradication of *M.tuberculosis*. Hence, an understanding of the state of key innate cellular immunological activities such as phagocytosis which involves migration, recognition, internalization and intracellular killing and factors that modulate these activities at PTB diagnosis and during anti-tuberculosis chemotherapy may aid in identification of targets for adjunct immunotherapy and vaccine development in PTB control and management.

1.2 Hypothesis

Phagocytic mechanisms and its plasma modulators are altered by zinc supplementation in pulmonary tuberculosis patients on anti-tuberculosis chemotherapy.

1.3 Aim of the study

To examine phagocytic mechanism and its plasma modulators in pulmonary tuberculosis patients on anti-tuberculosis chemotherapy and zinc supplement.

1.4 Objectives of the study

1. To measure the index of leucocyte migration (leucocyte migration inhibitory factor-LMIF) and phagocytic activity (nitroblue tetrazolium test-NBT) of leucocytes in PTB patients at diagnosis and during anti-TB chemotherapy compared with controls
2. To assess activities of superoxide dismutase (SOD), catalase (CAT), myeloperoxidase (MPO) and nitric oxide (NO) concentrations in plasma of PTB patients at diagnosis and during anti-TB chemotherapy compared with controls
3. To evaluate plasma concentrations of zinc (Zn), iron (Fe), copper (Cu) and vitamins A, C, D and E in PTB patients at diagnosis compared with controls
4. To examine plasma levels of IL-8 and IL-6 in PTB patients at diagnosis and during anti-TB chemotherapy compared with controls
5. To assess activities of SOD, CAT, MPO and NO concentrations in plasma of PTB patients at diagnosis and during anti-TB chemotherapy with zinc supplementation.
6. To assess the interations between phagocytic mechanism and its plasma modulators in PTB patients.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 TUBERCULOSIS

The infectious disease, tuberculosis (TB) is caused by infection with bacilli of the *Mycobacterium tuberculosis complex*. Members of the complex are *Mycobacterium tuberculosis*, *Mycobacterium bovis, Mycobacterium africanum, Mycobacterium microti, Mycobacterium caprae, Mycobacterium canetti, and Mycobacterium pinnipedi* (Boulahbal and Heifets, 2006; Grange, 2008). They are generally facultative intracellular pathogens and obligate aerobes; hence they grow better in oxygen rich tissues, which explain why pulmonary TB is more common form of TB. Nonetheless, recent advances are expanding our awareness of mycobacterial physiology in hypoxic conditions, particularly in regard to gene regulation, metabolism and energy homeostasis. Despite the high degree of DNA similarity within the mycobacterial complex, *M.tuberculosis* is the major cause of human tuberculosis (Niemann *et al.*, 2000), and molecular typing of *M. tuberculosis* complex among TB patients in Nigeria showed that the disease is mostly due to *M.tuberculosis,* followed by *M.africanum* infection (Cadmus *et al.*, 2006).

Tuberculosis has been with man through out known history (Daniel, 2006). Tuberculosis, historically named phthisis or consumption has been a constant threat to mankind, hitting hardest in densely populated urban settings (Herzog, 1998). The earliest evidence of TB in man date back to about 8000 BC and is presented by lesions in fossil bones (Herzog, 1998). Other early evidences of TB were found in pre-historic skeletal human remains dating from 7000 BC (Zink *et al.*, 2003). Severe TB disease was mentioned around 2000 BC in both India and the Americas, and references to “White plaque and White death” in 460 BC where Hippocrates opined that phthisis was a prevalent fatal hereditary disease. Though isolation in 1882, of tubercle bacillus by Robert Koch altered perception of the disease, and introduction of Bacille Calmette-Guerin (BCG) in 1921 followed by streptomycin, isoniazid and rifampicin mid 20th century raised optimism of disease eradication. Nonetheless, till date, TB remains a foremost public health challenge.

Though TB is curable, there is however no optimal and consistently effective vaccine for the control of infection with the causative agent. Hence best approaches for the disease containment aim to lower transmission via early identification and treatment of cases and preventive therapy for persons with latent TB. Whereas treatment of persons with active TB

is available in several countries, use of preventive therapy for latently infected individuals is yet to be implemented globally due to concerns with development of drug resistance from poor compliance as well as poor screening for latent TB in high-risk groups.

2.1.1 MYCOBACTERIUM COMPLEX

Mycobacteria are non-sporulating, non-motile, weakly gram-positive, acid-fast bacilli that appear as rods (straight or slightly curved) microscopically, 1-4 micrometers (μm) in length and 0.3-0.6 μm in width. Mycobacteria are of the Actinomycetales order which express rare mycolic acids within their cell wall (Barry *et al.*, 1998). The cell wall is a distinguishing features of this genus conferring them with acid-fastness, resistance to acidity/alkalinity, drying, extreme hydrophobicity, distinctive immunostimulatory properties and defiance of antibiotics (Daffe and Draper, 1998). They are not stained with standard stains because of impermeability and resistance of their lipoid (lipid-rich) cell wall. Acid-fast stains on the other hand utilize their capacity to retain dye following pre-treatment with acid-alcohol solution or mineral acid. Hence use of Ziehl-Neelsen (ZN stain) for its staining (AFB) (Hernandez-Pando *et al*., 2000; Glickman *et al.*, 2001).

The TB causing mycobacteria found in humans include *M.tuberculosis, M.africanum, M.bovis, M.canetti* and *M.microti* which, make up the *Mycobacterium tuberculosis* complex. Other disease causing *Mycobacteria* in humans are classed as non-tuberculous mycobacteria (NTM). Although NTMs occasionally cause illness with clinical presentation similar to tuberculosis within lungs, lymph nodes, bones or skin of host. However, most NTM are present in the environment, are mostly non-pathogenic in individuals with uncompromised immunity or healthy lung tissue. *Mycobacterium tuberculosis* and *Mycobacterium leprae* are however considered major human pathogens amongst the species of mycobacterium.

M.tuberculosis divides in 12- to 24-hours and around 21 days to culture on agar. The reasons for the slow growth vis-à-vis elongated culture period are not well understood but proposed theories include nutrient uptake limitation via their highly impervious cell wall with reduced RNA synthesis rate (Harshey and Ramakrishnan, 1977). Metabolic transition from aerobic (carbohydrate metabolizing) phase to a microaerophilic (lipid metabolizing) phase has been demonstrated in previous experimental studies (Bloch and Segal, 1956). The bacilli grows and expands within phagocytes, especially macrophages and monocytes, thus they are facultative intracellular bacteria. An evolution of *M.tuberculosis* from *M.bovis* during cattle

domestication was previously inferred (Stead *et al.*, 1995). However, genomic sequencing studies demonstrated that multiple DNA deletions in *M.bovis* while maintaining 99.95% identity with *M.tuberculosis* without any new genetic material (Brosch *et al.*, 2002), indicating a reverse case. Further genetic analyses implicate *M.canetti* (*Mycobacterium prototuberculosis*) as the origins of the *M.tuberculosis* complex (Gutierrez *et al.*, 2005).

2.1.2 EPIDEMIOLOGY OF TUBERCULOSIS

Approximately a third of the global population, which represents around 3 billion people, are projected to be infected with *M.tuberculosis* in the latent state (WHO, 2016). Between 5%-15% of these individuals advance to active symptomatic TB disease (Vynnycky and Fine, 1997), whereas others remain at risk of advancing to active TB for life (Andrews *et al.*, 2012). Recent estimates show that TB ranks ninth in cause of fatality globally and highest cause of fatality due to single infectious agent (WHO, 2017). Nonetheless, with timely diagnosis and proper treatment most TB associated deaths are preventable, which implies increased efforts towards closing the large gaps in detection and treatment (WHO, 2017). Globally, TB case notification rate has improved from 40% in 1995 and 56% in 2005 to 61% in 2016 while in Africa, case detection improved from 32% in 1995 to 50% in 2016 (WHO, 2017). In Nigeria, TB case detection rate improved from 8.9% in 1995 and 26% in 2005 to 51% in 2012 (WHO, 2013). However, there are still large gaps between case notification and estimated incidence in several regions globally.

Globally, about 10.4 million persons were sick with TB in 2016 (WHO, 2017). Western Pacific, Africa and South-East Asia were the foremost TB incidence contributors around the world, recording 1.8 million, 2.5 million and 4.6 million respectively. The scale of the global TB burden is expressed as incident cases per 100,000 population. A global TB burden of 142 per 100,000 in 2004 is comparable to 140 per 100,000 in 2016 (WHO, 2017), indicative of the need for increased efforts to control the disease. The 2016 estimates indicate 1.3 million TB deaths with 374,000 of these in people living with HIV/AIDS (WHO, 2017). Tuberculosis is therefore reported as the most common death cause among those living with HIV/AIDS and Africa, which has the highest HIV/AIDS prevalence accounts for the majority of TB-HIV/AIDS co-infection and mortality (WHO, 2017).

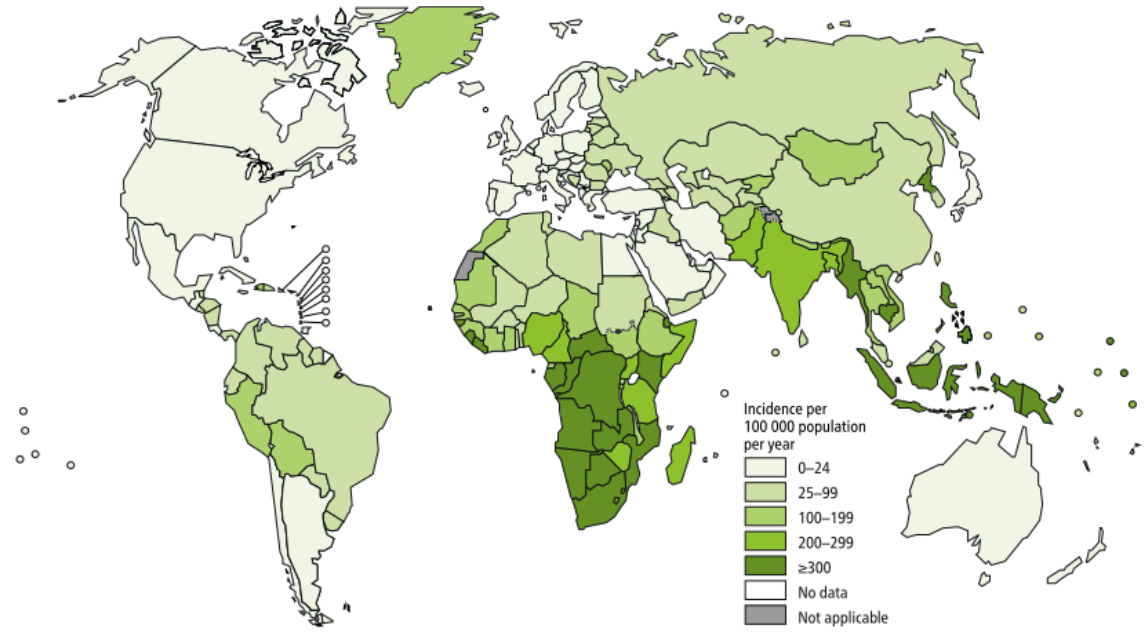


Figure 2.1: Estimated TB incidence rates, 2016

Source: WHO TB Report 2017

Figure 2.2: Estimated TB incidence in 2016, for countries with at least 100, 000 incident cases

Source: WHO TB Report 2017

Figure 2.3: Ten countries with the largest gaps between notification of new and relapse (incident) TB cases and estimated TB incidence in 2016

Source: WHO TB Report 2017

2.1.3 Tuberculosis Epidemiology in Nigeria

In the group of 30 high TB burden countries, Nigeria is ranked 4th. The World Health Organization estimated 407,000 incident cases of all forms of TB occurring in 2016, with 63,000 new cases being persons with HIV/AIDS (WHO, 2017). This results to an incidence of 219 per 100,000 population (WHO, 2017). It was also estimated that 16% of people with HIV/AIDS in Nigeria came down with TB in 2016 (WHO, 2017). Furthermore, 115,000 HIV-negative individuals and 39,000 HIV-positive individuals died from TB, representing 37.8% of TB incidence in 2016 (WHO, 2017). In Nigeria the rate TB increase is 7% annually as against 0.04% globally (Dauda, 2010).

Nigeria concluded its first National Tuberculosis prevalence assessment conducted by the National TB & Leprosy Control programme (NTBLCP) in November 2012 (FMOH, 2012). The survey determined PTB prevalence (microbiologically-confirmed: smear/culture confirmed) for people resident in the country and aged 15 years or more. Out of 113,247 volunteers, 77,797 (68.7%) were eligible in seventy clusters. About 44,186 volunteers

(56.8%) participated in the observational study, and 4,688 (10.6%) of these presented sputum for analysis. The mean number of study enrollees in each cluster was 631 (range: 279-819).

From all sputum samples analysed, there were 37 culture-positives with 107 smear-positives, that is 144 microbiologically-confirmed PTB cases. Tuberculosis prevalence per 100,000 population (≥15 years) was calculated as 524 (95% CI: 378-670) for microbiologically-confirmed and 318 (95% CI: 225-412) smear-positive only. Prevalence of smear-positive TB among males 484 (95% CI: 333-635) per 100,000, greater than females with an estimate of 198 (95% CI 108-289) per 100,000. The survey concluded that the TB burden in Nigeria is much greater than previously assumed with increased chances of ongoing transmission (FMOH, 2012) and despite ongoing DOTS implementation, DOTS services appears not to have gained full penetration into the community 10 years post implementation (FMOH, 2012).

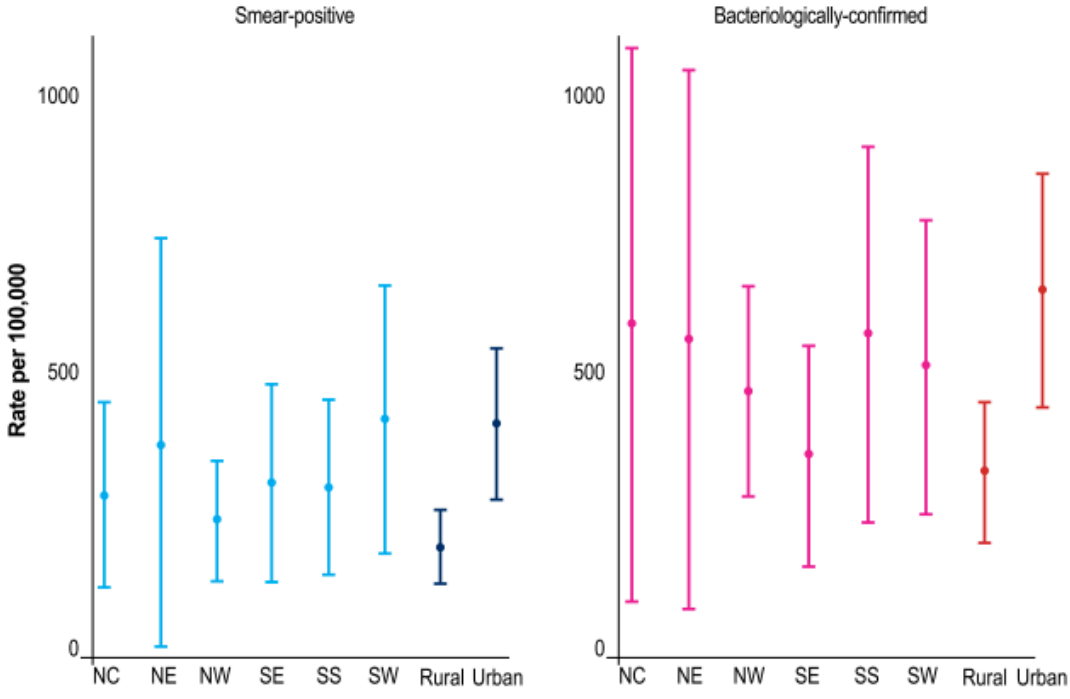


Figure 2.4: Zonal and Urban/Rural-specific TB prevalence rates per 100,000 in Nigeria
Source: NTBLCP database and 1st National TB prevalence survey, Nigeria

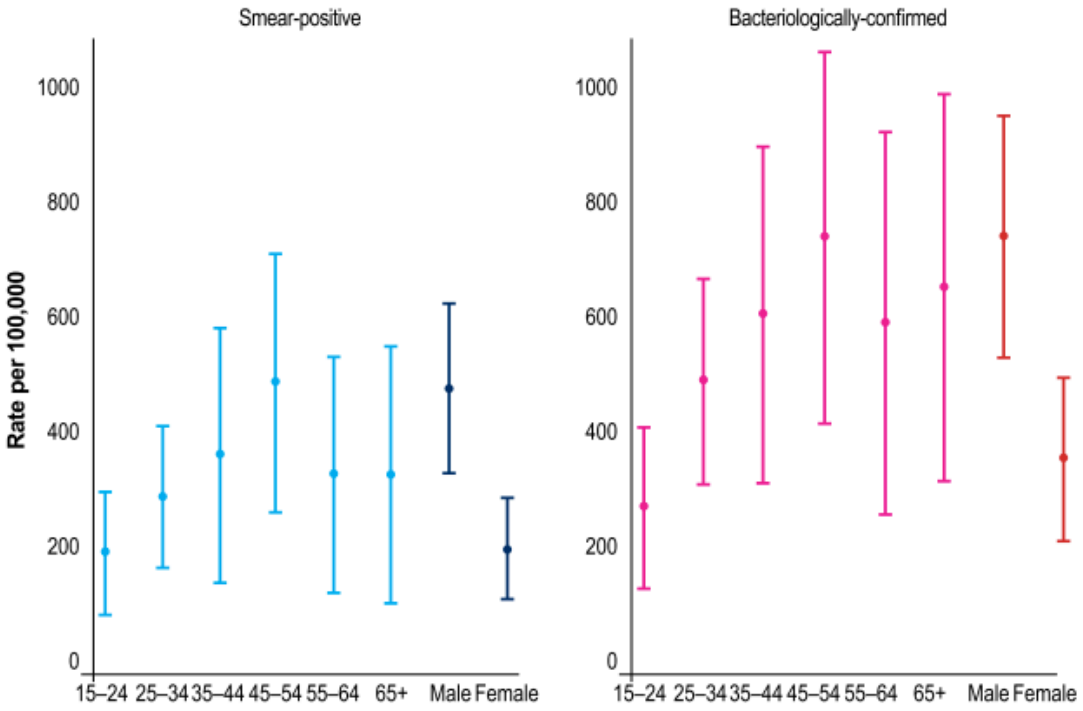


Figure 2.5: Age and sex-specific TB prevalence rates per 100,000 in Nigeria

Source: NTBLCP database and 1st National TB prevalence survey, Nigeria

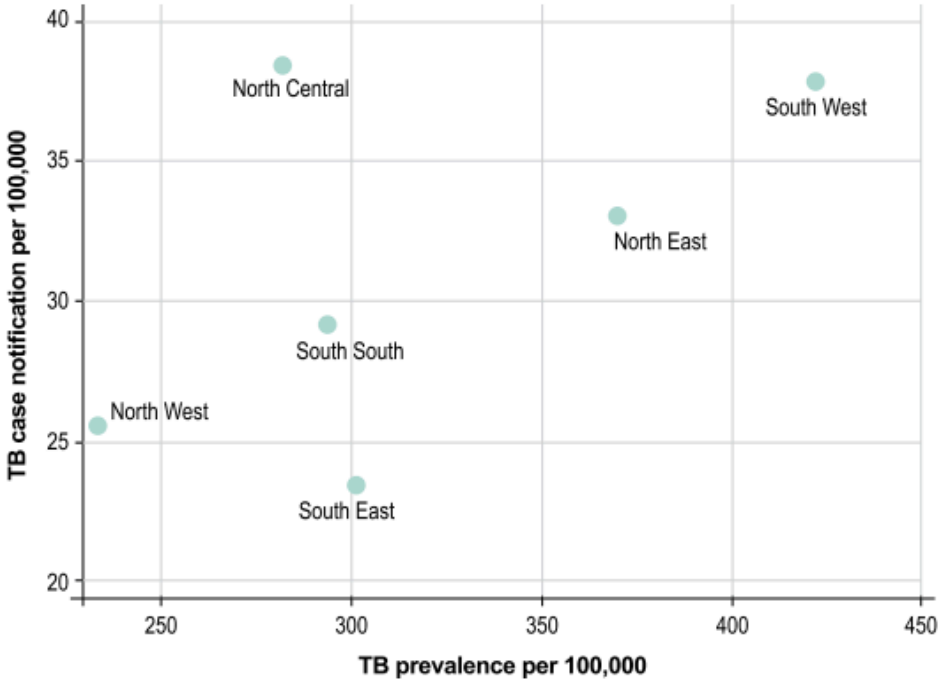


Figure 2.6: Scatterplot of smear-positive case notification rates against prevalence rated by zones in Nigeria

Source: NTBLCP database and 1st National TB prevalence survey, Nigeria

Drug-resistant TB

Drug resistance critically undermines tuberculosis eradication, both at the level of patient care and disease prevention. It imposes the chances of a return to the era where anti-TB treatments are not effective for the cure of tuberculosis, which makes it an utmost public health challenge in many regions. Drug resistant TB is classified into three broad categories; Rifampicin-resistant TB (RR-TB), Multi-drug resistant TB (MDR-TB) and Extensively-drug resistant TB (XDR-TB) (WHO, 2017). RR-TB is TB that is rifampicin resistant, MDR-TB is TB resistant to isoniazid and rifampicin, while XDR-TB is MDR-TB plus resistance to any of the fluoroquinolones or second-line injectables (capreomycin, amikacin or kanamycin) (WHO, 2017). There has been a worldwide manifestation of MDR-TB in the last 30 years (CDC, 1991; Frieden *et al.*, 1993; Rullan *et al.*, 1996; Ritacco *et al.*, 1997), XDR-TB (Gandhi *et al.*, 2006; Shah *et al.*, 2007; WHO, 2010) with emerging indications of strains resistant to all conventional anti-TB drugs (Migliori *et al.*, 2007; Shah *et al.*, 2009; Velayati *et al.*, 2009)

In 2016 an estimated 4.1% (95% CI 2.8-5.3%) of new cases and 19% (95% CI 9.8-27%) of previously treated cases had MDR/RR-TB globally (WHO, 2017). This represents an estimated 600,000 new MDR/RR-TB cases with MDR-TB responsible for 82% (490,000) of the new cases in 2016 (WHO, 2017). Approximately 240,000 fatalities from MDR/RR-TB were reported in 2016 (WHO, 2017). At the close of 2016, reports of XDR-TB was recorded in 123 member states of the WHO and the percentage of drug resistant cases that were XDR-TB was 6.2% (95% CI 3.6-9.5%). Nigeria was reported to have 2.2% and 9.4% MDR-TB rate among new and re-lapse TB respectively (WHO, 2011). Nigeria is one of 4 African countries recording the heightened TB drug resistance burden (WHO, 2011). According to the national TB drug resistance survey, MDR-TB prevalence rate of 2.9% and 14.3% among new and re-treatment TB patients was reported in 2012 (FMOH, 2012).

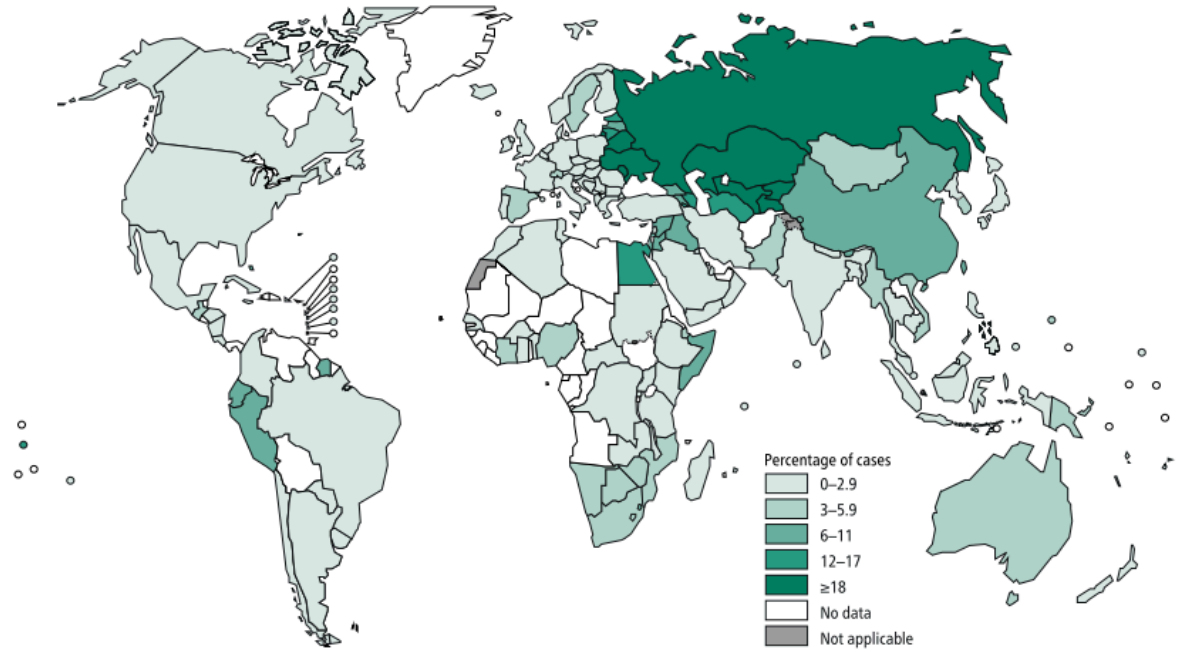


Figure 2.7: Percentage of new TB cases with MDR/RR-TB in 2016

Source: WHO TB report 2017

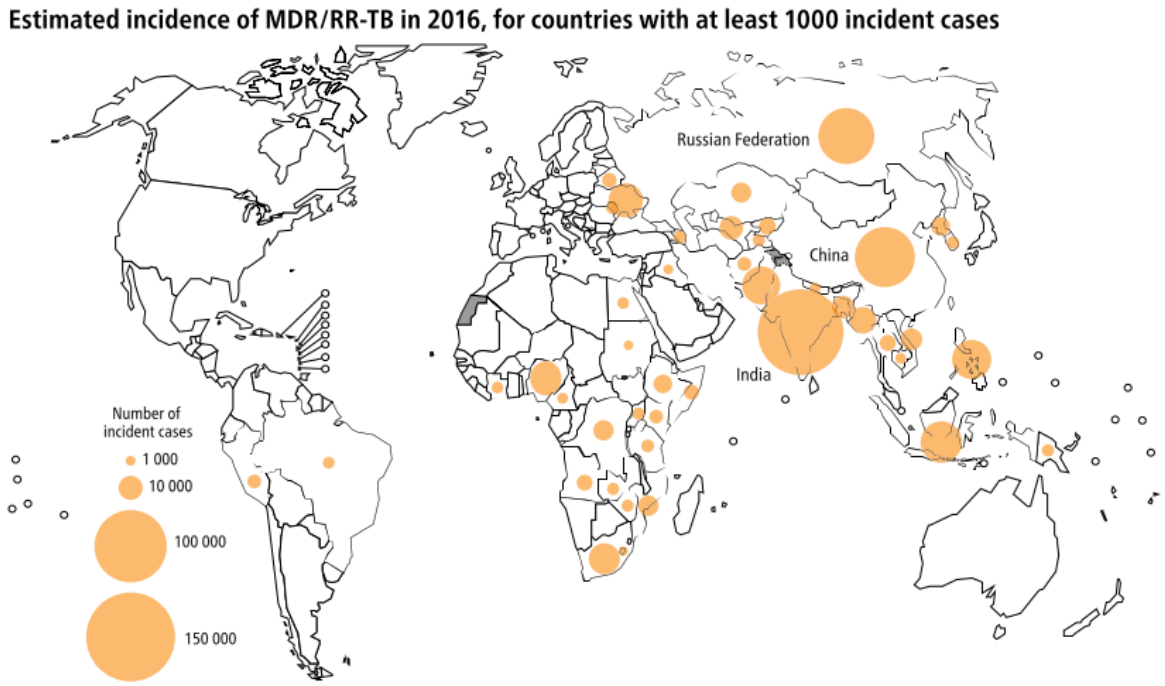


Figure 2.8: Estimated incidence of MDR/RR-TB in 2016, for countries with at least 1000 incident cases

Source: WHO TB report 2017

2.1.4 Tuberculosis Transmission

Tuberculosis is transmitted via the spread of the bacteria from person-to-person mostly by aerosolized particles. The sizes of *M.tuberculosis* droplets ranges from 0.65 to >7.0µm (Fennelly *et al.*, 2004). Whereas small *M.tuberculosis* particles transit past nasopharyngeal or tracheobronchial section toward the distal airways, the large particles may get stopped within the upper airway or oropharynx and lead to tuberculosis of the oropharynx (Fennelly and Jones-Lopez, 2015). These infectious *M.tuberculosis* containing droplets are expelled into the air via the respiratory tract pathways when an infected person coughs, sings, shouts, sneezes or undergoes any other energetic expiratory action that releases airway secretions, but coughing is most effectual in generation of *M.tuberculosis* infested aerosols (Turner and Bothamley, 2015). On the other hand, Zoonotic TB caused by *M.bovis* or *M.caprae* infection is contacted from cattle, commonly by ingestion of contaminated raw milk (Bos *et al.*, 2014). Humans remain the only recognized reservoir of *M.tuberculosis* as no environmental reservoirs have been identified (Cornas *et al.*, 2013).

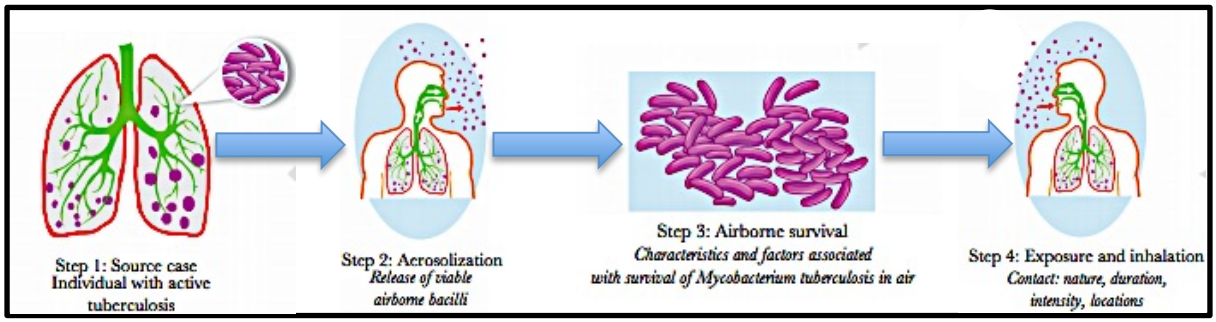


Figure 2.9: Schematic representation of tuberculosis transmission
(Turner and Bothamley, 2015)

Increasing evidence from countries with high tuberculosis burden, indicate an interplay of several factors in determining the intensity of tuberculosis transmission as against the simplistic model described above. It is opined that the veracity of local *M.tuberculosis* transmission depends on infection prevalence and infectiousness of individuals, susceptibility of contacts, the regularity and proximity of contact, nature of the pathogen (virulence), and environmental factors (crowding, ventilation and UV light exposure) (CDC, 2005). Pienaar *et al.* (2010) in their epidemiologic and modeling data indicated that the probability of spread of *M.tuberculosis* is proportional to exposure duration and inversely proportional to the volume of space within which exposure occurs.

2.1.5 *M.Tuberculosis* Infection

Epidemiological studies pose evidence for several potential outcomes following exposure to Mtb via aerosol including immediate clearance of the bacteria, development of primary TB or asymptomatic latent TB that has the propensity to transit to active disease (Dye *et al*., 1999). Early eradication of *M.tuberculosis* following exposure is particularly due to innate immune response or may also involve adaptive immune response, and exposure to *M.tuberculosis* in most non-vaccinated individuals does not lead to infection as the bacteria are cleared before an infection is manifested. It is acknowledged that about 50% of *M.tuberculosis* exposed persons show negative tuberculin skin test (TST) results, even among close household contacts (Morrison *et al.*, 2008). This implies eradication of *M.tuberculosis* by innate immune response. Also, among 95 contact investigation studies from low- and middle-income countries, *M.tuberculosis* infection prevalence among contacts was 51.5% (Fox *et al.*, 2013).

In the instance that the tubercle bacilli is not cleared, the model of human interaction with *M.tuberculosis* is represented with the binary outcome of either development of asymptomatic latent infection or active disease. Latently infected individuals can either remain asymptomatic throughout their lifetime or develop active disease at a remote time from the primary infection in a process known as reactivation. Epidemiologic studies have shown that only about 5% of healthy individuals resent with symptomatic TB disease upon first exposure to *M.tuberculosis* (Shiloh, 2016). Although it is has been difficult to clearly identify persons with an increased risk of infection, however certain host factors have been associated with likelihood of infection upon exposure. HIV infection, anti-TNF therapy, diabetes, renal dialysis, organ transplantation, sislicosis, malnutrition, illegitimate drug use, deleterious alcohol use and smoking have been correlated with TB infection susceptibility (Churchyard *et al.*, 2017).

Clinical course of *M.tuberculosis* Infection

Following transit through the upper airway, *M. tuberculosis* proliferates slowly, particularly in terminal alveoli (primary focus) and draining lymph nodes: these denote primary infection. About 8 weeks after infection, the primary focus becomes contained and condensed with central area of parenchymal necrosis (caseous necrosis) resulting from the actions of lymphocytes and macrophages. Specific TB immunity becomes evident at this point with positive tuberculin skin reaction (Grange *et al.*, 2011). There are two potential outcomes

consequent to the above sequence of interactions; the bacteria may cause primary symptomatic TB or bacilli develop dormancy and stays asymptomatic as latent tuberculosis infection (LTBI).

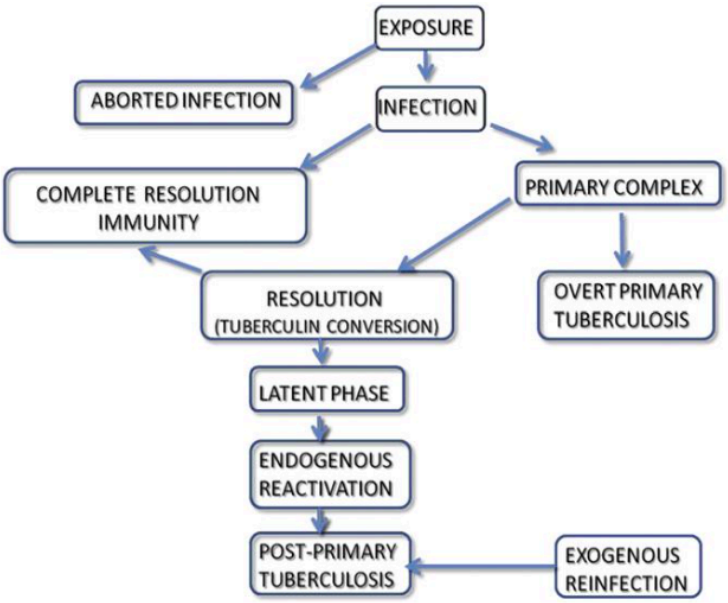


Figure 2.10: Flow chart of the 'timetable' of tuberculosis

Source: Grange *et al.* (2011)

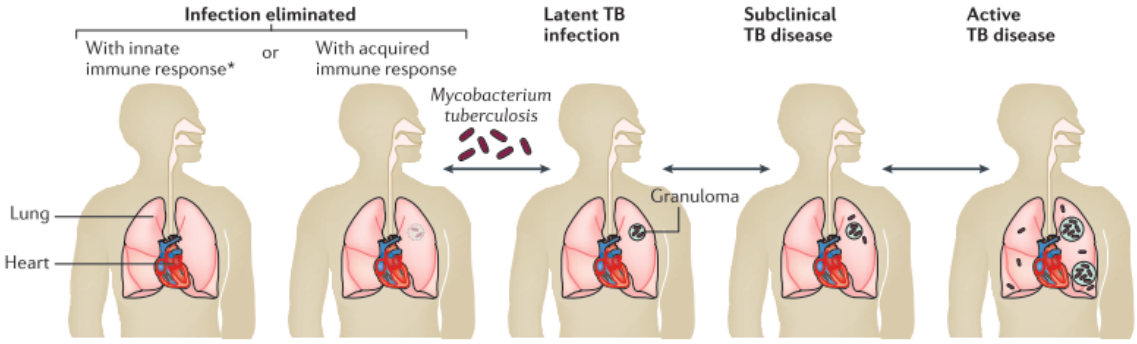


Figure 2.11: Clinical course of *M.tuberculosis* infection states

Source: Grange *et al.* (2011)

LTBI

Latent TB infection is widespread and roughly 2 billion individuals are latently infected around the globe (WHO, 2012). These subjects represent a huge reservoir for TB disease and transmission even without any new infection (Corbett *et al.*, 2003). Pre-existing immunity in

subjects with latent infection (or BCG vaccination) is posited to mediate containment of *M.tuberculosis* during re-infection, but does not provide full protection and might even cause a more violent tissue-destroying hypersensitivity reaction (CDC, 2010). Transition from latent TB to active TB is generally believed to be facilitated by factors that impair host immunity. In 22 high burden countries that account for 80% of the estimated total TB burden, Lönnroth and Raviglione (2008) estimated the percentage of TB cases that can be attributed to specific risk factors (population attributable fraction (PAF)): malnutrition; 34%, indoor air pollution; 26%, smoking; 23%, harmful alcohol use; 13%, HIV; 7%, and diabetes; 6%. Nonetheless, the likely import of these factors depend on their prevalence, which differ between countries and regions. In addition, genetic variation in *M.tuberculosis* strains correlate with virulence and transmission, while genetic variations between populations and individuals has been linked with TB susceptibility (Caws *et al.*, 2008; Dye and Williams, 2010). It is not clear how these factors combine or interact to influence the outcome of *M.tuberculosis* exposure and infection in individuals and regions.

Active TB disease

The synergy of macrophages, dendritic cells and T cells sufficiently controls bacterial growth, resulting in asymptomatic infection in most individuals. However, in some individuals, for reasons not entirely understood, infection progresses to clinical disease within weeks (primary disease) to years (reactivation). Active pulmonary TB could present as asymptomatic, mild to progressive dry cough, or multiple symptoms, including weight loss, frequent fatigue, fever, night sweats, pain in the chest, and a cough that produces bloody sputum. There may also be cases of extra-pulmonary tuberculosis characterized by spread of bacteria to other body organs. In 2016, extra-pulmonary TB represented 15% of the estimated 6.3 million incident cases. Several factors are associated with advancement to active disease from infection (Narasimhan *et al.*, 2013). These are generally conditions that alter immune response, with HIV co-infection being the most important of these. Diabetes, malnutrition, indoor air pollution, alcohol and tobacco smoke are factors that affect greater part of the population and fast-track advancement to active TB disease.

2.2 IMMUNOLOGY OF TUBERCULOSIS

OVERVIEW OF HOST DEFENSE AGAINST INVADING PATHOGENS

The human body has sophisticated ways of defending itself from invading foreign bodies. The immune system is made up of specialized cells of the innate immunity that ingest and

kill microorganisms. Some of these cells are capable of activating other immune cells to form a massive inflammatory reaction and long lasting immunological memory. For a long time the polymorphonuclear neutrophil (PMN) was considered a quite simple cell whose main objective was to phagocytose and efficiently kill microorganisms. During the last decades, the view on this cell type has changed and the PMNs are constantly attributed new functions such as recruitment and modulation of adjacent cells. Macrophages and DCs are professional phagocytes with capacity to ingest preys. In addition to ingestion and killing of pathogens, these cells attract and present peptides derived form the ingested prey to induce an immune response specifically directed to the specific pathogen-derived peptides, adaptive immunity. Macrophages and DC activation is thus necessary for the initiation of adaptive immunity in primary infection.

Tissue macrophages and DCs present antigens present at the infection site initiates the initial inflammatory reaction through the recruitment and activation of additional inflammatory cells such as PMNs, monocytes (subsequently differentiating into tissue macrophages) and lymphocyte. By migration to draining lymph nodes, DCs encounter an array of lymphocytes increasing the possibility of finding a lymphocyte that is specific for the antigen presented. It is crucial that cells of the innate immunity are sufficient to contain the initial infection since the adaptive T-cell response, B-cell activation and specific antibody production takes about two weeks to mobilize and reach sufficient clonal expansion at primary infection site.

HOST DEFENSE AGAINST MYCOBACTERIUM TUBERCULOSIS – UPPER AIRWAY.

M.tuberculosis passes into the alveoli in the lung following inhalation through the nose and mouth. The respiratory mucosa within the airway constitutes the initial defence against *M.tuberculosis* (Middleton *et al.*, 2002). This respiratory mucosa is comprised of (a) the epithelium, a layer of airway epithelial cells (AECs) forming a barrier preventing invasion; (b) the lamina propria, a layer of connective tissue and immune cells; (c) a covering of a highly complex substance known as airway surface liquid (ASL), containing mucus, IgA and a collection of other innate immune elements. Similarly located in key positions within the airway to tackle *M.tuberculosis* are bronchial- or nasal- linked lymphoid tissues that are important for *M.tuberculosis* antigen sampling (Lugton, 1999).

AECs identify pathogen-associated molecular patterns (PAMPs) on *M.tuberculosis* surfaces via pattern recognition receptors (Li *et al.*, 2012). Expression of these receptors by human AECs has been previously demonstrated and they also stimulate the production of cytokines and effector components to stage an effectual immune response. The AECs function as immune sentinels after *M.tuberculosis* exposure by antigen presentation to mucosa-associated invariant T cells (MAITs) (Harriff *et al.*, 2014) thereby enhancing their production of interferon gamma (IFN-γ) to rouse macrophages (Gold *et al.*, 2010), tumour necrosis factor alpha (TNF-α) and granzyme, towards *M.tuberculosis* elimination from host. Essentially, AECs regulate the content of airway surface liquid which contains anti-microbial peptides such as β-defensin 2, cathelicidin (LL-37), hepcidin and a diversity of cytokines and chemokines (Rivas-Santiago *et al.*, 2008; Sow *et al.*, 2011; Li *et al.*, 2012). *M.tuberculosis* that successfully goes through the barriers of the upper airways enters into the alveoli.

HOST DEFENSE AGAINST MYCOBACTERIUM TUBERCULOSIS – ALVEOLI.

The alveoli are comprised of a thin lining of type I and II epithelial cells and the immune cells, alveolar macrophages and dendritic cells (DCs). It is unclear if *M.tuberculosis* can infect type I epithelial cells which make up the alveolar walls and mainly responsible for gaseous exchange. On the contrary, *M.tuberculosis* infection of type II epithelial cells is widely reported, with *M.tuberculosis* DNA also detected from post-mortem analysis of these cells (Hernández-Pando *et al.*, 2000). Type II epithelial cells like AECs, also produce microbistatic and microbicidal substances (Rivas-Santiago *et al.*, 2005). Furthermore, type II epithelial cells generate and release hydrolytic enzymes, pulmonary surfactant and hydrolases. The surfactants are replete in fluid lining the epithelium and function in the early host–pathogen interaction (Watford *et al.*, 2001). Surfactant protein D prevents *M.tuberculosis* phagocytosis (Ferguson *et al.*, 1999). Conversely, hydrolases released by type II epithelial cells alter *M.tuberculosis* cell wall integrity thereby influence interactions with macrophages (Arcos *et al.*, 2011).

M.tuberculosis encounters dendritic cells (DCs) and macrophages within the alveoli (Tailleux *et al*., 2003; Wolf *et al.*, 2007). The macrophage serves as the prime host cell of *M.tuberculosis* which enters the macrophage through receptor-mediated phagocytosis. Complement and mannose receptors on the surface of the macrophage are the main receptor groups involved in *M.tuberculosis* phagocytosis (Schlesinger, 1993). The macrophages also express several other pattern-recognition receptors, including C-type lectin and Toll-like

receptors that identify *M.tuberculosis* components (Hossain and Norazmi, 2013). DCs perform a fundamental role in initiating adaptive immune response through priming of naïve lymphocytes (Banchereau *et al.*, 2000), and they are as well involved in induction and expansion of regulatory T cells (Treg) (Yamazaki and Steinman, 2009). Migration of DCs to the regional lymph nodes is important in the immune reaction to *M.tuberculosis* (Mihret, 2012), and infection of DCs by *M.tuberculosis* results in upregulation of the antigen presenting molecules, the major histocompatibility complex (MHC)-I and MHC-II in addition to co-stimulatory molecules (Henderson *et al.*, 1997; Mihret *et al.*, 2011).

After macrophage and DC infection, *M. tuberculosis* traverses the lung parenchyma, within which the infection process advances. This leads to recruitment of a high frequency of immune cells including neutrophils, interstitial macrophages, inflammatory monocytes and DCs. The recruited cells are infected by the expanding colony of *M.tuberculosis* resulting to early granuloma establishment. Recruitment of phagocytes confines and could eliminate infecting pathogens in other infections, however phagocyte recruitment in *Mycobacterial* infection really profits the pathogen by affording added cellular bodies for bacilli expansion (Davis and Ramakrishnan, 2009).

Pathogen recognition

Receptors identifying a variety of mycobacterial ligands contribute to human macrophage phagocytosis of *M.tuberculosis*. Collectins, C-type lectins, and toll-like receptors have been linked with recognition and ingestion of mycobacterial carbohydrates, lipoproteins and glycolipids (Philips and Ernst, 2011). During *M.tuberculosis* infection, mannose-binding lectin (MBL) identifies mannosylated lipoarabinomannan (ManLAM) and phosphatidylinositol mannosides (PIMs) (Philips and Ernst, 2011). Further to identification of *M.tuberculosis* ligands and intracellular ingestion, macrophage receptors are implicated in activation of intacellular pathways. Ligation of *M.tuberculosis* lipoarabinomannan and activation of macrophage peroxisome proliferator activated receptor gamma (PPARγ) expression in a phospholipase A2 and TLR-2 dependent manner by mannose receptor (MR), a trans-membrane C-type lectin has been suggested (Rajaram *et al.*, 2010; Liu *et al.*, 2015). *M.tuberculosis* is recognized by numerous receptors during human infection and no single receptor is entirely responsible for this (Sia *et al.*, 2015). On the other hand, DCs display mannose receptors, CD11b, CD11c, and DC- SIGN that are proficient in recognition of *M.tuberculosis* ligands. DC-SIGN is a prime receptor for *M.tuberculosis* ingestion by DCs through recognition of ManLAM (Geijtenbeek *et al.*, 2003; Tallieux *et al.*, 2003).

Antigen presentation

The dendritic cells are highly effective at antigen presentation to naïve T-cells, a vital source of IL-12 and induce anti-microbial and anti-tumor immune reactions. Previous studies indicate that DCs boost cellular immune reaction against *M.tuberculosis* infection (Demangel and Britton, 2000; Tascon *et al.*, 2000). DCs found in lung mucosa are dedicated for antigen ingestion. These DCs mature and move to lymphoid organs to activate T cells via expression of MHC and costimulatory molecules with release of cytokines upon interaction with pathogens (Banchereau and Steinman, 1998). Studies show that infection of monocyte derived dendritic cells (MDDCs) with *M.tuberculosis* results in up-regulation of MHC I and MHC II, CD40, CD54, CD58 and CD80, (Mihret *et al.*, 2011), a phenotype coherent with activation of DCs and increased generation of pro-inflammatory cytokines that effect maturation, migration and antigen processing and presentation. The DCs foster T cell activation with the initiation of efficient cell mediated immune response.

Furthermore, *M. tuberculosis* infected DCs induce T cells production of IFN-γ unlike monocyte derived macrophage (Giacomini *et al.*, 2001). Cross-talk between DCs and *M. tuberculosis* infected PMNs via the agency of DC-SIGN and Mac-1 induced DCs maturation that potentiates T-cell responses. T-cell activation and IFN-γ production could also amplify initial inflammatory processes with macrophage activation and bacterial elimination ensuing (Hedlund *et al.*, 2010). In mice, DCs depletion impaired their potential to mount effectual CD4 T cell response resulting in significant harm to bacterial replication containment, thereby presenting high bacterial load within the lungs and spleen (Tian *et al.*, 2005). The production of high levels of chemokines; CCL3, CCL4, CXCL8, CXCL9 and chemokine receptors; CCR7 by *M.tuberculosis* infected DCs have been demonstrated previously (Sasindran and Torrelles, 2011).

Immune cells recruitment

Neutrophils

Polymorphonuclear neutrophils (PMNs) are usually the first immune cells to permeate the lung post *M.tuberculosis* infection. They are also the most copious cell type present in bronchoalveolar lavage (BAL) and sputum of patients with PTB. Their recruitment to the lung are controlled by different cytokines and chemokines (Nouailles *et al.*, 2014; Niazi *et al.*, 2015), alarmins (S100A8/A9 proteins) (Gopal *et al.*, 2013) and inherently expressed miRNAs (miR-223) (Dorhoi *et al.*, 2013). They cause non-specific damage to bacteria and

host through the release of various molecular agents during respiratory bursts, including elastase, collagenase and myeloperoxidase. Neutrophils therefore represent an effective population of effector cells that carry out both anti-mycobacterial and immunopathological activities during *M.tuberculosis* infection (Dallenga and Schaible, 2016) due to their ability to also secrete enzymes that result in damage of pulmonary parenchyma (Hesse *et al.*, 2001; Elkington *et al.*, 2007). Previous studies showed inverse correlation between the pulmonary TB development and peripheral blood number of neutrophils in contacts of active TB patients. Additionally, in vitro diminution of whole blood neutrophil resulted in failure to contain *M.tuberculosis* growth (Martineau *et al.*, 2007).

In *M.tuberculosis* infection, apoptotic neutrophils are ingested by macrophages thereby leading to blocking of bacilli reproduction within the macrophages (Tan *et al.*, 2006). Programmed death ligand 1 (PD-L1) expression on neutrophil surfaces was also reported to be primarily accountable for elevated PD-L1 expression in whole blood of PTB patients, implying that neutrophils could also serve an immune-regulatory function during *M.tuberculosis* infection (McNab *et al.*, 2011). Furthermore, a loss of autophagy-related gene 5 (Atg5) in neutrophils has been reported to sensitize mice to *M.tuberculosis* (Kimmey *et al.*, 2015) given that neutrophils are implicated in stimulation of adaptive immunity and are important for granuloma cavitation in *M.tuberculosis* infection (Blomgran and Ernst, 2011; Ong *et al.*, 2015).

Monocyte/Macrophages

Monocytes circulate in blood and are recruited to location of infection or tissue damage upon stimulation. During this process, the cell gain qualities such as adherence to and migration into the tissue, increased phagocytic receptor expression and morphological characteristics for macrophage (Guirado *et al.*, 2013). Studies show that diverse macrophage subsets and diverse phagocytes are important at initiation of infection, development of granuloma and cytokine expression and response (Russell *et al.*, 2009; Galagan *et al.*, 2013; Srivastava *et al.*, 2014). Nonetheless, macrophages generally control *M.tuberculosis* growth by these mechanism; surface binding of *M.tuberculosis* to macrophages and ingestion, phagosome-lysome fusion, mycobacterial growth inhibition/killing (Raja, 2004).

Binding and ingestion of *M. tuberculosis* by monocytes / macrophages:

The complement receptors (CR1, CR2, CR3 and CR4), mannose receptors (MR) and other cell surface receptors perform vital roles in attachment of *M.tuberculosis* to phagocytes bearing the receptors (Dheda *et al.,* 2010). The interface between mannose receptors on phagocytic cells and mycobacterial cell wall is facilitated via the mycobacterial surface glucoprotein, lipoarabinomannan (LAM) (Nigou *et al.,* 2001). Prostalglandin E2 (PGE2) and interleukin-4 (IL-4) up-regulate complement and mannose receptor expression and function in this binding process. But interferon gamma (IFN-γ) produced by lymphocytes decrease expression of these receptors thereby amounting to reduced adherence of *Mycobacteria* to macrophages (Dheda *et al.,* 2010).

Phagosome-lysome fusion:

Phagocytized bacilli are degraded by intra-lysosomal acidic hydrolases upon phagolysosomal fusion. This constitutes a significant antimicrobial mechanism of phagocytes. However mycobacterial sulphatides which are derivatives of multiacylated tetralose 2-sulphate can inhibit phagolysomal fusion (Goren, 1977). *M.tuberculosis* also blocks phagosomes maturation after entry into the host macrophage thereby promoting a milieu that supports bacillary replication (Lee *et al*, 2009). In vitro studies also showed that *M.tuberculosis* produces copius amount of ammonia in culture that could account for some of the inhibitory effect on macrophages (Gordon *et al*., 1980).

Macrophage action after *M.tuberculosis* engulfment:

Macrophages generate antimicrobial reactive oxygen and nitrogen species (ROS and RNS) via activity of the enzymes NADPH oxidase and inducible nitric oxide synthase (iNOS) (Ehrt and Schnappinger, 2009). The anti-mycobacterial effector action of macrophages is in the generation of ROS and RNS, as well as other means involving cellular enzymes and cytokines.

Reactive Oxygen Intermediates (ROI):

Macrophages utilise the NADPH oxidase NOX2 to produce ROS from O_2 for killing pathogens. Upon activation, the enzymes complex subunits of the NADPH oxidase transfers cytosolic NADPH electrons to O_2 within the phagosome, thereby yielding superoxide (O_2-), which is converted to hydrogen peroxide (H_2O_2) through dismutation within the phagosome. H_2O_2 can further react with O_2- to generate other ROS with the ability to kill the intra-

phagosomal pathogen (Flannagan *et al.,* 2009; Robinson, 2009). This hydrogen peroxide (H_2O_2), produced by macrophages through oxidative burst is the first in the array of effector molecules that mediates the mycobactericidal effect of mononuclear phagocytes (Rohde *et al*., 2007; Dheda *et al.,* 2010). *M.tuberculosis* infection promotes proliferation of macrophages within the lungs with resultant H_2O_2 production (Dheda *et al*., 2010). Superoxide anion produced in the phagocytes are meant to sterilize the bacteria, but *M.tuberculosis* evades killing by these anions through its increased expression of bacterial catalase and superoxide dismutase (SOD) that directly scavenge the oxygen radicals (Casbon *et al.,* 2012).

Reactive Nitrogen Intermediates (RNI):

Upon activation by T-cell derived cytokines IFN-γ and TNF-α, phagocytes produce nitric oxide (NO) and its associated RNIs through the agency of inducible Nitric Oxide Synthase (iNOS2), which uses L-arginine as substrate. This iNOS, also termed NOS2 in phagocytes, synthesized following microbial stimulation of macrophages, catalyses the production of nitrogen radicals on the cytoplasmic portion of the phagosome. There are 2 sub units of iNOS, which synegistically generate NO and citrulline from L-arginine and O_2. Further reaction of NO with oxygen radicals leads to conversion to other toxic reactive nitrogen intermediates (RNI) within the phagosomes (Flannagan *et al.,* 2009).

The import of toxic nitrogenous oxides in host defense against *M.tuberculosis* are well recognized (Benoit *et al*., 2008; Pawlowski *et al.*, 2012). *M.tuberculosis* replicates more rapidly in genetically modified iNOS gene knockout mice than wild type, thus implies the importance of NO in host defense (MacMicking *et al.,* 1997). The function of RNI in human models of infection has been widely discussed and it differs from mice infection models. 1, 25-dihydroxy vitamin D3 (1, 25-$(OH)_2D_3$) was reported to prompt expression of NOS2 and *M.tuberculosis* anti-inhibitory function in human HL-60 macrophages–like cell line (Liu and Modilin, 2008). This distinguishes NO and other RNIs as potential anti-mycobacterial effectors. This is supported by report of mycobactericidal activity parallel to the expression of NOS2 in IFN-γ stimulated human macrophages cultured with lymphocytes (Bikle, 2008).

Macrophages Apoptosis:

Macrophage defense also involves apoptosis or programmed cell death. Apoptosis was observed in *M.tuberculosis* infection to be prompted in a dose dependent manner in BAL

cells from TB patients, especially HIV co-infected patients (Placido *et al.,* 1997). There is clear prominence of apoptosis in the epitheloid cells within the granuloma, which is confirmed by condensed chromatin as viewed by a light microscope (Keane *et al.,* 1997). The macrophage apoptosis results to induced viability of *M.tuberculosis* with formation of vesicles containing *M.tuberculosis* antigens. Dendritic cells engulf the vesicles and present them as antigens through MHC-II, MCH-I and CD1 in an extremely efficient process (Schaible *et al.,* 2003; Winau *et al.,* 2006). It is postulated that this process called cross-priming plays a key part in T-cell stimulation.

T-cells

Containment of *M. tuberculosis* infection within its host imperatively requires cell mediated immune response; hence the stimulation of $CD4^+$ and $CD8^+$ T-cells observed in active TB in human and experimental mice infection (Cooper, 2009). $CD4^+$ T cells of type Th1 are the most critical (North and Jung, 2004). In experimental mice infection, adaptive immune response was mounted two weeks after infection with *M.tuberculosis*, which is followed by reduction in bacterial replication (Ducati *et al.*, 2006; Dheda *et al.*, 2010). *M.tuberculosis* antigen is presented by infected DCs and macrophages to T-cell via MHC 1 to CD8+ cells, via MHC II to CD4+ cells, which lead to stimulation and proliferation of lymphocytes. In addition, CD restricted T-cells can be activated upon presentation of glycopid antigens by DC while γδ T-cells require presentation of phospholigands. Consequent to *M.tuberculosis* infection, memory T-cells are also formed. These cells collectively contribute to protective immunity against TB through the production of IFN-γ or existing cytotoxic activity (Raja 2004; Dheda *et al*., 2010).

Cytokines (IL-7, IL-12, IL-15, IL-23, and TNF-α) are produced by infected macrophages and DCs resulting to recruitment of more leucocytes to the infection site (Dheda *et al.,* 2010). This creates a cytokine environment that determines if the CD4+T cell can employ a T_h1 or T_h2 response. A T_h1 response implies release of pro-inflammatory cytokines principally IFN-γ which promotes intra-macrophage *M.tuberculosis* killing through NO and ROS action (Russell, 2007; Rohde *et al.,* 2007; Dheda *et al.,* 2010). However, a T_h2 response implies release of anti-inflammatory cytokines including IL-4, IL-5, IL-10 and IL-13 that promote B lymphocyte stimulation with antibody production. Regulatory T-cell (T-reg) have also been identified in *M.tuberculosis* infection. These T-reg cells also produce anti-inflammatory cytokines particularly IL-10 and hence suppress microbial mechanism within macrophages in

TB patients (Dheda et al., 2010). Specific activation of cytotoxic T cells ($CD8^+$) was reported to induce *M. tuberculosis* killing via perforin and granulysis-mediated pathways wherein infected macrophages undergo cell death. Cell death occurs through induction of apoptosis via the extrinsic pathway involving fas ligands (Weerdenburg *et al.,* 2010; Dheda *et al.,* 2010). Defective killing capacity of cytotoxic T cells has been reported in TB patients (Brighenti & Andersson, 2012).

The granuloma

Granuloma formation is the hallmark of *M.tuberculosis* infection. The granuloma is an inflammatory mononuclear cell infiltrate that restrains *M.tuberculosis* proliferation but it could also provide a survival niche for the bacilli. The TB granuloma is extremely dynamic and is influenced by immune response components as well as pathogenic virulence (Ehlers and Schaible, 2012). During active TB disease necrotic (caseous) granuloma may be formed. A necrotic granuloma has an outer lymphocyte niche made up of T and B cells with macrophage-rich mid-section surrounding an amorphous caseous necrosis center (Mattila *et al.*, 2013).

Macrophages in granulomas serve both as anti-mycobacterial effectors as well as growth niche for *M.tuberculosis*. Immunohistological study of granulomatous lesions from *M.tuberculosis* infected cynomolgus macaques, with both phenotypical and functional markers indicates that macrophages with anti-inflammatory properties are confined to outer sections of granuloma, whereas the inner sections comprise macrophages with pro-inflammatory, probably bactericidal, properties. Also, TB lesions exhibit a gradient of anti- and pro-inflammatory characteristics, where anti-inflammatory $CD163^+$ $iNOS^+$ $Arg1^{high}$ macrophages are localized in outer borders while pro-inflammatory $CD11c^+$ $CD68^+$ $CD163^{dim}$ $iNOS^+$ $eNOS^+$ $Arg1^{low}$ macrophages are localized within the mid region. This increases the chance of mounting antibacterial activities away from surrounding host tissue. This supports the postulations of ordered microenvironments within granulomas that stabilize antimicrobial and anti-inflammatory immune activities to control damage to surrounding lung tissue (Mattila *et al.*, 2013) and in consonance with previous study that demonstrated spatial organisation of inflammatory signaling in human tuberculosis granuloma (Marakalala *et al.*, 2016). In this study, laser-capture microdissection, mass spectrometry and confocal microscopy were applied to design detailed molecular maps of human granulomas. It was observed that granuloma centers exhibit pro-inflammatory properties depicted by

antimicrobial peptides, ROS and pro-inflammatory eicosanoids while the surrounding to the caseum had a relatively anti-inflammatory feature (Marakalala *et al.*, 2016).

2.4 TB DISEASE REACTIVATION

Our current understanding of the relationship between host immune responses and the transition of *M.tuberculosis* from a latent state to active TB has remained largely obscure. The underlying mechanisms by which identified risk factors promote this transition are just partly understood. Appropriate comprehension of these mechanisms would be of utmost significance, as this would facilitate: identification as well as targeting of these mechanisms in patients with latent TB who could advance to active disease, thereby preventing avoidable potentially noxious and expensive courses of chemoprophylaxis given to those who may not need it due to reduced risk of advancement; monitoring and successful projection of time when *M.tuberculosis* could shift from latency into active disease in these individuals; development of immunological intervention strategies to enhance immune control of *M.tuberculosis*.

For many years, entry into and persistence of latent infection has been ascribed largely to the immune system, however, recent data indicates that *M.tuberculosis* also contributes actively to this phenomenon. Hypoxia, carbon monoxide and nitric oxide within infection loci are prevalent during *M.tuberculosis* latency, this leads the bacilli to activate the regulon controlled by DosR-DosS signal transduction system, which promotes a switch to the use of alternative energy sources (Sherman *et al.*, 2001; Park *et al.*, 2003). This switch in gene expression during dormancy and shutting off certain other transcriptional activities that are hitherto active in the replicative phase entails that certain mycobacterial epitopes are expressed while others are shut off during latency (Black *et al.*, 2009; Govender *et al.*, 2010). Two gene clusters have been associated with *M.tuberculosis* shift from dormancy, whose regulation is proposed to contribute to determination of infection outcome. They encode five proteins whose actions are similar to the "resuscitation-promoting factor (Rpf)" of *M.luteus* to promote recovery after a nutrient-starved latent phase (Chao, 2010). Deletion of these Rpf-like genes from *M.tuberculosis* has been shown to impair recovery from latency (Tufariello *et al.*, 2006; Russell-Goldman *et al.*, 2008) in a mice model. Additionally, transcription of an 88 toxin-antitoxin gene pair system is pivotal to the sustenance of latency or progression to outright replication and virulence (Ramage *et al.*, 2009).

Notwithstanding the bacterial influences involved in exit from latency, two well-documented host mechanisms tend to heighten chances of disease reactivation. These mechanisms are; qualitative and quantitative reduction of $CD4^+$ T cells, and impairment of TNF-α signaling. Hence, immune deficiencies resulting in loss of $CD4^+$ T cell are associated with raised *M.tuberculosis* reactivation risk (Kwan and Ernst, 2011). Nonetheless, TB reactivation probability with HIV is not only linked with reduced $CD4^+$ T-cell counts, given most HIV infected persons advance to active TB before their $CD4^+$ T-cell counts decline to less than 350-200/µl. This is supported by discriminatory targeting of TB-specific $CD4^+$ T-cells, instability of $CD4^+$ T cells with distorting of polyfunctionality or skewing of cytokine production patterns demonstrated in HIV co-infection (Day *et al.*, 2008; Geldmacher *et al.*, 2010; Marin *et al.*, 2012; Perreau *et al.*, 2013). Previous studies show that TNF-α neutralization considerably increases the chance of TB reactivation (Harris and Kaene, 2010). Other reported mechanisms have also been insinuated to promote transition from latency, including programmed death receptors and ligands (PD-1/PD-L1). Raised blood expression of PD-L1 has been demonstrated in active PTB (McNab *et al.*, 2011). Furthermore, PD-1 expression has been related with varying functional profiles of $CD8^+$ antigen specific CTLs in PTB (Rozot *et al.*, 2013), hence indicating different functional predominance in these differing states in addition to crosstalk between innate and adaptive immune response.

2.5 CYTOKINES AND TB

Cytokines and chemokines released by immune cells perform key roles in host protection against *M.tuberculosis* (Russell, 2007; Cooper and Khader, 2008). Previous reports have demonstrated that differential induction of these substances could be associated with immunopathology, inflammation, granuloma formation, disease latency and reactivation, which are determinants of disease outcome (Wang *et al.*, 2010). The relevance of cytokines and chemokines in the control of TB hinges mainly on the necessity for cross-communication across cells for effectual migration and precise direction in the execution of host defense. However, cytokines release in response to *M.tuberculosis* infection may create a milieu that allows propagation of bacilli since *M.tuberculosis* has adapted mechanisms to escape or influence the immune responses to favor its survival and transmission.

Interleukin-6 (IL-6)

Interleukin-6 is a pleiotropic cytokine, with both pro- and anti- inflammatory characteristics.

It is secreted in reaction to inflammatory stimuli with involvement in cell differentiation, proliferation and apoptosis (van Snick, 1990). IL-6 was originally discovered as a B-cell differentiating factor but further investigation showed that it lacked interferon (IFN) activity (Teranishi *et al.*, 1982; Sehgal *et al.*, 1986). Human IL-6 has a helix bundle structure comprising 4 long α-helices and its gene contains 5 exons and 4 introns and maps to locus 7p2. Although stimulation of different cells including T cells, B cells, granulocytes, and smooth muscle cells has been shown to induce IL-6 secretion, the main sources of IL-6 are monocytes/macrophages, fibroblasts and endothelial cells (Akdis *et al.*, 2011). Synthesis and secretion of IL-6 is stimulated during inflammation by various stimuli including other cytokines- IL-1, TNF-α and IFN-γ (Shalaby *et al.*, 1989; Sanceau *et al*., 1989). IL-6 signals through soluble and membrane bound receptors (IL-6R), and downstream by a phosphorylation cascade involving Janus kinase (JAK), mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription (STAT) pathways (Heinrich *et al.*, 1998; 2003).

IL-6 is crucial for resistance against *M.tuberculosis* and is vital for producing protective T_h1 immune reaction (Leal *et al.*, 1999). In vivo studies also supports a protecting role for IL-6 in stimulation of early protective responses facilitated via IFN-γ in *M.tuberculosis* infection (Ladel *et al.*, 1997; Saunders *et al.*, 2000). Cavitary TB patients were reported to express reduced IL-6 levels in their bronchial alveolar lavage (BAL) fluid compared to non-cavitary disease TB patients, specifying IL-6 as a probable marker of controlled (non-cavitary) TB (Nolan *et al*., 2013). Additionally, mechanistic data from animal studies and humans suggest an association between IL-6 and effective early expression of lung protection through a blend of coordinated mononuclear inflammation and prompt accumulation of lymphocytes (Domingo-Gonzalez *et al*., 2016). Though its effects are uncertain, they may be dire subsequent to high dose exposure or in immunodeficiency (Domingo-Gonzalez *et al*., 2017).

Interleukin 8 (IL-8)

IL-8, or CXCL8 from most recent nomenclature, exemplifies the classical chemokine of the CXC sub-family (Remick, 2005). Its active form has 72 amino acids though IL-8 gene transcription encodes a 99 amino acid polypeptide, which is cleaved proteolytically to the biologically active 72 amino acids protein (Waugh and Wilson, 2008). Though a broad range of cell types synthesizes IL-8, its principal cellular sources are characteristically monocytes and macrophages. IL-8 is primarily responsible for recruitment of monocytes and neutrophils.

Cell recruitment takes place via development of a chemotactic gradient that induces movement of inflammatory cells to a zone of elevated chemokine concentration (Gimbrone *et al.*, 1989). This gradient serves both to attract cells to site of inflammation as well as retain them at the site (Gimbrone *et al.*, 1989). IL-8 promotes activation of monocytes and neutrophils in addition to recruitment (Remick, 2005; Waugh and Wilson, 2008).

Physiological functions of IL-8 are facilitated by IL-8 binding to 2 cell surface receptors, CXCR1 and CXCR2, which are G-protein-coupled receptors (Holmes *et al.*, 1991; Murphy and Tiffany, 1991). IL-8 associated signals are transmitted across the membrane via ligand-induced structural transformations that expose intracellular loop epitopes and the carboxy-terminal tail. These epitopes foster coupling to functional hetrotrimeric G proteins (Schraufstatter *et al.*, 2001; Moser and Loetscher, 2002).

In *M.tuberculosis* infection, IL-8 is synthesized by multiple cell types- alveolar epithelial cells, macrophages, monocytes and fibroblast, and plays a principal part in leukocytes recruitment (Friedland *et al.*, 1992; Zhang *et al.*, 1995; Lin *et al.*, 1998; O'Kane *et al.*, 2007). Previous reports demonstrated that IL-8 attracts neutrophils and T cells to infection locus either directly or indirectly, and it has also been implicated in monocyte recruitment (Gerszten *et al.*, 1999). In vivo studies reported that IL-8 is crucial for typical host immune response to *M.tuberculosis* while anti-IL-8 impedes granuloma development (Larsen *et al.*, 1995). Bronchoalveolar lavage (BAL) fluid of TB patients has been shown to contain IL-8 in concentrations that correlate with leukocyte numbers (Kurashima *et al.*, 1997; Sadek *et al.*, 1998). Increased IL-8 mRNA has also been shown in *M.tuberculosis*-infected tissue (Bergeron *et al.*, 1997).

2.6 NBT AND TB

Phagocytosis causes an increase in metabolic activity resulting in increased oxygen utilization and activation of the pentose phosphate cycle, a phenomenon referred to as respiratory burst (Slauch, 2011). This results in the release of highly reactive oxygen radicals with bacteriostatic and bacteriocidal potential (Slauch, 2011). Nitroblue tetrazolium (NBT) test is an indirect marker of the oxygen-dependent bactericidal killing in phagocytes (Hellum, 1977; Nakagawara *et al.*, 1989). Nitroblue-tetrazolium (NBT) is a yellow water-soluble dye that is converted to a dark-blue water insoluble diformazan upon reduction (Berridge *et al.*, 2005). The NBT test measures the proportion of phagocytic cells that have ingested and

produced oxygen radicals that reduce NBT dye. This implies that the dye serves as a visible marker of the phagocytic process. Also, the NBT test may be stimulated; in which case, blood is incubated with endotoxin before adding NBT dye. Here, the test examines the capacity of phagocytes to respond to standard stimulus, making it a measure of circulating concentration of functional phagocytes (Segal, 1974).

Elevated NBT scores were previously demonstrated in miliary tuberculosis and in tuberculosis meninigitis (Park, 1971). Also, NBT was previously proposed as a useful diagnostic aid in tuberculosis (Mandell and Fuller, 1972). Ryden and Silverman (1974) went on to demonstrate positive NBT results in TB patients, which may progress to negative as patients recover depicting clinical utility in monitoring disease prognosis with treatment. They further stated that NBT results in TB patients were not affected by the presence of diabetes mellitus, prior steroid therapy, alcoholism or syphilis. However these are in contrast to normal scores reported in different forms of active mycobacterial infections (Park *et al.*, 1968; Matula and Peterson, 1971; Gordon *et al.*, 1973).

2.7 RESPIRATORY BURST IN TB PATIENTS

The generation of reactive oxygen and nitrogen species during chemotactic locomotion and phagocytosis by activated neutrophils and macrophages involved in host defense, is a vital part of their physiological function. These reactive species perform a crucial part in killing of intracellular bacteria including *M.tuberculosis* and are mostly produced via the respiratory burst pathway. There have been previous reports of negative influence of disease severity on ROI production via the respiratory burst pathway in TB patients. Depressed ROI production in untreated patients, further exacerbation in advanced patients and near absence in HIV coinfected patients are documented (Fiorenza *et al.*, 2003). In murine macrophages, reactive nitrogen species were implicated as the principal process by which *M.tuberculosis* destruction is achieved (Chan *et al.*, 1992). Although they further suggested an unlikely contribution of ROI to *M.tuberculosis* killing owing to no effect of superoxide dismutase and catalase on anti-mycobacterial activity of macrophages, Lau et al. (1998) reported recurrent intractable tuberculosis in chronic granulomatous disease (CGD) patients due to defective oxygen radical generation by neutrophils.

2.8 ROLE OF MICRONUTRIENTS IN PHAGOCYTOSIS

Phagocytosis is a component of several cellular processes spanning a large range of physiological and biological functions. Several studies have demonstrated that vitamins and some trace metals alter macrophage phagocytosis and its constituent steps significantly. Both human and animal studies have particularly implicated vitamins A, C, D and E, as well as the trace metal Zn in altered macrophage phagocytosis (Erickson *et al.*, 2000). Enhanced substrate adherence, migration, chemotaxis, ingestion, ROI production were demonstrated following in vitro treatment of peritoneal macrophages with vitamins C, D and E (Del-Rio *et al.*, 1998). Rat Kupffer cells and peripheral blood monocytes treated with vitamin A also showed improved phagocytic activity and ROI production (Hoglen *et al.*, 1997; Xu *et al.*, 1993). Diminished phagocytosis was also reported in human monocytes due to zinc deficiency (Kruse-Jarres, 1989)

2.9 TB DIAGNOSIS

Rapid and accurate diagnosis of tuberculosis is a key global health measure to control TB disease. However, the diagnostic tool applied for TB is determined by the reason for testing; detection of LTBI, active TB disease or drug resistant TB. The diagnostic tools discussed below are based on the Nigerian context.

LTBI

Although there are 2 tests available for the identification of LTBI; tuberculin skin test (TST) and Interferon gamma release assay (IGRA), TST is the most commonly used test for LTBI in Nigeria. The TST is performed using the Mantoux technique. It involves intradermal injection of 5 tuberculin units of purified protein derivative (PPD)-S or 2 tuberculin units of PPD-RT23. Individuals with antigen related cell-mediated immunity, present a delayed-type hypersensitivity reaction in 48–72 hours. TST interpretation considers the size of induration, the pre-test probability of *M.tuberculosis* infection and the active TB disease risk.

Though TST has various merits, mostly in low-income situations, such as reduced equipment and reagent costs as well as limited laboratory and skill requirement, it also has drawbacks. Firstly, its specificity is hampered by late (i.e. post-infancy) or repeated BCG vaccination. Also, exposure to non-tuberculous *Mycobacteria* (Farhat *et al.*, 2006). Secondly, it has limited predictive value (Pai *et al.*, 2014) since majority of individuals with positive TST do not necessarily advance to active TB disease.

Active TB disease

Tuberculosis diagnosis is based on "possible exposure, clinical history, indicative clinical findings, classical radiological features and positive microbiological tests" (WHO, 2010). Medical history and physical examination is combined with four diagnostic techniques: imaging techniques (chest X-rays and PET-CT), microbiological methods (sputum smear and culture) and molecular tests.

Medical history and physical examination

The WHO recommends TB investigations in persons presenting persistent cough of 2 weeks or more. Nonetheless, additional generalized symptoms include weight loss, appetite loss, low-grade fever, night sweats, chills and easy fatigability. Expectorations such as those generating mucoid sputum and further hemoptysis (blood) can be considered as a clinical predictor in even in smear-negative TB patients (Kanaya *et al.*, 2001).

Imaging techniques

The WHO recommends imaging investigations particularly chest radiography to identify chest and lung lesion when there is any 'suggestive appearance of TB'. Since chest X-ray non-specific for TB, it is requested with sputum smear microscopy in many national TB programmes. Due to the challenge of non-specificity, chest X-ray is regarded as an added diagnostic tool. However, chest X-ray becomes important for confirmation when smear microscopy is negative or infant cases (TCTA, 2006). It is also mostly suitable in populations with high occurrence of HIV. Infiltrates are more diffuse and situated in lower lung regions in HIV co-infection (Tshibwabwa-Tumba *et al.*, 2012). Early stage diagnosis of pleural effusion and pericardial effusion, when clinical symptoms are minimal is based on Chest X-ray outcomes. It is also important in diagnosis of miliary TB where there are appearances of distinctive nodulary infiltrations disseminated in pulmonary fields (Burrill *et al.*, 2007).

Microbiological methods

sputum smear examination and microscopy

Ziehl – Nelson (ZN) smear microscopy

Sputum smear microscopy is in common use for TB diagnosis and was used by Robert Kosch 125 years ago. It has been the major means of diagnosing pulmonary tuberculosis in low- and middle-income settings (TB CARE I, 2014), which account most TB cases. It is a easy, fast and low-cost method that is largely specific in regions with high TB prevalence (Reider *et al.,*

2007). It also detects most infectious patients and generally relevant in diverse populaces with distinctive socio-economic distribution (Reider *et al.,* 2007; WHO, 2012).

The World Health Organization (WHO, 2012) and the International Union against Tuberculosis and Lung Disease (The Union) (Reider *et al.,* 2007) guidelines stipulates that the crucial step in the examination of patients queried for PTB is microscopic examination of their sputum samples. The International Standard for Tuberculosis Care emphatically outlines that every patient (children and adults capable of producing sputum) queried for PTB must have at least two (2), to three (3), sputum samples for microscopic investigation (WHO, 2013; TB CARE I, 2014). Nevertheless, sputum smear microscopy has important limits to its performance. Its sensitivity is highly undermined at bacterial load below10,000 organism/ml sputum sample. There is also poor applicability in extra-pulmonary tuberculosis, pediatric tuberculosis and HIV coinfection (Luelmo, 2004). Furthermore, owing to the necessity of serial sputum examinations, persons who are unable to return for repeated sputum examination become "diagnostic defaulters" (Harries *et al.*, 1998).

The test previously required the submission of 3 samples collected over two days from a patient suspected for PTB. However, the progressive improvement of quality assurance programmes for smear microscopy has led to attempts to reduce "diagnostic defaulting" due to multiple samples collection over two days. The viability of diagnosing PTB by collection of two sputum samples on a single day (1-day protocol), compared to the policy of collecting samples on consecutive days (2-day protocol) has been weighed. It was posited that due to no statistical difference in diagnostic performance of 2-day protocol from 1-day protocol, the 1-day protocol should be adopted to reduce default (Shingadia and Novelli, 2003). The WHO has recently redefined a new sputum smear-positive PTB as the presence of single AFB one or more sputum smear examination from two sputum samples obtained from a TB suspect (WHO, 2010).

Front loaded smear microscopy:

In 2007, WHO recommended that countries with well functioning facilities with good quality assurance could reduce the number of samples collected from patients from three to two bacillary load sputum samples which would be sufficient to diagnose a case of TB. The front-loaded smear microscopy techniques takes the second sputum sample one hour after the first, this is eliminating drop out from the second visit by the patient. The WHO through

preliminary studies provided evidences to validate the techniques in a study of 923 symptomatic patients with chronic cough. The result of TB detection using this techniques compared with the conventional method was similar. Significant advantage of same day result for persons with TB symptoms is preventing drop-out from diagnosis schedule (Millen *et al.,* 2008).

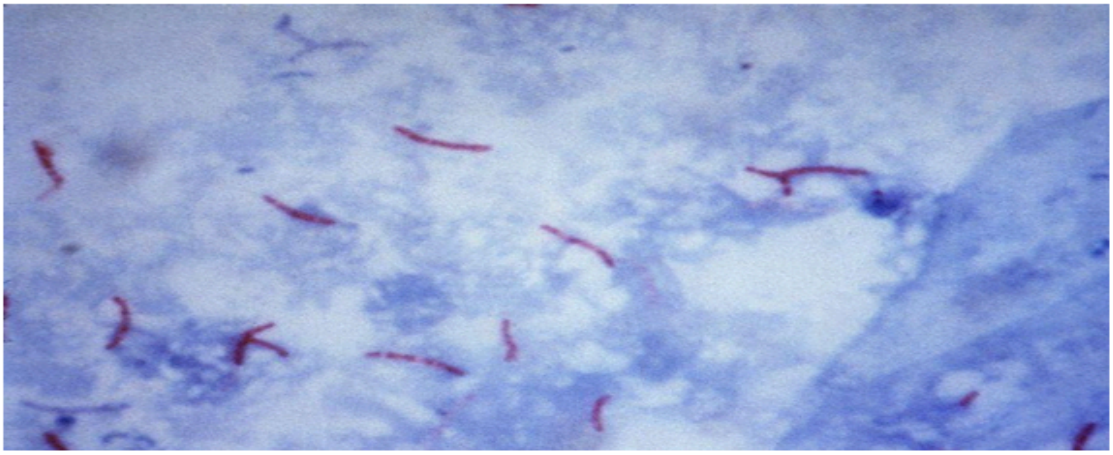


Figure 2.12: *Mycobacterium tuberculosis* (red bacilli) visualized using the Ziehl-Neelsen stain for acid fast bacilli in a sputum sample
Source: CDC Public Health Library

Sputum Culture

The microbiological culture entails multiplication of microbial organisms on particular growth culture media in precisely regulated laboratory conditions in a bid to identify infectious organism(s). The isolation and culture of tubercle bacillus is the 'gold standard' for conclusive diagnosis of tuberculosis with 100% specificity and no missing of any PTB case (Tiwari *et al.*, 2007). However, it requires four to eight weeks because of slow growth of *M.tuberculosis*. Culture is also useful in differentiating *M.tuberculosis* from other mycobacteria. Other limiting factors for isolation are paucibacillary samples mainly in childhood lesions and the requirement of greater than 100 bacilli per mL of sample compared with 5,000-10,000 bacilli per mL sputum smear sample (Aderaye *et al.*, 2007).

MGIT 960 mycobacterium detection system (Becton Dickinson) and BACTEC 460 radiometric TB systems (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD, USA) are the commonly used *M.tuberculosis* culture methods (Rodriguez *et al.*, 2007; Tiwari *et al.*, 2007).

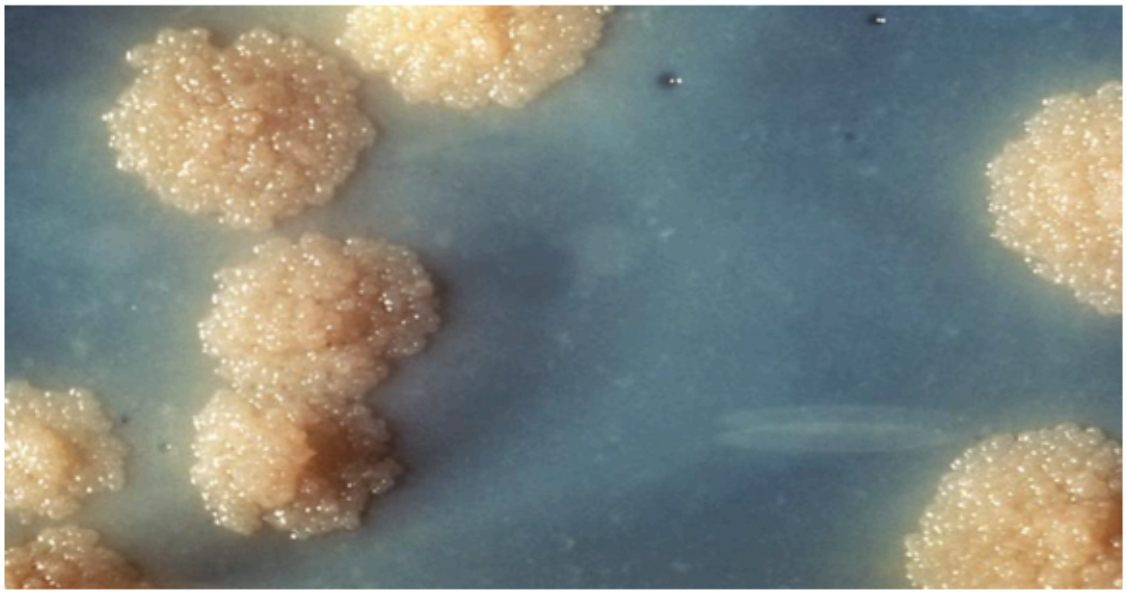


Figure 2.13: *Mycobacterium tuberculosis* culture showing colourless rough colonial morphology

Source: CDC Public Health Library

TB Drug resistance

There are phenotypical, culture-based and molecular-based techniques for TB drug resistance diagnosis. The employment of Xpert MTB/RIF for active TB diagnosis has significantly improved detection of drug-resistant TB within diverse settings (Raizada *et al.*, 2014; Sachdeva *et al.*, 2015). The GeneXpert system is an automated closed platform which carries out real-time polymerase chain reaction (Rt-PCR), giving result within 2 hours. GeneXpert detects *M.tuberculosis* complex and rifampicin (RIF) resistance in the bacilli. One-use cartridges are utilized with test automation following a manual sample liquefaction step. It is easy to use and specialist training is not necessary, just basic computer operation technique. It has higher sensitivity compared to smear microscopy and is close to culture (Helb *et al.*, 2010; Armand *et al.*, 2011; Boehme *et al.*, 2011; Theron *et al.*, 2011). Furthermore, the assay is useful in diagnosis of extrapulmonary tuberculosis utilising specimens from various extrapulmonary part, with sensitivity of 53%–95% (Lawn and Nicol, 2011). It also differentiates non-tuberculous mycobacteria or normal respiratory tract flora (Blakemore *et al.*, 2010). In HIV patients before antiretroviral therapy, case detection increased from 28% using smear microscopy to 73% (Lawn *et al.*, 2011). Nonetheless, there are reports of false-

positive cases of RIF resistance (Lawn *et al.*, 2011; Marlowe *et al.*, 2011; Tang *et al.*, 2017) in settings with low prevalence of RIF resistance.

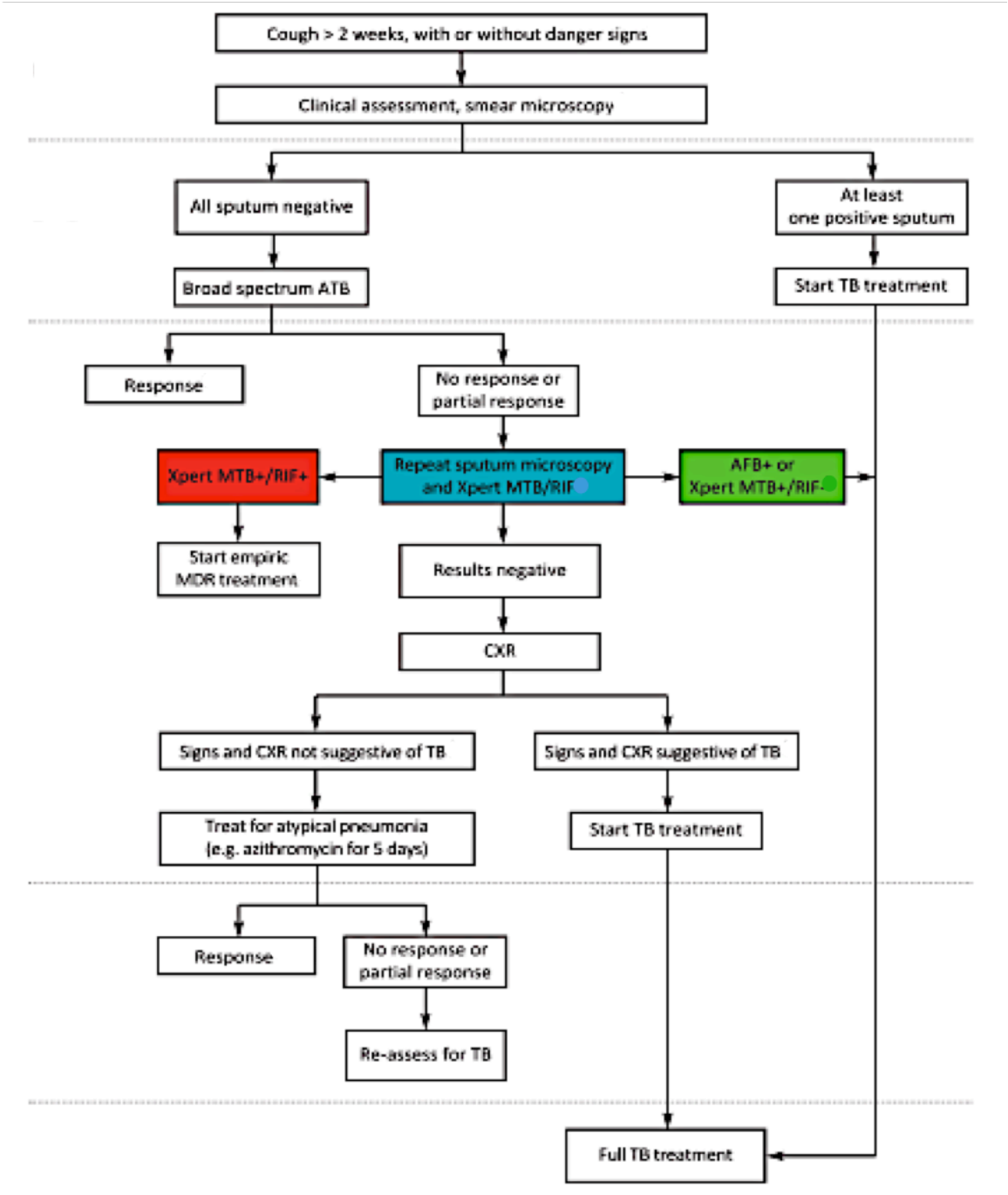


Figure 2.14: Flow chart showing the standard protocol for TB diagnosis

Source: CDC Public Health Library

2.10 TB MANAGEMENT

BCG vaccination and chemoprophylaxis are unsatisfactory TB control measures thereby29 leaving anti-tubercular (anti-TB) chemotherapy as the only option. The essential (first-line) anti-TB drugs are isoniazid (H), rifampicin (R), pyrazinamide (Z), ethambutol (E), and streptomycin (S) (WHO, 2010). Their use in combinations (multi-drug therapy) is aimed at achieving cure without relapse, preventing death, impeding transmission by depleting infection source pool, and averting the occurrence and spread of drug resistance (Du-Toit *et al.*, 2006; WHO, 2010). The fixed-dose combinations (FDCs) have equivalent efficacy to separate tablet combinations (Bartacek *et al.*, 2009) and are therefore preferred because of several advantages such as increased patient acceptance, discouragement of selective ingestion of the drugs and possible monotherapy, and prescription error (WHO, 2010). Other strategies introduced to improve TB treatment are the "patient kit" which ensures that the full treatment for a patient is available for the desired treatment duration; the "standard regimen" which introduced a standard (same) treatment for each patient registration group (category) (WHO, 2010).

The standard therapies of TB are usually split into 2 phases, an initial intensive phase followed by a continuation phase. For new patients, the regimen (2HRZE/4HR) involves of an intensive phase with daily doses of rifampicin, isoniazid, pyrazinamide, and ethambutol for 2 months, and 4 months continuation phase of rifampicin and isoniazid preferably daily or 3 times per week (WHO, 2010). Three times weekly regimen through out the course of the treatment is an acceptable alternative in non HIV-prevalent areas provided that treatment is directly observed (WHO, 2015). This therefore implies that Nigeria with an HIV/AIDS prevalence rate of 3.1% as at 2007 (KFF, 2010), is not eligible to use this alternative regimen. Furthermore, in settings with proven or unknown isoniazid resistance level, HRE instead of HR should be used at the continuation phase.

The DOTS in Nigeria uses 2HRZE/4HR or 2HRZE/6HE regimens for category 1 (new cases) – 6HE is self administered while 4HR should be observed daily (FMOH, 2010). For retreatment cases (category 2), the 2SRHZE/1RHZE/5RHE is used (Adamu *et al.*, 2008). The current WHO guideline recommends that all re-treatment cases undergo specimen culture and drug sensitivity testing (DST) for at least isoniazid and rifampicin before treatment if the Rapid molecular-based DST is available, otherwise standard empirical treatment (category 2) should be commenced and modified with the result of the conventional culture when

available (WHO, 2010). This recommendation is in response to the report of the Global Project on Anti-tuberculosis Drug Resistance Surveillance which showed a high global mean multi-drug resistance (MDR) of 15.3% (95% CI: 9.6-21.1) for previously treated cases when compared to 2.9% (95% CI: 2.2-3.6) among new cases. For the African region, the report showed a men MDR of 1.5% (95% CI: 1.0-2.0) for new cases, and 5.8% (95% CI: 3.9-7.7) for previously treated cases (WHO, 2008).

2.11 NUTRITION AND TUBERCULOSIS

Consumption is a terminology that became synonymous with tuberculosis from ancient history. This association originates from the severe weight loss vis-à-vis nutritional deficiency observed in TB patients. Nonetheless, the association between tuberculosis and malnutrition is rather complicated since malnutrition is a known TB risk factor that predisposes infected individuals to advance to clinical disease and clinical disease can also aggravate malnutrition (Macallan, 1999). Prior to the introduction of anti-tuberculosis chemotherapy, diet rich in proteins, calories, fats, vitamins and minerals was normally regarded as a vital measure in tuberculosis treatment (Macallan, 1999). However, development of anti-tuberculosis drugs, has transformed disease management that the role of nutrition is given minimal attention in regard to treatment advances (Gupta *et al.*, 2009). Notwithstanding the intricate link between nutrition and tuberculosis, accumulating evidences still favor the suggestion that malnutrition may contribute to the elevated TB mortality and morbidity in populations exposed to food unavailability (Gupta *et al.*, 2009). Active TB disease could trigger or aggravate pre-existing under-nutrition through decreased appetite, malabsorption, or increased catabolism (Schaible and Kaufmann, 2007). Heightened predominance of under-nutrition in TB patients has been previously reported and is related to deaths (Zachariah *et al.*, 2002; Van-Lettow *et al.*, 2004), and increased risk of relapse (Khan, 2006).

Breakdown of multiple demographic and nutrition surveys projected 10-20% African adults 20-49 years of age are malnourished, with protein-calorie malnutrition being reported as the common form of malnutrition (Nube and van den Boom, 2003; Koethe *et al.*, 2009). Reduced expression of interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), as well as mycobactericidal substances that selectively hamper cell-mediated immune response which is important for containing and restricting *M.tuberculosis* has been reported in protein-calorie malnutrition (Hood, 2013).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study design and study population

This study is a longitudinal case control study. Convenience sampling was utilized for recruitment of consenting participants that satify the inclusion criteria. PTB cases were recruited before commencement of treatment and followed-up during 6-months of anti-TB treatment.

One hundred and sixty (160) participants were enrolled for this study. They included 50 multi-drug resistant TB (MDR-TB) patients, 60 drug-sensitive TB (DS-TB) patients and 50 non-TB apparently healthy individuals who served as controls. MDR-TB patients comprised of 32 (64.0%) males and 18 (36.0%) females, DS-TB patients comprised of 34 (56.7%) males and 26 (43.3%) females while controls comprised of 27 (54.0%) males and 23 (46.0%) females. Study participants were aged between 20 – 60 years and all females were non-pregnant, non-lactating women.

MDR-TB was diagnosed as isoniazid and rifampicin resistant strains of *M.tuberculosis* using GENE Xpert as well as Chest X-ray and clinical history. Study participants with MDR-TB were enrolled from the DOTS at the MDR-TB Centre, University College Hospital (UCH) Ibadan, Nigeria. Diagnosis of DS-TB was based on sputum smear microscopy, chest X-ray and clinical history. The participants with DS-TB were enrolled from the DOTS at the Medicine Out-Patient Clinic, University College Hospital, Ibadan, Nigeria. A Consultant Chest Physician confirmed all diagnosis of MDR-TB and DS-TB.

3.1.1 Inclusion criteria for cases

- Diagnosis of symptomatic (active) pulmonary tuberculosis
- Adults aged 18 years and above
- Ability to give consent

3.1.2 Inclusion criteria for control

- Absence of active PTB
- Adults aged 18 years and above
- Ability to give consent

3.1.3 Exclusion criteria for cases and controls

- History of HIV/AIDS
- Severe illness or any recent history of chest disease
- Inability to give consent

3.2 Diagnosis of Tuberculosis

A chest physician obtained clinical history of patients. Patients presenting with the typical TB symptoms; protracted cough (≥2 weeks) with sputum production, with or without haemoptysis, were further examined for other less obvious symptoms; persistent fatigue, weight loss, shortness of breath, chest pain, anorexia, moderate fever and nights sweats. These patients were given sterile sputum tubes to produce sputum for microbiology analysis and chest X-ray of patients requested.

Sputum Collection

Two sputum samples were collected; a morning sample (collected prior to morning meal or any dosing) and a spot sample. Study participants rinsed their mouth with water to reduce salivary contamination of sputum. Sputum produced was collected into 20ml sterile disposable polypropylene tubes. Study participants were asked to deliver sample same day as collected.

Microbiology Analysis

Sputum Digestion-Decontamination

Sputum digestion and decontamination was carried out using NaOH/N-acetyl-L-cysteine(NALC)-Na citrate solution.

Procedure

A daily preparation of NaOH/NALC-Na citrate working solution was made as follows;
100ml 6% NaOH was mixed with 100 mL 2.9% Na citrate after which 1g NALC powder was dissolved in the mixture to make 0.5% NALC. Same volume of NaOH/NALC-Na citrate solution was added to sputum sample (1:1 volume) and the suspension was vortex until liquefied (15-30 sec). The mixture was incubated for 15 minutes. Phosphate buffer (pH 6.8) was added to the mixture making up the volume to 50 mL. (Addition of phosphate buffer was done before 20 minutes after addition of digestion-decontamination solution, since mycobacteria can be killed off if exposed to NaOH longer than 20 minutes). This was mixed

by inversion and centrifuged at 3000 xg for 15 minutes at 4^{o}C. Supernatant was decanted carefully without unsettling sediments at bottom of the tube and phosphate buffer (pH 6.8) was added to the pellets to make final volume of 2.5 mL. Sediment was resuspended by gentle aspiration using a pipette and suspended pellet was utilized for smear preparation for Acid fast bacilli (AFB) microscopy and culture.

AFB

Procedure

100µl of well-mixed resuspended pellet from the digested-decontaminated sputum sample was transferred onto glass slide and spread over an area approximately 1 x 2 cm. Smear was air-dried for 30 minutes and slide was heat-fixed by passing it over blue flame of Bunsen burner 3 times. Slide was flooded with carbol fuchsin on a staining rack and heated to steaming with the flame from a Bunsen burner. Stain was washed off with 3% acid-alcohol and allowed to stand for 2 minutes. Acid-alcohol was rinsed off slide with distilled water and tilted to drain. Slide was flooded with methylene blue and allowed to stand for 2 minutes. Methylene blue was washed off with distilled water, tilted to drain and slide was air-dried. Smear was examined with 100X oil objective, examining at least 10 fields. AFB was identified as bright red short rods to long filaments against the background material counterstained blue.

Culture

Procedure

Digested-decontaminated sputum deposit was inoculated on two slopes of egg-based medium. Tubes were incubated for 1 week at 36°C in a slanted position, with screw-caps loose. Screw-caps were tightened and tubes were set upright, after 1 week of incubation. Tubes were examined weekly for colony formation. Cultures were kept for 8 weeks.

GENE Expert

The GeneXpert (GX)-IV machine was used for detection of *M.tuberculosis* in sputum and determination of Rif resistance. The test is a Real-time PCR (Amplification and detection) with automated data analysis and result interpretation.

Procedure

Two (2) volumes of sample reagent was poured into 1 volume of sputum sample in sputum container. The lid of sputum container was replaced and the mixture was shaken briskly with back and forth movements for 15 times. Mixture was incubated at room temperature for 10 minutes and shaken briskly with back and forth movements for 15 times. Mixture was incubated for an additional 5 minutes at room temperature to attain perfect fluidity with no visible clumps of sputum. 2ml of mixture was transferred to GeneXpert cartridge using a plastic transfer pipette. Cartridge was closed firmly and inserted into the GeneXpert (GX)-IV machine. Test was started and results obtained recorded.

3.3 Ethical consideration

Ethical approval was obtained from the University College Hospital/University of Ibadan Joint Ethics Committee (UI/EC/13/0340)

3.4 Data collection

A short structured questionnaire (Appendix I) was used to obtain sociodemographic, some clinical and nutritional information.

3.5 Sample collection, processing and storage

Blood and sputum samples were obtained from TB patients before commencement of chemotherapy, at 2 months, 4 months and 6 months of anti-TB chemotherapy whereas blood sample was obtained from the controls once.

Blood sample collection

Ten milliliters (10 mL) of blood was drawn from the antecubital vein of study participants and dispensed into lithium heparin containing sample tubes. Blood sample was centrifuged at 5000 rpm for 5 minutes to obtain plasma, which was stored in aliquot at -20^{o}C until analysed.

Cell sediments left after obtaining plasma was mixed with equal volume of 6% dextran solution and the mixture was incubated at 37^{o}C for 60 minutes in an upright position. Leucocyte-rich supernatant was decanted into plain tube and centrifuged at 5000 rpm for 5 minutes. Supernatant was decanted and cell sediment obtained was washed by suspending in 4ml of Ringers solution followed by centrifugation at 5000 rpm for 5 minutes. This was repeated twice to make three washes. Cells were resuspended in Ringers solution, counted

and adjusted to 0.5 x 10^6 cells/mL. Cell suspension was used for leucocyte migration inhibitory factor (LMIF) test.

3.6 Anthropometric indices

Height

The height of each participant was measured in meters with subjects standing bare footed on a stadiometer.

Body weight and Percentage body fat (PBF)

Body weight and PBF were measured with a bioelectrical impedance analysis (BIA) scale (Intelli Scale BS0114, China) which was placed on a flat surface. Study participants wore light clothing and were without shoes before they stood on the scale. Body weight was measured in kg while PBF was measured in percentage.

Mid upper arm circumference (MUAC)

MUAC was measured using a non-stretchable measuring tape. Tape was extended around the mid upper arm of participant while the arm was straight and relaxed.

Waist circumference (WC)

WC was measured using a non-stretchable measuring tape placed at the horizontal plane midway between the lowest rib and iliac crest.

Hip circumference (HC)

HC was measured using a non-stretchable measuring tape extended around the widest circumference of the hip of study participants.

Indices and Ratios

The following indices and ratios were calculated using their respective formulae;

Body mass index (BMI) = $\frac{\text{Weight (kg)}}{\text{Height}^2\ (\text{m}^2)}$

Fat Mass Index (FMI) = $\frac{\text{Fat mass (kg)}}{\text{Height}^2\ (\text{m}^2)}$

Fat mass = PBF (%) x Body weight (kg)

Fat Free Mass Index (FFMI) = $\frac{\text{Fat free mass (kg)}}{\text{Height}^2\ (\text{m}^2)}$

Fat Free Mass = Body weight – Fat mass

Waist circumference to Hip circumference ratio (WHR) = $\frac{\text{Waist circumference (cm)}}{\text{Hip circumference (cm)}}$

3.7 Biochemical Analysis

Nitroblue tetrazolium dye reduction test (NBT)

Modified semi-quantitative NBT procedure as described by Feigin *et al.* (1971) was used.

Principle

The addition of NBT to blood results in the precipitation of an NBT/heparin and/or fibrinogen complex which enters neutrophils and monocytes by phagocytosis. This yellow soluble dye complex is then converted to a dark blue insoluble diformazan (formazan) upon reduction by the oxidants superoxide and hydrogen peroxide of the respiratory burst pathway within the phagocyte.

Procedure

NBT solution (50 µL) was transferred to a vial and 25 µL of well-mixed heparinized blood was added after which 25 µL of stimulant solution (bacterial endotoxin) was also added and mixed gently (mixing was gentle but adequate). Mixture obtained was incubated for 10 minutes at 37^{o}C followed by a further 10 minutes at room temperature. The vial content was mixed again and a moderately thick smear was prepared from the mixture. Smear was air-dried and treated with Wright stain by flooding with 1ml of stain for 30 seconds. Distilled water (1ml) was added to the flooded smear and the mixture was allowed to stand for 30 seconds. Smear was rinsed with water and allowed to air-dry. Stained smear was viewed with

a microscope using oil immersion objective and 200 neutrophils were counted. Neutrophils showing formazan deposits were recorded as positive

%NBT was calculated as;

$$\frac{\text{Number of neutrophils showing formazan deposit}}{\text{Total number of neutrophils counted}} \times 100$$

Leukocyte Migration Inhibitory Factor (LMIF) test

LMIF assay was carried out as described by Hudson and Hay (1976).

Principle

This test assesses the ability of lymphocytes to secrete leucocyte migration inhibitory factor (LMIF) in response to antigenic stimulation. Lymphocytes stimulated *in vitro* release factors called lymphokines, which inhibits the migration of granulocytes within culture medium. Migration inhibition due to antigenic stimulation is then measured as an index of migration without antigenic stimulation.

Procedure

Heparinized blood (10 mL) was centrifuged at 1500rpm for 15minutes to obtain plasma and cell sediments. Cell sediments obtained was mixed with equal volume of 6% dextran solution and the mixture was incubated at 37^{o}C for 60 minutes in an upright position. Leucocyte-rich supernatant plasma was transferred to a plain tube and centrifuged at 300 x g for 5 minutes. Sedimented cells were washed 3 times in Ringer solution. After the third wash, sedimented cells were resuspended in Ringer solution, counted in haemocytometer and adjusted to 0.5 x 10^6 cells/mL. Capillary tubes (haematocrit) were filled two-third full with the cell suspension and sealed with plasticine at one end. Cells were packed by centrifugation at 850 x G for 10 minutes and capillary tubes were cut at the cell-medium interface. A small drop of silicone grease was put in one corner of each well of improvised migration chamber and cut capillary was carefully placed in the chamber by anchoring the sealed end in silicone grease. Wells were immediately topped up with medium (Ringers solution) or antigen (BCG) in medium solutions in duplicates. A drop of streptomycin was added to the well and chambers were covered with sterile culture plate sealing tapes to prevent contamination and avoid air bubbles. Migration chambers were incubated at 37^{o}C for 24 hours.

Image of migration field was projected on the screen of an immunodiffusion plate reader (Osram 64425; Behring Institut) and traced on a piece of graphic paper
Counting the number of small squares enclosed the area of the traced portion was assessed.

The percentage leucocyte migration index was calculated as

$$\% \text{ migration index} = \frac{A}{M} \times 100$$

A = Area of migration in antigen in medium solution
M = Area of migration in medium-only solution

Total protein estimation

Total protein concentration was estimated using Biuret's method as described by Gornall *et al.* (1949).

Principle

In alkaline solution, polypeptyides form coloured complex with cupric ion in alkaline solution. Biuret reagent forms a complex with protein having maximum absorbance at 540nm. This procedure was calibrated using standard Bovine Serum Albumin (BSA) curve.

Procedure

Bovine serum albumin (BSA), 0.1, 0.2, 0.3, 0.4 and 0.5 mL were dispensed into five different test tubes for standard series. Serial dilution of BSA was done by adding 0.9, 0.8, 0.7, 0.6, 0.5 ml of distilled water to the test tubes as arranged to achieve concentrations from 1 g/L to 5 g/L. Distilled water (0.8 mL) was dispensed into sample test tubes and 0.2 mL of each sample was dispensed into the test tubes to yield 1:5 dilutions. Biuret reagent (3 mL) was added and mixture incubated for 30 minutes at room temperature. Absorbance was read at 540 nm and protein concentrations of the samples were deduced from standard curve. Concentrations were multiplied by the dilution factor to attain actual concentration in g/L

Hydrogen peroxide (H_2O_2) estimation

H_2O_2 was estimated using Wolff's method (1994).

Procedure

Serial dilution of H_2O_2 was made by adding 0.95 mL, 0.90 mL, 0.85 mL, 0.80 mL, 0.70 mL, 0.60 mL, 0.50 mL of distilled water to 0.05 mL, 0.10 mL, 0.15 mL, 0.20 mL, 0.30 mL, 0.40 mL, 0.50 mL of H_2O_2 to yield 10, 20, 30, 40, 60, 80, and 100 µmoles of H_2O_2 respectively. Phosphate buffer (2.5 mL), 250 µL of AFS, 100 µL of sorbitol, 100 µL of xylenol orange and 25 µL of H_2SO_4 were dispensed into all test tubes and 50 µL of standard/sample was dispensed into the tubes appropriately. The samples were mixed by vortexing and incubated for 30 minutes at room temperature. Absorbance at 560 nm was read, standard curve was plotted and concentrations of the samples were extrapolated from the standard curve.

Determination of catalase enzyme activity

Catalase activity was determined using the method of Sinha (1971).

Principle

Heating of dichromate in acetic acid in the presence of H_2O_2 results in the formation of perchromic acid as an unstable intermediate with eventual reduction to chromic acetate. Chromic acetate produced is measured at 570 nm using a spectrophotometer.

Procedure

Samples were diluted 50 fold by adding 0.1 mL of sample to 4.9 mL of ddH_2O. Serial dilution of H_2O_2 was made by adding 0.95 mL, 0.90 mL, 0.85 mL, 0.80 mL, 0.70 mL, 0.60 mL, 0.50 mL of ddH_2O to 0.05 mL, 0.10 mL, 0.15 mL, 0.20 mL, 0.30 mL, 0.40 mL, 0.50 mL of H_2O_2 to yield 10, 20, 30, 40, 60, 80, and 100 µmoles of H_2O_2 respectively. H_2O_2 (2 mL) and 2.5 mL of phosphate buffer (assay mixture) were dispensed into separate test tubes and 0.5 mL of the diluted sample/standard was dispensed into the designated tubes containing assay mixtuire. 2 mL of dichromate/acetic acid solution was dispensed into another set of test tubes and 1 mL of sample/standard and assay mixture was blown into it at 60 second interval. The mixture was heated for 10 minutes at 100^0C and cooled at room temperature. The absorbance of clear fluid was read at 570 nm. The standard curve was plotted and concentrations of the sample were extrapolated from the curve. Concentrations were

multiplied by the dilution factor and catalase activity was calculated as H_2O_2 consumed/mg protein

Determination of superoxide dismutase (SOD) activity

The SOD activity was determined using the method of Misra and Fridovich (1972).

Principle

The method is based on the principle that SOD inhibits the autoxidation of epinephrine at pH 10.2.

Procedure

Samples were diluted ten (10) fold by adding 0.1 mL of sample to 0.9 mL of ddH_2O. Carbonate buffer (2.5 mL) was dispensed into all the test tubes and 0.2 mL of sample was added to the appropriate tubes. Adrenaline (0.3 mL) was added to all the tubes and the solution was mixed by inversion. The absorbance was taken at 480 nm using a spectrophotometer and absorbance values were recorded every 30 seconds until 150 seconds (i.e 0, 30, 60, 120 and 150 seconds). The blank contained 2.5 ml of buffer, 0.3 ml of adrenaline and 0.2 ml of ddH_2O. Concentrations were multiplied by the dilution factor.

SOD activity was calculated as:

Change (Δ) in absorbance = A_{150}-A_0/2.5

% inhibition (x) = 100 – 100 (ΔAbs of sample/ΔAbs of blank)

SOD activity (% inhibition) = x/50

Determination of myeloperoxidase (MPO) activity

MPO activity was determined using the method of Bergmeyer (1974).

Principle

Decomposition of H_2O_2 by peroxidase, with guaiacol as hydrogen donor, produced tetraguaiacol which was measured at 436 nm and at 25^0C.

Procedure

Potassium phosphate buffer (2.80 mL), 0.05 mL of guaiacol and 0.05 mL of H_2O_2 were dispensed into test tubes for sample and blank. The mixture was equilibrated and the

absorbance was monitored at 436 nm until it became constant. Sample (0.1 mL) and 0.1 mL of distilled water were dispensed into test tubes for sample and blank respectively. The mixture was mixed by inversion and at exactly 1 minute, the absorbance was read at 436 nm.

$$\text{Volume activity (U/ml)} = \frac{(\text{Absorbance of test} - \text{Absorbance of blank}) \times 4 \times \text{total volume}}{\text{micromolar extinction co-efficient of tetraguaiacol (25.5)} \times \text{sample volume}}$$

Nitric oxide (NO) estimation

NO estimation was done using Griess reagent as described by Green *et al.* (1982).

Principle

The method is based on diazotization reaction.

Procedure

Nitrite solution (100 µM) was prepared by diluting 0.1M nitrite standard 1:1000 with distilled water. Six serial dilutions (50, 25, 12.5, 6.25, 3.13 and 1.56 µM) were done with distilled water. Sulfanilamide and NED solutions were equilibrated to room temperature for 15 to 30 minutes. Sample/standard (50 µL) was dispensed into the microtitre wells appropriately while a microtitre well containing distilled water served as blank. Sulfanilamide (50 µL) was dispensed into the microtitre wells using multi-channel pipette. The mixture was incubated at room temperature, protected from light, for 10 minutes. NED (50 µL) was dispensed into all the wells and incubated at room temperature, protected from light, for 10 minutes. Purple colour developed and absorbance was read at 520 nm wavelength within 30 minutes. A standard curve was plotted and the concentrations of samples extrapolated from the curve.

Estimation of Interleukin 6 and 8 concentrations

Plasma levels of IL-6 and IL-8 were determined using ELISA kits supplied by Invitrogen (USA). The ELISA test is based on the principle of antigen-antibody interaction. Human IL-6 and IL-8 specific antibodies were pre-coated on respective microtitre plates. Standards, quality controls and test samples are added to appropriate wells and biotinylated detection antibodies were added to the wells subsequently to form a sandwich of capture antibody-antigen-detection antibody. Avidin-Biotin-Peroxidase Complex (ABC) is added and unbound conjugates are washed off. Horseradish peroxidase (HRP) is used to visualize HRP enzymatic

reaction, which catalyzes the conversion of tetramethyl benzidine (TMB) from a colourless solution to a blue coloured product. The intensity of colour formed is proportional to the concentration of IL-6 or IL-8 in the well.

Procedure

Samples were diluted in 10 fold by adding 20 µL of sample to 180 µL of sample diluent. Diluted samples and standards (100 µL) were dispensed appropriately into the microtitre wells, plate was sealed and incubated at 37^0C for 90 minutes. The cover was removed, content discarded and blotted unto paper towels. Biotinylated anti-human IL-6/IL-8 antibody working solution (100 µL) was dispensed into each well and incubated at 37^0C for 60 minutes. Microtitre plate was washed manually 3 times with PBS and 100 µL of streptavidin-HRP working solution was dispensed into each well and incubated at 37^0C for 30 minutes. Microtitre plate was manually washed 5 times with PBS and 90 µL of TMB colour developing agent was dispensed into all the wells and incubated at 37^0C in dark for 20 minutes. Stop solution (100 µL) was added and the colour changed to yellow immediately. Absorbance was read at 450 nm with a microplate reader within 30 minutes after adding the stop solution. Standard curve was plotted and the concentrations of samples were extrapolated from the curve. Concentrations obtained were multiplied by the dilution factor.

Vitamin estimation

Plasma concentrations of vitamins A, C, D and E were determined using HPLC as described by Kandar and Zakova (2009). Appropriate internal standards were used. Vitamin D is particularly light sensitive, hence, necessary precaution was taken to minimize exposure of standard and plasma samples to daylight by covering the sample bottles with aluminum foil.

Procedure

Internal standard (10 µL) was added to 500 µL of sample/standards. The mixture was vigorously mixed for 10 min and 500 µL of cold ethanol was carefully added. The mixture was incubated at 4^0C for 10 minutes and then centrifuged at 22000 x g for 10 minutes at 4^0C. The supernatant was filtered through a 0.20 µm nylon filter (4 mm diameter) and suctioned into HPLC using 95% methanol in deionized water (v/v) as the mobile phase and the flow rate was kept constant at 0.5 mL/min. The amount of analyte was quantified from a peak area ratio of analyte/internal standard using Clarity chromatography software. The concentration of analyte in the samples was determined from the calibration curve.

Trace elements estimation

Plasma concentrations of iron (Fe), zinc (Zn) and copper (Cu) were determined with Beck 200 Flame Atomic Absorption Spectrophotometer (AAS) using direct methods as previously described (Kaneko, 1999). The atoms of the element vaporized and absorbed light of the same wavelength as that emitted by the element when in the excited state.

Procedure

Samples were diluted with 0.1N hydrochloric acid (1:20 v/v). Working standard solutions were prepared from stock standard and the required concentrations used to standardize respective trace elements. AAS flame was ignited and lamp turned on. De-ionized water was used to blank the equipment. Respective standards were aspirated to standardize the equipment. This process was repeated periodically to assess intra-assay drift. Diluted samples were aspirated into the equipment. Results were obtained from read-out

Concentration was calculated as:

Reading × dilution factor × standard concentration

3.8 Statistical analysis

Data were statistically analysed using the SPSS statistical software version 22.0. After assessing all the variables for normality, one way ANOVA was used to determine the differences between normally distributed variables while Kruskal Wallis test was used to determine the differences between non-normally distributed variables in TB patients (MDR-TB and DS-TB) before commencement of chemotherapy and controls. Inter-group comparisons was carried out using Duncan post hoc test for normally distributed variables while Mann Whitney U test was used for non-normally distributed variables. Differences between pre-treatment, 2 months, 4 months and 6 months of treatment were compared using repeated measures ANOVA and Friedman test for normal and non-normally distributed variables respectively. Pairwise comparison of 2 months, 4months and 6 months of treatment with pre-treatment was carried out using Duncan post hoc test for normally distributed repeated measures while Wilcoxon test was used for non-normally distributed repeated measures. Relationship between the variables was determined using Spearman rank correlation. Differences between MDR-TB patients who had sputum smear converted at 2 months were compared with non-converted using independent Student's t-test and Mann

Whitney U test for parametric and non-parametric variables respectively. *P*-values less than 0.05 were considered significant.

CHAPTER FOUR

RESULTS

4.1 BEFORE ANTI-TB TREATMENT

4.1.1 Nutritional indices

Anthropometry

The mean mid upper arm circumference (MUAC), body weight, body mass index (BMI), percent body fat (PBF), fat mass index (FMI), fat free mass index (FFMI) and hip circumference (HC) were significantly lower in MDR-TB and DS-TB patients before anti-TB treatment when compared with controls (Table 4.1). However, the mean MUAC, body weight, BMI, PBF, FMI and HC were significantly lower in MDR-TB patients when compared with DS-TB patients before anti-TB chemotherapy (Table 4.1).

Furthermore, there were higher proportions of under-weight patients in the MDR-TB and DS-TB groups whereas there was no under-weight among the controls (Table 4.1).

4.1.2 Modulators of innate cellular immunity

Micronutrients and Total protein

The mean plasma levels of Zn, total protein and vitamins C and E were significantly lower in DS-TB patients before chemotherapy when compared with controls (Table 4.2). Also, the mean plasma levels of Fe, Zn and vitamin A were significantly lower while the mean plasma levels of total protein and vitamins C and E were significantly higher in MDR-TB patients before chemotherapy compared with controls (Table 4.2). However, the mean plasma levels of Fe, Zn and vitamin A were significantly lower while the mean plasma levels of total protein and vitamins C and E were significantly higher in MDR-TB patients compared with DS-TB patients before anti-TB chemotherapy (Table 4.2).

4.1.3 Mechanisms of innate cellular immunity

Leucocyte migration indices

The median plasma level of IL-8 was significantly higher in DS-TB patients before anti-TB chemotherapy compared with controls (Figure 4.1). However, the median plasma levels of IL-6 and IL-8 and the mean %LM were significantly higher in MDR-TB patients compared with DS-TB patients before anti-TB chemotherapy as well as controls (Figure 4.1, Figure 4.2 and Figure 4.3).

Leucocyte intracellular killing

The median plasma SOD activity was significantly lower while the median plasma CAT activity was significantly higher in DS-TB patients before anti-TB chemotherapy compared with controls (Table 4.3). Also, plasma H_2O_2 and NO levels in DS-TB patients before anti-TB chemotherapy were significantly lower compared with controls (Table 4.3).

The median plasma CAT and MPO activities were significantly lower in MDR-TB patients compared with controls (Table 4.3). Furthermore, plasma NO level in MDR-TB patients before anti-TB chemotherapy was significantly lower compared with controls (Table 4.3)

Plasma CAT and MPO activities were significantly lower in MDR-TB compared with DS-TB patients before anti-TB chemotherapy (Table 4.3). Also, MDR-TB patients had significantly higher plasma H_2O_2 level compared with DS-TB patients before anti-TB chemotherapy (Table 4.3). Plasma NO level in MDR-TB patients was significantly lower compared with DS-TB patients before anti-TB chemotherapy (Table 4.3).

4.2 DURING 6 MONTHS OF TREATMENT

4.2.1 Mechanisms of innate cellular immunity

Leucocyte migration indices in DS-TB patients

There was a significant increase in mean %LM at 4 months of anti-TB chemotherapy compared with baseline (Figure 4.6).

Leucocyte intracellular killing in DS-TB patients

There was a significant decrease in %NBT at 2 months of anti-TB chemotherapy compared with baseline (Table 4.4). In contrast, there were significant increases in plasma MPO activity and NO level while plasma CAT activity decreased significantly at 2 months and 4 months of anti-TB chemotherapy compared with baseline (Table 4.4). Furthermore, plasma CAT activity, H_2O_2 and NO levels increased significantly at 6 months of anti-TB chemotherapy compared with baseline (Table 4.4).

Leucocyte migration indices in MDR-TB patients

Plasma level of IL-6 decreased significantly at 2 months of anti-TB chemotherapy while plasma level of IL-8 increased significantly at 4 months of anti-TB chemotherapy when compared with baseline (Figure 4.7, Figure 4.8). At 6 months of anti-TB chemotherapy, plasma level of IL-6 and %LM decreased significantly compared with baseline (Figures 4.7 and 4.9).

Leucocyte intracellular killing in MDR-TB patients

There were statistically significant increases in plasma CAT and MPO activities, plasma NO level at 2 months of anti-TB chemotherapy compared with baseline (Table 4.5). There were significant increases in plasma CAT and MPO activities at 4 months of anti-TB chemotherapy compared with baseline (Table 4.5). Plasma levels of H_2O_2 and NO increased significantly at 4 months of anti-TB chemotherapy compared with baseline (Table 4.5). At 6 months of anti-TB chemotherapy, %NBT, plasma H_2O_2 level, plasma CAT and MPO activities increased significantly while plasma SOD activity decreased significantly compared with baseline (Table 4.5).

4.3 DURING SUPPLEMENTATION OF DS-TB PATIENTS

Leucocyte intracellular killing

There were significant increases in plasma SOD and MPO activities while plasma CAT activity decreased significantly at 2 months of anti-TB chemotherapy and zinc supplementation compared with baseline (Table 4.6).

At 4 months of anti-TB chemotherapy and zinc supplementation, there was a significant decrease in plasma CAT activity whereas plasma MPO activity was significantly increased compared with baseline (Table 4.6).

At 6 months of anti-TB chemotherapy with zinc supplementation, plasma SOD activity, H_2O_2 and NO levels decreased significantly while plasma MPO activity increased significantly compared with baseline (Table 4.6).

4.4 CORRELATIONS

Correlation between mechanisms of innate cellular immunity in pulmonary tuberculosis patients and healthy controls

There were significant negative correlations between %NBT and plasma H_2O_2 levels in MDR-TB and DS-TB patients (Table 4.7). %NBT correlated negatively with plasma SOD activity in DS-TB patients (Table 4.7). Plasma CAT activity correlated positively with %LM in MDR-TB patients (Table 4.7). Plasma SOD activity and H_2O_2 level correlated negatively with %LM while plasma NO level correlated positively with %LM in DS-TB patients (Table 4.7).

There were significant negative correlations between plasma IL-6 and plasma CAT activities while plasma NO correlated positively with plasma IL-6 in both MDR-TB and DS-TB

patients (Table 4.7). Plasma IL-6 correlated positively with plasma H_2O_2 while plasma SOD activity correlated negatively with plasma IL-6 in DS-TB patients (Table 4.7). However, plasma IL-6 had negative correlation plasma CAT activity in MDR-TB patients (Table 4.7). Similarly, plasma IL-8 correlated negatively with plasma H_2O_2 level in DS-TB patients (Table 4.7).

Correlation of mechanisms with modulators of innate cellular immunity in MDR-TB patients

There were significant positive correlations between plasma Fe level and %NBT, plasma SOD activity and H_2O_2 level while plasma Fe level had negative correlation with %LM, plasma IL-8 and IL-6 (Table 4.8). Plasma Cu level had positive correlation with %NBT, plasma CAT activity and H_2O_2 level while %LM, plasma IL-8 and IL-6 had negative correlation with plasma Cu level (Table 4.8). Plasma Zn level had negative correlation with %NBT, %LM, plasma IL-8 and IL-6 while plasma CAT activity had positive correlation with plasma Zn level (Table 4.8).

Plasma vitamin A level had negative correlation with %LM while plasma SOD activity had positive correlation with plasma vitamin A level (Table 4.8). Plasma vitamin C level had significant negative correlations with plasma SOD activity while %LM and plasma IL-6 had positive correlation with plasma vitamin C level (Table 4.8). Plasma vitamin D level had positive correlation with %LM (Table 4.8). There was a significant negative correlation between plasma vitamin E level and plasma H_2O_2 level (Table 4.8). Furthermore, plasma TP had negative correlation with %NBT, plasma MPO activity, plasma H_2O_2 and NO levels while it had positive correlation with plasma SOD activity (Table 4.8).

Correlation of mechanisms with modulators of innate cellular immunity in DS-TB patients

There were significant positive correlations between plasma Fe level and %NBT, plasma SOD activity and H_2O_2 level while plasma Fe level correlated negatively with %LM and plasma IL-6 level (Table 4.9). Plasma Cu level had positive correlation with %NBT, plasma SOD and MPO activities while it had negative correlation with %LM, plasma IL-8 and IL-6 (Table 4.9). There were significant negative correlations between plasma Zn level and %NBT, %LM, plasma IL-8, IL-6 and NO levels whereas plasma SOD activity and H_2O_2 level had positive correlation with plasma Zn level (Table 4.9).

Plasma vitamin A level had negative correlation with %NBT and %LM while plasma H_2O_2 had positive correlation with plasma vitamin A level (Table 4.9). There were significant positive correlations between plasma vitamin C level and %NBT, %LM, plasma IL-8, IL-6 and NO levels while plasma SOD activity and H_2O_2 level had negative correlation with plasma vitamin C level (Table 4.9). Plasma IL-8 and NO levels had positive correlation with vitamin D level (Table 4.9). There were significant negative correlations between plasma vitamin E level and %NBT, plasma H_2O_2 level whereas it had positive correlation with %LM, plasma IL-8 and IL-6 (Table 4.9). Plasma TP had negative correlation with plasma IL-6 level, CAT activity, H_2O_2 level and NO levels (Table 4.9).

4.5 ANTI-TB TREATMENT OUTCOME MEASURES

Treatment outcome in MDR-TB patients

Of the 50 MDR-TB patients, 49 patients (98%) completed the six months intensive phase of directly observed therapy (DOT) while 1 patient (2%) absconded form the treatment facility. Four (4) deaths (8.16%) was recorded among the forty nine patients that completed the intensive phase of anti-TB chemotherapy (Figure 4.12).

Among the 45 MDR-TB patients that completed intensive phase chemotherapy, sputum culture conversion was observed in 30 patients (66.67%) at 2 months of anti-TB chemotherapy, 11 patients (24.44%) at 3 months of anti-TB chemotherapy and 4 patients (8.89%) at 4 months of anti-TB chemotherapy (Figure 4.10).

Treatment outcome in DS-TB patients

Although there was no treatment default recorded among DS-TB patients during 6 months of ant-TB chemotherapy, a single case of mortality (1.67%) was recorded (Figure 4.13). Among the 59 DS-TB patients that completed the 6 months of anti-TB chemotherapy, sputum smear conversion was observed in all these patients (100%) at 2 months of anti-TB chemotherapy (Figure 4.12).

Modulators and mechanisms of innate cellular immunity in MDR-TB patients whose sputum culture converted compared with non-converted at 2 months of anti-TB treatment

The mean body weight, BMI, PBF, FMI, FFMI and plasma vitamin D level were significantly higher in patients whose sputum culture converted compared with patients that

did not convert at 2 months of anti-TB chemotherapy (Table 4.11). In contrast, the mean plasma vitamin E level was significantly lower in MDR-TB patients whose sputum culture converted compared with patients that did not convert at 2 months of anti-TB chemotherapy (Table 4.11). There were no significant differences in mechanisms of innate cellular immunity in MDR-TB patients whose sputum culture converted compared with the patients that did not convert at 2 months of anti-TB chemotherapy (Table 4.12)

Table 4.1: Gender, age and anthropometric indices of pulmonary tuberculosis patients at diagnosis compared with controls

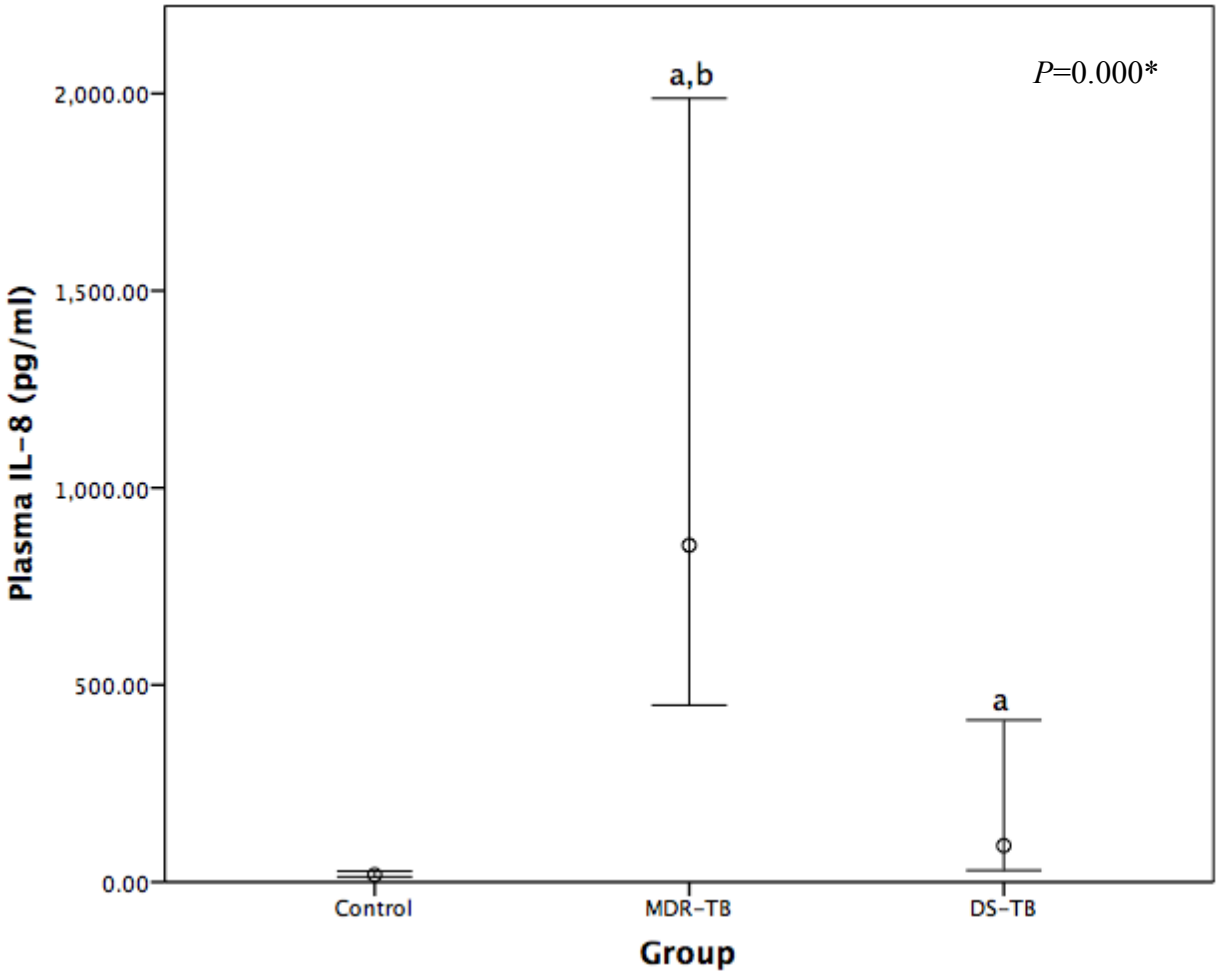
Variables	MDR-TB (n=50)	DS-TB (n=60)	Controls (n=50)	F-value	*P*-value
Male	32 (64.0)	34 (56.7)	27 (54.0)	[c]0.943	0.638
Female	18 (36.0)	26 (43.3)	23 (46.0)		
Age *(years)*	34.80±11.87	36.66±12.37	22.06±8.54	1.429	0.243
UAC *(cm)*	21.29±3.00[a,b]	22.91±3.50[a]	27.13±2.66	45.777	0.000*
Weight *(kg)*	47.91±8.96[a,b]	53.70±11.29[a]	68.41±10.90	47.291	0.000*
BMI *(kg/m^2)*	17.12±3.01[a,b]	19.17±3.60[a]	24.14±3.77	49.738	0.000*
Under-weight	36 (72.0)	26 (43.3)	0 (0.0)	[d]79.571	0.000*
Normal weight	14 (28.0)	32 (53.3)	33 (66.0)		
Over-weight	0 (0.0)	2 (3.3)	17 (34.0)		
PBF *(%)*	13.37±6.76[a,b]	19.40±8.34[a]	24.69±7.06	25.427	0.000*
FMI *(kg/m^2)*	2.44±1.65[a,b]	3.91±2.53[a]	6.10±2.41	29.899	0.000*
FFMI *(kg/m^2)*	14.68±1.90[a]	15.26±2.02[a]	18.05±2.40	34.525	0.000*
WC *(cm)*	71.60±8.05[a]	74.66±8.77	77.57±10.54	4.784	0.010*
HC *(cm)*	83.14±6.66[a,b]	87.77±7.78[a]	94.74±13.48	15.967	0.000*

*Significant at $p<0.05$
[a]Significantly different from Control
[b]Significantly different from DS-TB
[c]Chi-square test
[d]Fisher's Exact test

Table 4.2: Plasma levels of micronutrients and total protein in patients with pulmonary tuberculosis at diagnosis compared with controls.

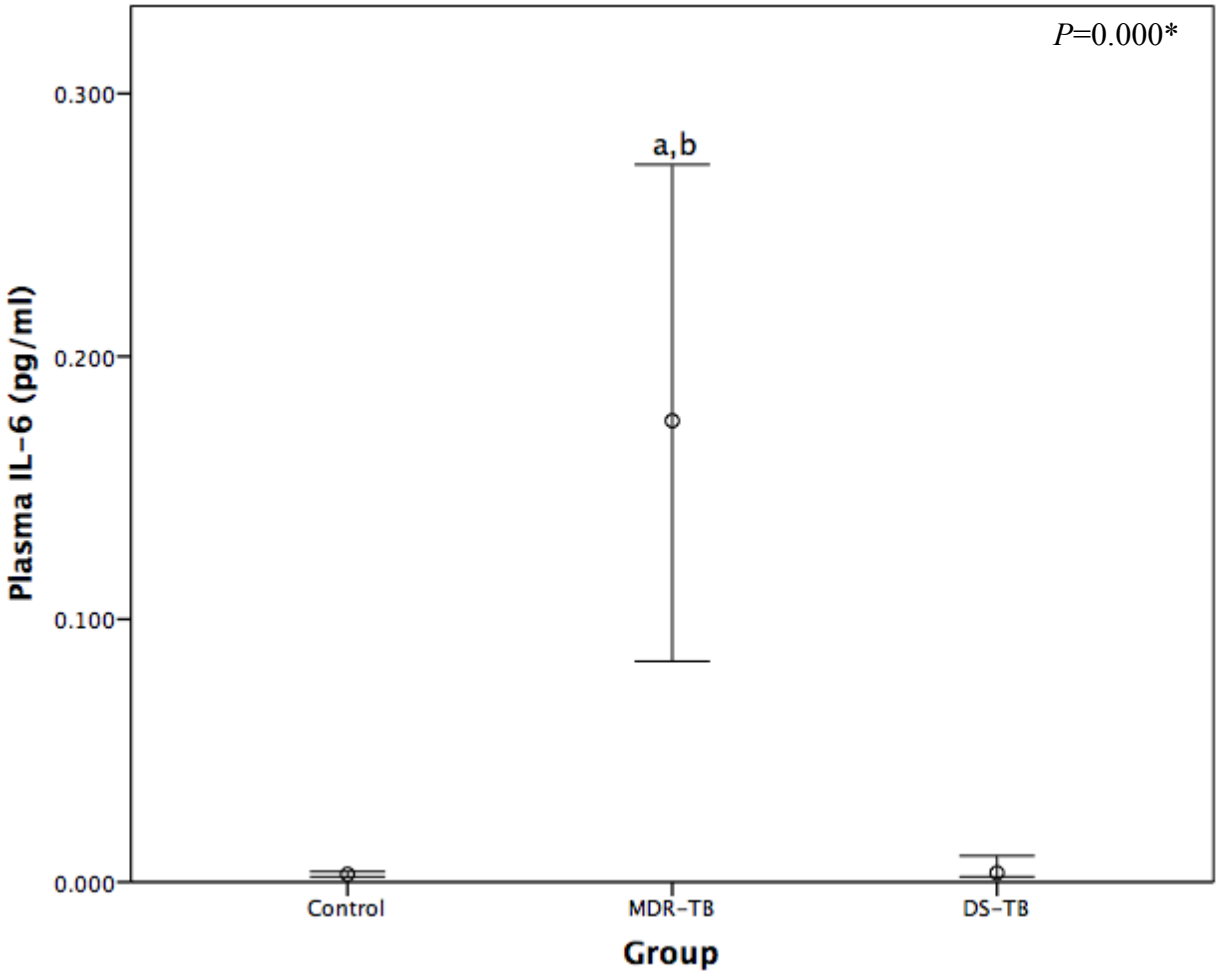
Variables	MDR-TB (n=50)	DS-TB (n=60)	Controls (n=50)	F-value	*P*-value
Fe *(µg/dl)*	92.17±10.47[a,b]	109.24±18.33	123.26±5.94	7.491	0.002*
Zn *(µg/dl)*	62.57±4.63[a,b]	81.25±23.56[a]	118.27±9.85	7.231	0.002*
Cu *(µg/dl)*	96.34±9.59	103.23±23.34	107.18±7.38	0.931	0.402
Vit A *(µg/dl)*	49.81±11.43[a,b]	71.59±12.36	80.78±20.28	13.083	0.000*
Vit C *(mg/dl)*	4.80±1.04[a,b]	0.72±0.13[a]	3.55±2.25	42.903	0.000*
Vit D *(pg/ml)*	56.51±12.29	56.70±10.28	58.72±17.05	0.105	0.900
Vit E *(mg/dl)*	2.16±0.59[b]	1.05±0.19[a]	1.74±0.90	14.668	0.000*
Total protein *(mg/dl)*	73.11±10.70[b]	56.08±5.74[a]	67.50±22.63	16.807	0.000*

*Significant at $p<0.05$
[a]Significantly different from Control
[b]Significantly different from DS-TB



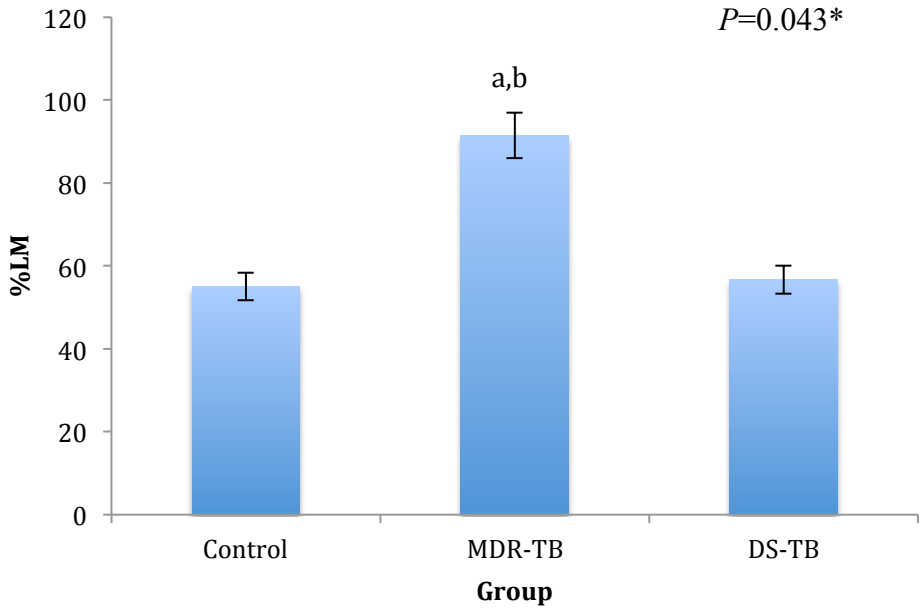
[a]Significantly different from Control
[b]Significantly different from DS-TB

Figure 4.1: Plasma IL-8 level in TB patients and controls



[a]Significantly different from Control
[b]Significantly different from DS-TB

Figure 4.2: Plasma IL-6 levels in TB patients and controls



[a]Significantly different from Control
[b]Significantly different from DS-TB

Figure 4.3: Percent Leucocyte Migration in TB patients and controls

Table 4.3: Mediators of leucocyte intracellular killing in pulmonary tuberculosis patients at diagnosis and controls

Variables	MDR-TB (n=50)	DS-TB (n=60)	Controls (n=50)	*P*-value
%NBT	88.62±1.38	86.19±10.44	83.33±7.58	0.218
SOD *(U/ml)*	0.17(0.11-0.24)	0.15(0.11-0.19)[a]	0.19(0.14-0.26)	0.010*
CAT *(U/mg protein)*	0.02(0.02-0.03)[a,b]	0.04(0.03-0.06)[a]	0.03(0.02-0.05)	0.000*
MPO *(U/ml)*	7.57(6.45-8.07)[a,b]	9.33(7.70-10.95)[a]	8.27(7.23-9.59)	0.000*
H_2O_2 *(µmol/l)*	310.1(290.7-327.1)[b]	251.9(214.2-325.7)[a]	311.0(228.5-336.0)	0.001*
NO *(µmol/l)*	6.85(5.52-11.45)[a,b]	10.02(6.25-15.78)[a]	12.75(9.47-16.08)	0.000*

*Significant at $p<0.05$
[a]Significantly different from Control
[b]Significantly different from DS-TB

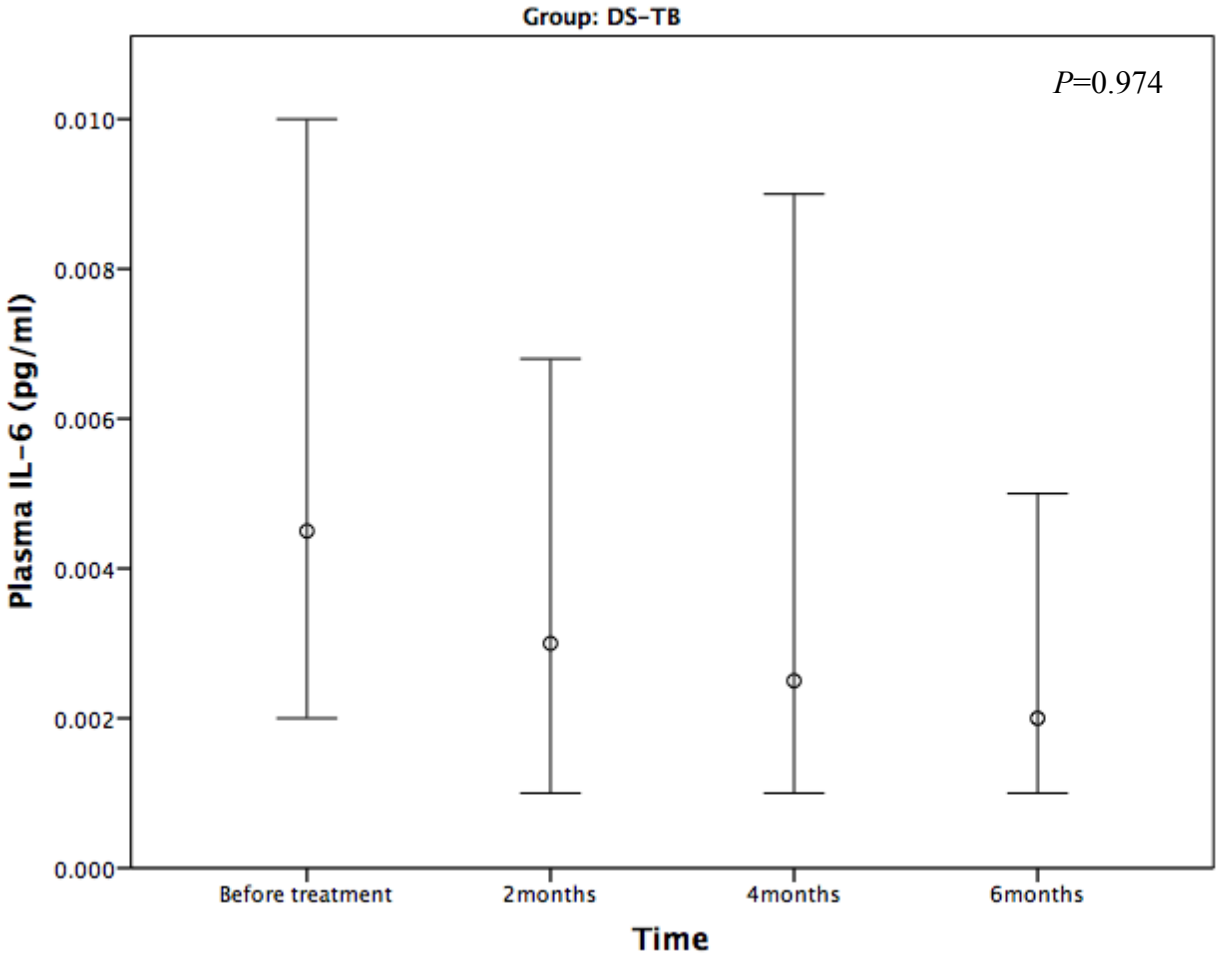


Figure 4.4: Plasma IL-6 level in DS-TB patients before and during ant-TB treatment

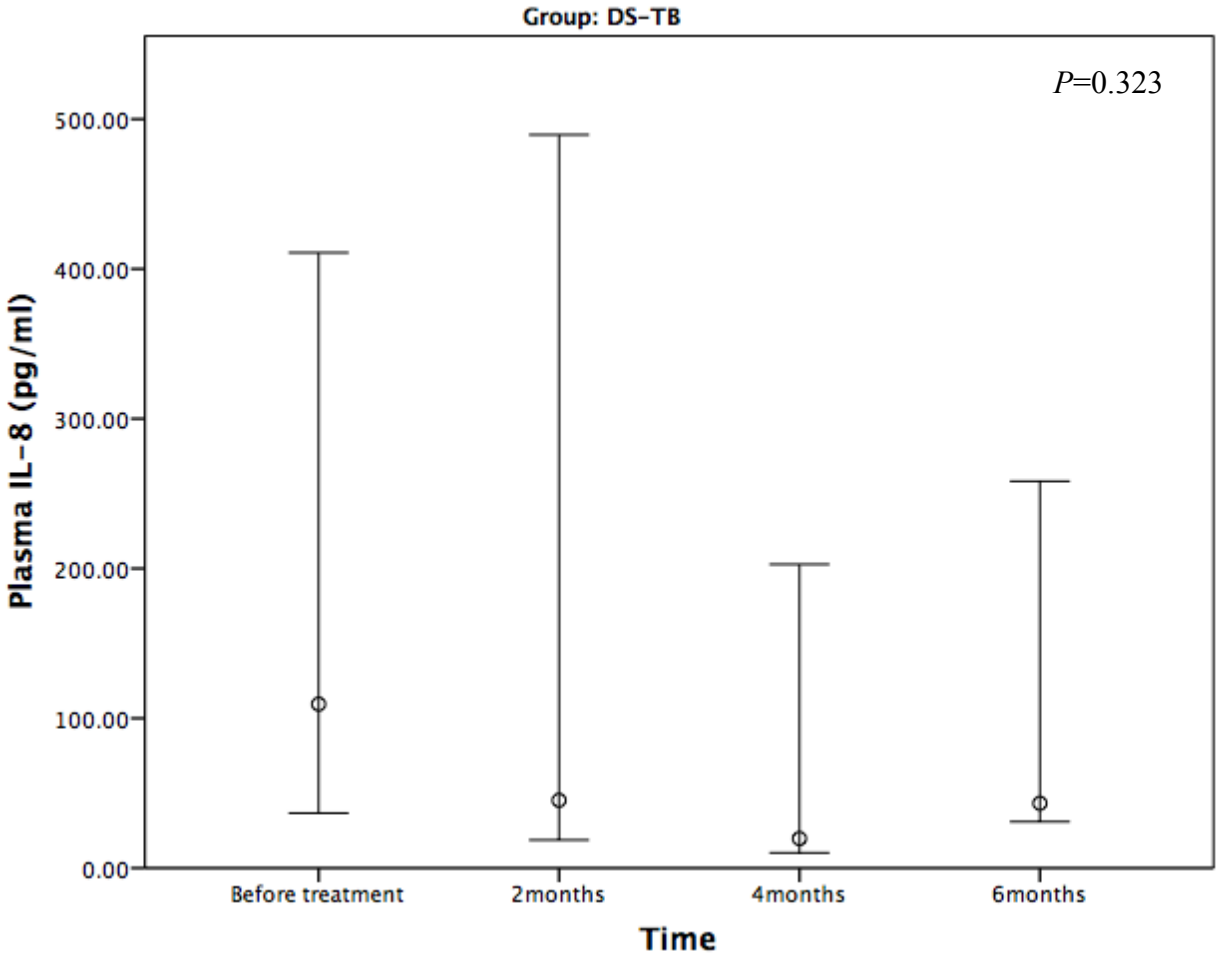
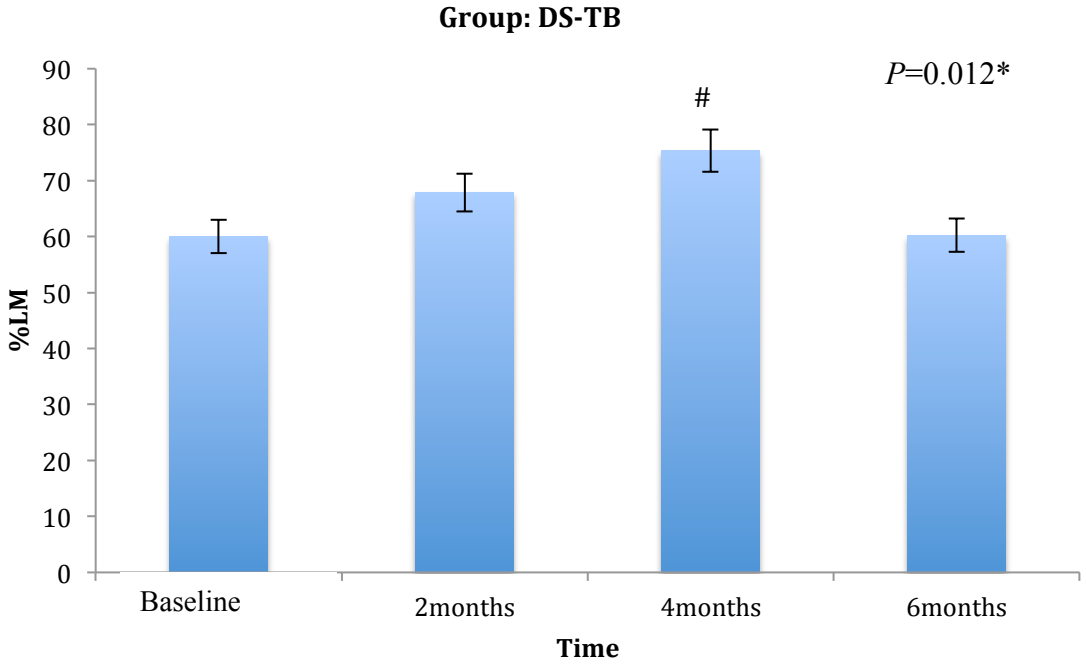


Figure 4.5: Plasma IL-8 level in DS-TB patients before and during ant-TB treatment



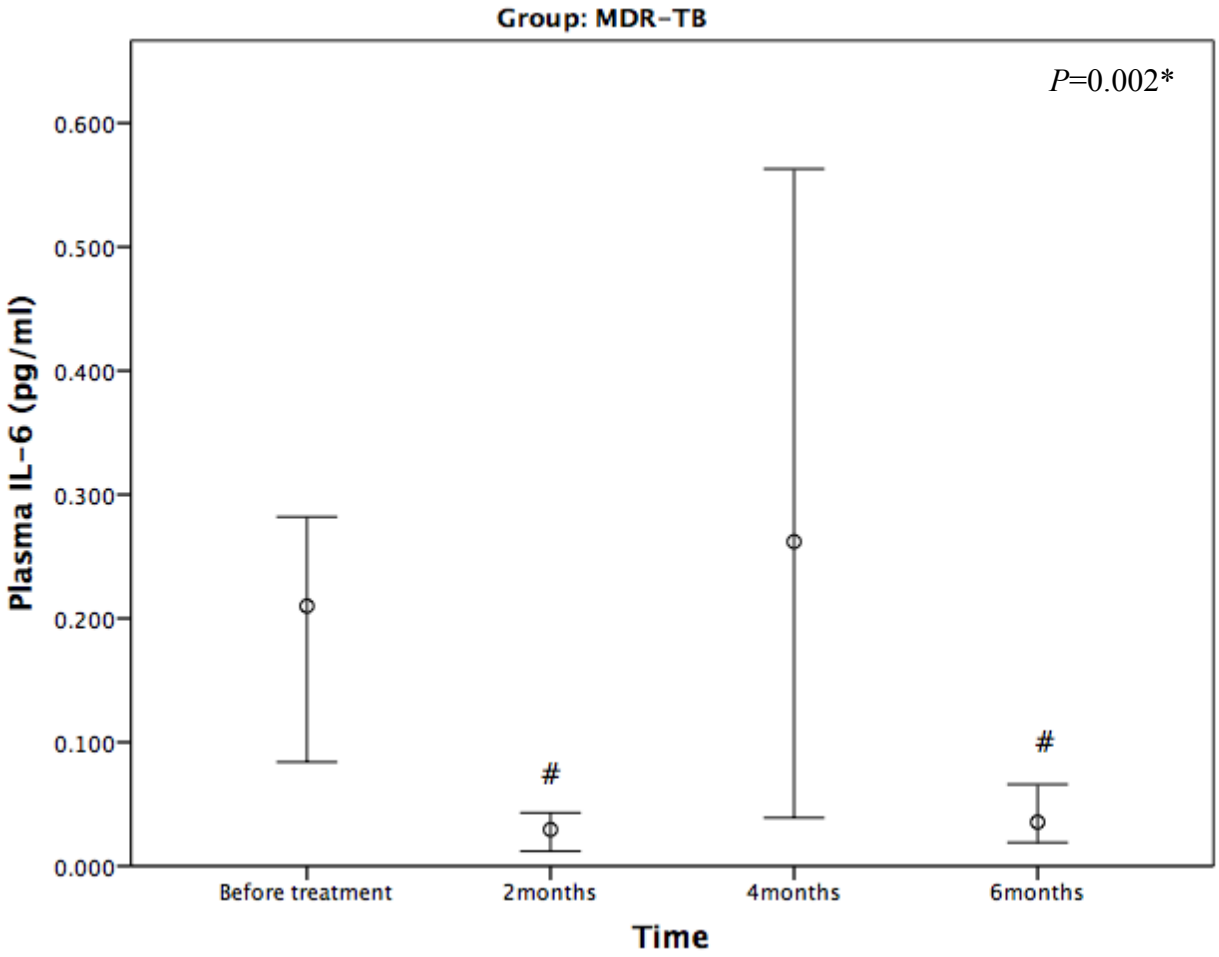
*Significant at p<0.05
#Significantly different from Baseline

Figure 4.6: Percent LM in DS-TB patients before and during ant-TB treatment

Table 4.4: Mediators of leucocyte intracellular killing in DS-TB patients at diagnosis compared with 2, 4 and 6months of anti-TB treatment.

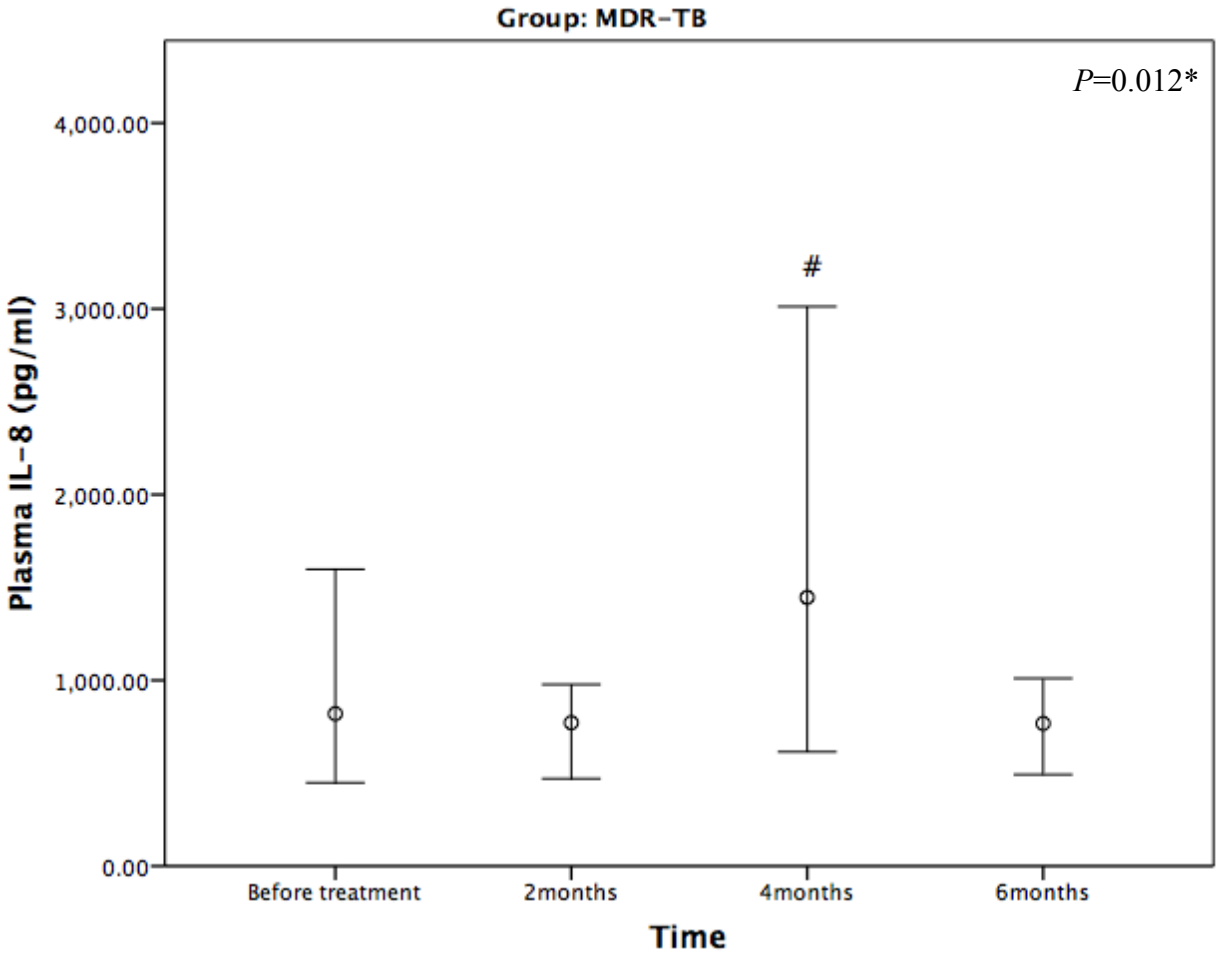
Parameters	Baseline (n=26)	2months (n=26)	4months (n=26)	6months (n=26)	*P*-value
%NBT	85.80±6.28	72.32±13.59[#]	80.53±8.61	85.30±3.88	0.035*
SOD *(U/ml)*	0.14(0.11-0.22)	0.15(0.11-0.23)	0.14(0.10-0.21)	0.14(0.10-0.22)	0.061
CAT *(U/mg protein)*	0.04(0.02-0.07)	0.02(0.01-0.03)[#]	0.03(0.02-0.05)[#]	0.05(0.02-0.08)[#]	0.000*
MPO *(U/ml)*	9.84(8.70-10.48)	11.20(10.0-11.7)[#]	10.5(9.9-11.5)[#]	9.6(8.9-10.2)	0.000*
H_2O_2 *(µmol/l)*	320.7(296.7-346.4)	318.6(299.1-346.1)	312.5(301.9-329.7)	374.8(344.7-398.9)[#]	0.008*
NO *(µmol/l)*	9.93(7.41-15.40)	10.59(7.05-16.09)[#]	11.41(8.66-15.81)[#]	12.74(8.40-19.79)[#]	0.000*

*Significant at $p<0.05$
[#]Significantly different from Baseline



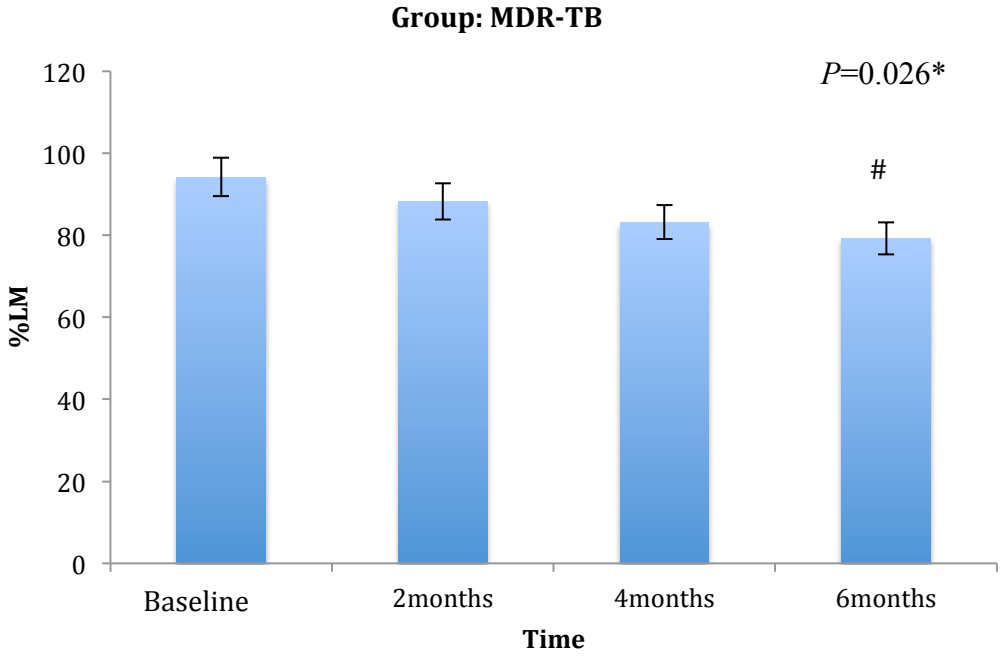
*Significant at p<0.05
#Significantly different from Before treatment

Figure 4.7: Plasma IL-6 level in MDR-TB patients before and during ant-TB treatment



*Significant at p<0.05
#Significantly different from Before treatment

Figure 4.8: Plasma IL-8 level in MDR-TB patients before and during ant-TB treatment



*Significant at p<0.05
#Significantly different from Baseline

Figure 4.9: Percent Leucocyte migration in MDR-TB patients before and during ant-TB treatment

Table 4.5: Mediators of leucocyte intracellular killing in MDR-TB patients at diagnosis compared with 2, 4 and 6months of anti-TB treatment.

Parameters	Baseline (n=45)	2months (n=45)	4months (n=45)	6months (n=45)	*P*-value
%NBT	85.66±4.38	86.11±5.36	91.62±3.41	92.26±2.05[#]	0.041*
SOD *(U/ml)*	0.18(0.12-0.30)	0.16(0.11-0.22)	0.18(0.11-0.25)	0.12(0.09-0.22)[#]	0.036*
CAT *(U/mg protein)*	0.02(0.02-0.04)	0.04(0.02-0.05)[#]	0.05(0.03-0.06)[#]	0.04(0.02-0.06)[#]	0.003*
MPO *(U/ml)*	7.38(6.44-7.96)	7.82(7.23-8.59)[#]	7.96(6.42-8.98)[#]	8.36(7.44-9.12)[#]	0.020*
H_2O_2 *(µmol/l)*	312.1(289.9-327.5)	317.1(305.6-329.0)	318.5(307.9-342.9)[#]	323.2(310.5-343.9)[#]	0.042*
NO *(µmol/l)*	6.70(5.39-12.16)	11.48(8.82-16.87)[#]	9.55(6.35-17.79)[#]	8.71(5.46-13.95)	0.001*

*Significant at $p<0.05$
[#]Significantly different from Baseline

Table 4.6: Mediators of leucocyte intracellular killing in DS-TB patients using zinc supplement at diagnosis compared with 2, 4 and 6 months of anti-TB treatment.

Parameters	Baseline (n=24)	2months (n=24)	4months (n=24)	6months (n=24)	*P*-value
SOD *(U/ml)*	0.14(0.11-0.17)	0.17(0.14-0.19)[#]	0.14(0.10-0.16)	0.11(0.09-0.14)[#]	0.000*
CAT *(U/mg protein)*	0.05(0.04-0.05)	0.03(0.02-0.03)[#]	0.03(0.03-0.04)[#]	0.05(0.05-0.06)	0.000*
MPO *(U/ml)*	8.50(6.85-11.49)	17.30(13.59-21.81)[#]	17.52(14.17-23.98)[#]	17.61(15.75-27.95)[#]	0.000*
H_2O_2 *(µmol/l)*	213.3(207.0-223.0)	220.2(211.4-230.3)	207.3(199.5-214.6)	203.9(194.7-211.6)[#]	0.000*
NO *(µmol/l)*	22.1(17.0-32.0)	27.4(15.5-37.2)	22.5(17.4-31.2)	18.1(14.2-29.2)[#]	0.000*

*Significant at $p<0.05$
[#]Significantly different from Baseline

Table 4.7: Correlation of mechanisms of innate cellular immunity in pulmonary tuberculosis patients and controls

Correlating pair		MDR-TB (n=50) (r-value, *P*-value)	DS-TB (n=60) (r-value, *P*-value)	Controls (n=50) (r-value, *P*-value)
%NBT	**SOD**	-0.003, 0.984	-0.286, 0.011*	-0.052, 0.820
	MPO	0.101, 0.121	0.099, 0.571	0.136, 0.291
	CAT	0.065, 0.111	0.053, 0.763	-0.063, 0.774
	H_2O_2	0.272, 0.000*	0.242, 0.000*	0.152, 0.388
	NO	0.144, 0.022*	0.132, 0.024*	0.055, 0.809
%LM	**SOD**	-0.085, 0.171	-0.243, 0.006*	-0.030, 0.628
	MPO	-0.009, 0.888	0.015, 0.785	0.035, 0.572
	CAT	0.171, 0.006*	0.016, 0.780	-0.016, 0.789
	H_2O_2	-0.097, 0.117	-0.307, 0.000*	0.212, 0.066
	NO	0.055, 0.379	0.241, 0.000*	-0.079, 0.195
Plasma IL-6	**SOD**	-0.042, 0.500	-0.235, 0.000*	-0.117, 0.059
	MPO	0.035, 0.569	-0.055, 0.326	-0.014, 0.827
	CAT	-0.175, 0.004*	-0.045, 0.429	0.011, 0.856
	H_2O_2	0.068, 0.274	0.324, 0.000*	0.047, 0.449
	NO	0.146, 0.017*	0.261, 0.000*	-0.214, 0.001*
Plasma IL-8	**SOD**	-0.035, 0.576	-0.103, 0.073	-0.003, 0.967
	MPO	0.025, 0.687	-0.044, 0.439	0.086, 0.172
	CAT	-0.104, 0.095	-0.004, 0.949	0.055, 0.377
	H_2O_2	0.080, 0.198	-0.157, 0.006*	-0.085, 0.175
	NO	0.103, 0.075	0.048, 0.406	-0.052, 0.530

*Significant at $p<0.05$

Table 4.8: Correlation of modulators of innate cellular immunity with mechanisms of innate cellular immunity in MDR-TB patients

		^{p}Fe	^{p}Cu	^{p}Zn	^{p}Vit A	^{p}Vit C	^{p}Vit D	^{p}Vit E	^{p}TP
%NBT	r	0.404	0.365	-0.318	-0.066	0.116	0.098	-0.066	-0.230
	P	0.000*	0.000*	0.000*	0.289	0.067	0.120	0.297	0.000*
%LM	r	-0.209	-0.302	-0.226	-0.205	0.375	0.227	0.087	-0.081
	P	0.000*	0.000*	0.000*	0.001*	0.000*	0.000*	0.163	0.196
^{p}IL-8	r	-0.137	-0.212	-0.154	0.076	0.017	0.039	-0.081	-0.082
	P	0.027*	0.001*	0.013*	0.224	0.786	0.536	0.193	0.188
^{p}IL-6	r	-0.175	-0.144	-0.358	-0.017	0.171	0.067	-0.027	-0.060
	P	0.004*	0.019*	0.000*	0.780	0.005*	0.275	0.667	0.334
^{p}SOD	r	0.191	0.027	-0.032	0.255	-0.162	-0.063	-0.094	0.178
	P	0.002*	0.666	0.602	0.000*	0.008*	0.304	0.129	0.002*
^{p}MPO	r	-0.087	-0.089	-0.030	0.008	-0.080	0.077	0.013	-0.343
	P	0.160	0.151	0.627	0.897	0.195	0.212	0.831	0.000*
^{p}CAT	r	-0.070	0.135	0.181	-0.034	-0.057	0.043	-0.035	-0.104
	P	0.259	0.028*	0.003*	0.582	0.354	0.490	0.569	0.080
$^{p}H_2O_2$	r	0.316	0.180	0.082	0.027	-0.079	-0.011	-0.167	-0.313
	P	0.000*	0.003*	0.184	0.659	0.199	0.856	0.007*	0.000*
^{p}NO	r	0.062	0.000	0.116	-0.056	-0.005	0.050	0.007	-0.130
	P	0.316	0.996	0.058	0.360	0.930	0.420	0.908	0.028*

*Significant at $p<0.05$
^{p}*Plasma*

Table 4.9: Correlation of modulators of innate cellular immunity with mechanisms of innate cellular immunity in DS-TB patients

		^{p}Fe	^{p}Cu	^{p}Zn	^{p}Vit A	^{p}Vit C	^{p}Vit D	^{p}Vit E	^{p}TP
%NBT	r	0.337	0.357	-0.337	-0.197	0.398	0.114	-0.145	-0.112
	P	0.000*	0.000*	0.000*	0.001*	0.000*	0.051	0.013*	0.055
%LM	r	-0.303	-0.235	-0.255	-0.152	0.383	0.073	0.289	-0.100
	P	0.000*	0.000*	0.000*	0.008*	0.000*	0.203	0.000*	0.078
^{p}IL-8	r	-0.110	-0.224	-0.166	-0.080	0.293	0.148	0.130	-0.085
	P	0.056	0.000*	0.004*	0.163	0.000*	0.010*	0.023*	0.138
^{p}IL-6	r	-0.231	-0.123	-0.181	-0.055	0.344	0.081	0.240	-0.242
	P	0.000*	0.031*	0.001*	0.338	0.000*	0.156	0.000*	0.000*
^{p}SOD	r	0.141	0.191	0.184	0.060	-0.147	0.033	0.000	0.055
	P	0.013*	0.001*	0.001*	0.291	0.010*	0.561	0.995	0.308
^{p}MPO	r,	0.060	0.151	0.048	0.065	0.058	-0.041	-0.022	0.023
	P	0.290	0.008*	0.403	0.254	0.308	0.470	0.700	0.670
^{p}CAT	r	-0.054	0.051	0.084	-0.041	-0.031	0.038	0.035	-0.165
	P	0.346	0.373	0.139	0.476	0.588	0.501	0.542	0.002*
$^{p}H_2O_2$	r	0.193	0.192	0.237	0.114	-0.247	-0.073	-0.187	-0.121
	P	0.001*	0.001*	0.000*	0.046*	0.000*	0.202	0.001*	0.025*
^{p}NO	r	-0.078	-0.029	-0.142	-0.084	0.150	0.129	0.041	-0.199
	P	0.169	0.606	0.012*	0.141	0.008*	0.024*	0.468	0.000*

*Significant at $p<0.05$
^{p}*Plasma*

Table 4.10: Correlation of modulators of innate cellular immunity with mechanisms of innate cellular immunity in controls

		^{p}Fe	^{p}Cu	^{p}Zn	^{p}Vit A	^{p}Vit C	^{p}Vit D	^{p}Vit E	^{p}TP
%NBT	r	0.128	0.179	0.018	-0.081	0.278	0.152	0.060	-0.190
	P	0.043*	0.005*	0.773	0.202	0.000*	0.016*	0.349	0.003*
%LM	r	-0.114	-0.163	-0.066	-0.066	0.075	0.111	0.111	-0.002
	P	0.070	0.009*	0.290	0.290	0.232	0.077	0.076	0.977
^{p}IL-8	r	0.008	-0.098	-0.146	-0.117	-0.004	0.065	-0.061	-0.039
	P	0.996	0.118	0.020*	0.062	0.955	0.300	0.332	0.530
^{p}IL-6	r	-0.145	-0.029	-0.036	-0.107	0.032	0.076	-0.097	0.130
	P	0.019*	0.636	0.568	0.086	0.607	0.219	0.120	0.036*
^{p}SOD	r	0.007	0.077	-0.085	0.136	0.068	0.032	0.104	-0.019
	P	0.912	0.213	0.172	0.028*	0.272	0.608	0.095	0.743
^{p}MPO	r	0.039	0.105	0.087	-0.080	-0.044	-0.093	-0.194	-0.455
	P	0.532	0.091	0.160	0.196	0.482	0.133	0.002*	0.000*
^{p}CAT	r	0.167	0.189	0.127	-0.011	-0.168	-0.174	-0.006	0.047
	P	0.007*	0.002*	0.041*	0.862	0.007*	0.005*	0.929	0.423
$^{p}H_2O_2$	r,	0.103	-0.022	0.004	0.068	0.068	0.019	-0.007	-0.127
	P	0.097	0.725	0.955	0.276	0.272	0.763	0.908	0.031*
^{p}NO	r	0.084	0.000	0.014	0.150	0.022	-0.101	0.108	0.094
	P	0.176	0.996	0.821	0.016*	0.724	0.106	0.083	0.112

*Significant at $p<0.05$
^{p}*Plasma*

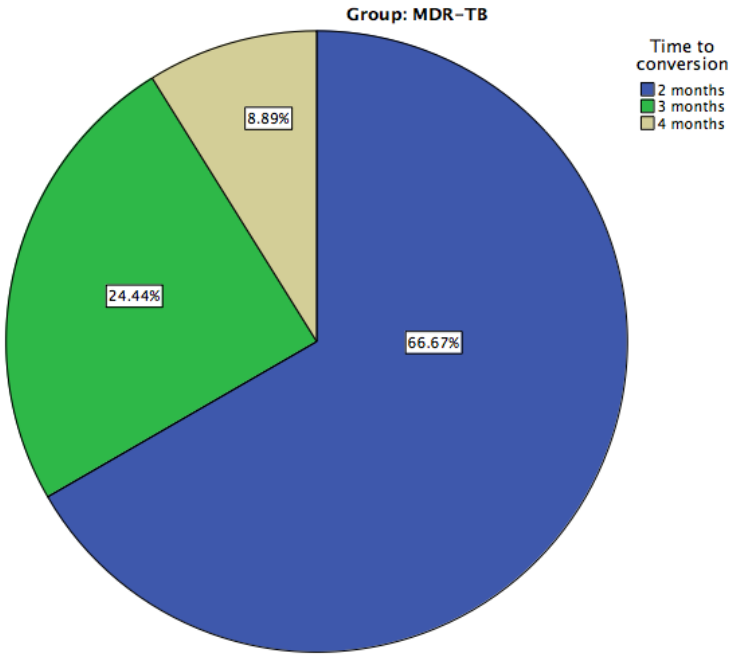


Figure 4.10: Time to culture conversion among MDR-TB patients during 6 months of anti-TB treatment (n=45)

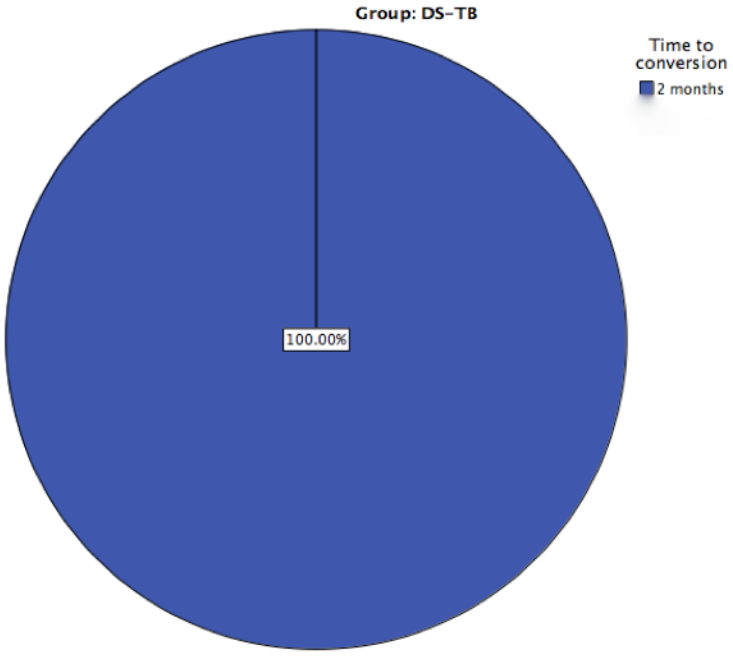


Figure 4.11: Time to sputum smear conversion among DS-TB patients during 6 months of anti-TB treatment (n=59)

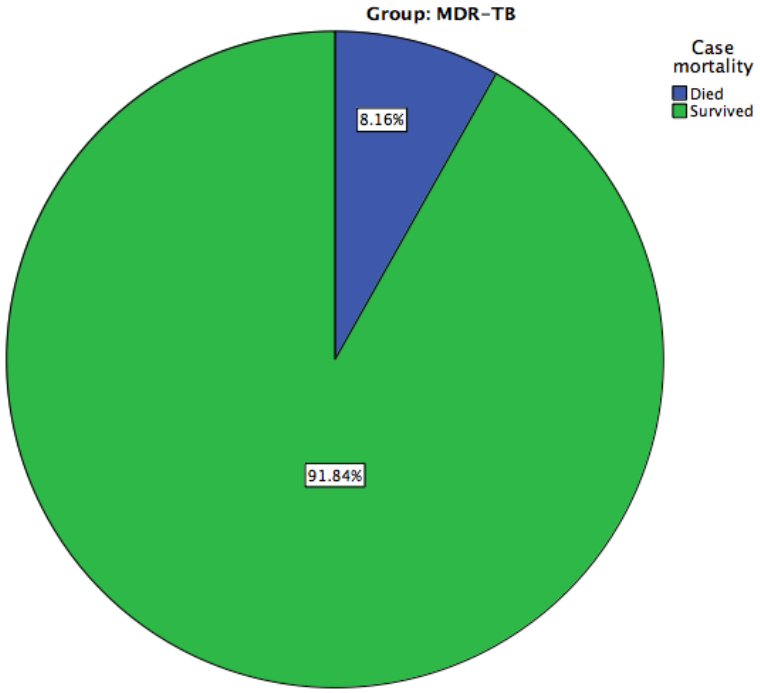


Figure 4.12: Mortality among MDR-TB patients during 6 months of anti-TB treatment (n=50)

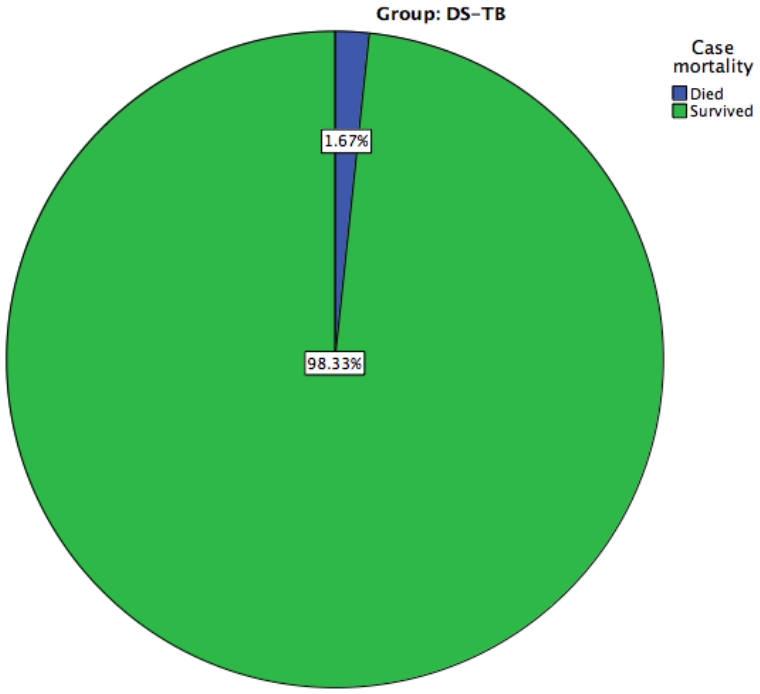


Figure 4.13: Mortality among DS-TB patients during 6 months of anti-TB treatment (n=60)

Table 4.11: Anthropometry and plasma levels of micronutrients in MDR-TB patients whose sputum culture converted compared with non-converted at 2 months of anti-TB chemotherapy

Variables	Converted at 2 months (n=28)	Non-converted at 2 months (n=16)	t-value	*P*-value
Anthropometry				
UAC *(cm)*	22.61±2.57	23.19±2.82	-0.696	0.490
Weight *(kg)*	51.70±9.31	41.77±4.92	3.940	0.000*
BMI *(kg/m^2)*	18.84±2.91	14.58±2.08	5.110	0.000*
PBF *(%)*	19.96±1.59	8.43±1.16	5.110	0.000*
FMI *(kg/m^2)*	3.94±2.28	1.29±0.84	4.446	0.000*
FFMI *(kg/m^2)*	14.90±1.50	13.30±1.56	3.331	0.002*
WC *(cm)*	77.14±8.25	74.91±6.70	0.923	0.362
HC *(cm)*	86.68±7.56	87.44±6.33	-0.339	0.736
Micronutrients				
Fe *(µg/dl)*	92.78±12.87	87.55±7.82	1.490	0.144
Zn *(µg/dl)*	69.68±4.49	67.79±4.54	1.338	0.188
Cu *(µg/dl)*	101.22±4.08	99.98±5.27	0.871	0.389
Vit A *(µg/dl)*	57.77±11.94	53.51±8.52	1.254	0.217
Vit C *(mg/dl)*	1.96±0.49	2.21±0.48	-1.640	0.109
Vit D *(pg/ml)*	72.25±29.12	54.35±14.60	2.292	0.027*
Vit E *(mg/dl)*	0.36±0.13	1.06±0.19	-14.492	0.000*

*Significant at $p<0.05$

Table 4.12: Indices of phagocytosis of MDR-TB patients whose sputum culture converted compared with non-converted at 2 months of anti-TB chemotherapy

Variables	Converted at 2 months (n=28)	Non-converted at 2 months (n=16)	*P*-value
Indices of leucocyte migration			
IL-6 *(pg/ml)*	0.01(0.003-0.03)	0.04(0.02-0.05)	0.062
IL-8 *(pg/ml)*	470.6(385.1-871.8)	795.8(489.0-1432.4)	0.171
%LM	81.60±9.27	85.14±4.85	0.164
Mediators of leucocyte intracellular killing			
%NBT	90.64±7.13	86.55±9.49	0.113
SOD *(U/ml)*	0.15(0.11-0.21)	0.17(0.12-0.25)	0.252
CAT*(U/mg protein)*	0.04(0.02-0.07)	0.02(0.02-0.04)	0.157
MPO *(U/ml)*	8.00(7.35-8.57)	8.05(7.51-8.95)	0.721
H_2O_2 *(µmol/l)*	315.62(293.9-324.9)	319.1(305.6-357.2)	0.185
NO *(µmol/l)*	11.54(7.64-17.13)	11.18(9.42-15.71)	0.572

*Significant at $p<0.05$

CHAPTER FIVE

5.0 DISCUSSION

The immunological response to *M.tuberculosis* infection is a vital determinant of the course of both infection as well as the associated disease. Understanding TB immunity and other host factors associated with immune response that determine development of active disease is a major challenge applicable to a large proportion of persons with latent TB due to TB reactivation. TB reactivation from dormancy currently accounts for bulk of the incident TB cases in adults, and it is a major public health concern as estimates show that over 3 billion of the world's population harbor *M.tuberculosis* in latency.

5.1 BEFORE ANTI-TB TREATMENT

5.1.1 ANTHROPOMETRIC INDICES

Nutrition is a critical risk factor in many infectious diseases including tuberculosis. It is of significant import in the control and management of infectious diseases, with malnutrition associated with more severe infectious disease forms including tuberculosis (Sinclair *et al.*, 2011). This study observed under-nutrition (BMI<18.5kg/m^2) in 56.4% of TB patients before commencement of anti-TB treatment. This agrees with the findings of Dodor (2008) and Zachariah *et al.* (2002) who reported under-nutrition in 51% and 57% of TB patients in Ghana and Malawi respectively. However, it is lower than 71.6% reported by Kennedy *et al.* (1996) in Tanzanian TB patients. This is further supported by lower mean MUAC, weight, BMI, PBF and HC in DS-TB and MDR-TB patients, lower mean WC in DS-TB patients compared with controls also observed in this present study. These observations indicate decline of both fat and fat-free (lean) mass in these patients, which could be due to a metabolic shift in favour of increased catabolism and increased protein loss (negative nitrogen balance), or decreased nutrient supply due to anorexia and/or malabsorption. Previous studies of protein and energy metabolism in infection demonstrated metabolic changes in acute, sub-acute and chronic infectious diseases characterized by increased energy demand and expenditure with increased oxidation of fat and breakdown of lean tissue for energy (Macallan *et al.*, 1998; Paton *et al.*, 2001).

Macallan *et al.* (1998) further hypothesized that there is a block to the anabolic response to nutrition in TB patients, which hampers utilization of exogeneous nutrient supply in these patients. These may explain the decline of fat and fat-free mass in TB patients observed in this study. It also supports the age-long association between TB and wasting as well as

previous reports of protein-calorie malnutrition in TB patients (Karyadi *et al.*, 2000; Kassu *et al.*, 2006; Dodor *et al.*, 2008; Cegielski *et al.*, 2012; Mupere *et al.*, 2014). However, due to the viscous cycle of TB and under-nutrition, it is difficult to establish if under-nutrition preceded active TB or active TB preceded under-nutrition in our study population. Previous studies have suggested that under-nutrition alters effectiveness of anti-TB medication, TB vaccine efficacy and increases mortality of TB patients (Sushama and Lekshmi, 2002; Cegielski and McMurray, 2005; Compher, 2005; Schaible and Kaufmann, 2007). The elevated prevalence of under-nutrition among TB patients in this study indicates that under-nutrition remains a major concern in active TB and there is need for nutritional assessment of TB patients at diagnosis as well as nutritional support during treatment.

5.1.2 MODULATORS OF INNATE CELLULAR IMMUNITY

Micronutrients play important roles in several metabolic pathways, function as anti-oxidants, and form essential factors in both intra-cellular and inter-cellular signaling pathways of many immunocompetent cells, with T lymphocytes and monocytes subsets particularly sensitive to changes in micronutrient levels (Wintergerst *et al.*, 2007). Zinc is important for normal growth and functioning of cells mediating non-specific immunity. In addition, the macrophage that plays a pivotal role in host defense against *M.tuberculosis*, is adversely affected by zinc deficiency (Byrd *et al.*, 2002). Lower zinc levels in MDR- and DS-TB patients before treatment observed in this study is in concert with previous studies (Karyardi *et al.*, 2000; Ciftci *et al.*, 2003; Kassu *et al.*, 2006; Rohini *et al.*, 2013). Reduced plasma Zn level observed in this study could be due to increased redistribution of zinc from circulation to other tissues, decreased hepatic synthesis of α2-macroglobulin (a zinc carrier protein) or an increase in synthesis of metallothionein, which transports zinc to the liver. It may also be due to increased zinc utilization by the immune cells. IFN-γ secretion, a major feature of active TB disease, has been previously associated with high expression of zinc transporters on T_H1 cells which ultimately leads to increased zinc demand and utilization (Aydemir *et al.*, 2009).

Similarly, lower plasma iron concentration was seen in MDR-TB patients compared with controls. This observation supports the report of Kassu *et al.* (2006) and Karyadi *et al.* (2000) among Ethopian and Indonesian TB patients respectively. This decrease may be due to increased blood loss from hemoptysis in MDR-TB patients, an attempt by the body to create an iron-scarce environment thereby limiting iron availability for use by *M.tuberculosis* for growth and expansion, or due to mild to moderate anemia which was previously associated

with active TB disease (Das *et al.*, 2003). Although iron was reported to play a role in myeloperoxidase-dependent production of hypochlorous acid and was implicated in the pathways leading to the *in vitro* bactericidal effect of vitamin C on *M.tuberculosis*, there is the need for caution in recommending iron supplementation in TB patients as increased dietary iron was connected with a 3.5-fold increase in odds of developing active TB and a tendency towards higher mortality among TB patients (Gangaidzo *et al.*, 2001).

Vitamin A was reported to inhibit multiplication of *M.tuberculosis* within macrophages *in vitro* (Crowle and Ross, 1989; Anand *et al.*, 2008) and plays a key function in lymphocyte proliferation and maintenance of epithelial tissues activities (Chandra, 1991). In this present study, plasma vitamin A concentration decreased in MDR-TB patients compared with controls. This may be explained by increased utilization and urinary excretion with reduced supply due to reduced intake and absorption. Although a previous study reported that vitamin A supplementation with anti-TB treatment had some negative effects in children with tuberculosis (Hanekom *et al.*, 1999), another study on vitamin A and zinc supplementation in adults reported improved treatment outcomes in TB patients (Karyadi *et al.*, 2002). These conflicting reports suggest the need for further studies on vitamin A supplementation in TB patients.

The etiology of lung fibrosis and dysfunction in TB involves induced production of oxidative free radicals, which promote tissue injury and inflammation (Madebo *et al.*, 2003; Vijayamalimi and Manoharan, 2004). Antioxidants in different forms scavenge free radical and suppress the actions of ROS, protecting the host from tissue injury and inflammation. Vitamins C (ascorbic acid) and E (α-tocopherol) are potent vital hydrophilic and lipophilic antioxidants respectively. Vitamin C scavenges superoxide radical, hydrogen peroxide and thiol radicals and is a potent quencher of singlet oxygen while vitamin E converts superoxide radical, hydroxyl and lipid peroxyl radicals (Vijayamalimi and Manoharan, 2004). It has been demonstrated that vitamin E acts as a mobilizable antioxidant released from tissue stores and diverted to the lungs of TB patients during oxidative stress and radical mediated pulmonary fibrosis (Chow *et al.*, 1993). This leads to formation of tocopherol radical, which is reversed back to tocopherol by ascorbic acid (vitamin C), thereby conferring vitamin E sparring ability on vitamin C (Winkler *et al.*, 1994). In addition to this antioxidant function in infection, vitamin C kills *M.tuberculosis* via the Fenton reaction pathway while vitamin E (α-tocopherol) has also been shown to act as a pro-oxidant upon oxidation to α-tocopheroxyl

with the potential to augment bacterial clearance (Bhattacharyya and Banerjee, 2013; Vilcheze *et al.*, 2013).

This study found lower plasma levels of vitamins C and E in DS-TB patients compared with controls while plasma vitamins C and E levels were higher in MDR-TB patients compared with controls. Lower plasma levels of vitamins C and E found in this study corroborates the reports of Karyadi *et al.* (2000) and Rohini *et al.* (2013). The observed decreased plasma vitamins C and E in DS-TB patients may be due to increased redistribution and utilization with inadequate intake to replenish their levels in circulation whereas increased plasma levels of these vitamins in MDR-TB patients may be due to increased intake particularly of vitamin C supplements, which was observed to be common practice among MDR-TB patients before commencement of anti-DR-TB treatment following failure of previous anti-TB treatment. Vitamin C supplements are readily accessible and there is a common perception that they boost immunity.

Changes in plasma proteins are seen in different physiological and pathological conditions, with these changes reflecting the net effects of the rate of protein synthesis, protein catabolism and protein loss. This present study found decreased plasma total protein in DS-TB patients whereas plasma total protein increased in MDR-TB patients before anti-TB treatment compared with controls. Decreased plasma total protein found in this present study agrees with previous studies (Adedapo *et al.*, 2006; Akiibinu *et al.*, 2007) where it was attributed to increased protein breakdown associated with fever or oxidative protein damage in these patients. On the other hand, increased plasma total protein observed in MDR-TB patients in this present study is in consonance with the reports of Yamanaka *et al.* (2001) and Jemikalajah *et al.* (2014) but at variance with the reports of Adedapo *et al.* (2006) and Akiibinu *et al.* (2007). The disparity may be due to the population of tuberculosis patients, as these previous studies did not clearly specify the population of TB patients (DS-TB or MDR-TB) studied. Increased plasma total protein observed in this study may be due to increases in globulins in this population as previously reported (Adedapo *et al.*, 2006; Akiibinu *et al.*, 2007; Jemikalajah *et al.*, 2014). Nagayama *et al.* (1999) and Paton *et al.* (2004) stated earlier that hyperglobulinaemia in TB is among predictive factors for residual thickening in tuberculous pleurisy while Arinola and Igbi (1998) reported high levels of IgG and IgM in PTB patients.

5.1.3 MECHANISMS OF INNATE CELLULAR IMUNITY

5.1.3.1 LEUCOCYTE MIGRATION INDICES

Phagocytosis, a hallmark of anti-bacterial defense, plays an important role in protection against *M.tuberculosis*. Phagocytosis can be divided into phases, which include leucocyte migration to the infected foci, engulfment and intracellular killing. These phases employ various mechanisms that are controlled by a combination of factors to ensure clearance of foreign body including *M.tuberculosis*. Phagocytes are attracted to infection site, activated and retained by cytokines, leading to engulfment, phagolysosomal fusion and intracellular killing of ingested agent.

This present study found increased plasma IL-8 level in MDR-TB and DS-TB patients before treatment compared with healthy controls while plasma IL-6 and %LM with BCG antigen were increased in MDR-TB patients before treatment compared with healthy controls. Increased plasma IL-8 in TB patients observed in this present study is in consonance with previous studies (Miller *et al.*, 1993; Hilda *et al.*, 2014; Krupa *et al.*, 2015) and could be indicative of active production and release of this chemoattractant for the recruitment of immune cells in TB patients. IL-8 functions as an effective chemoattractant for leukocytes recruitment to inflammatory loci in the inflammatory process (Ma *et al.*, 2003). The up-regulation of IL-8 gene expression in human macrophages infected with *M.tuberculosis* through protein tyrosine kinases and NF-κB, has been previously reported to be a component of the macrophage activation sequence (Ameixa and Friedland, 2002; Nau *et al*., 2002). Anti-IL-8 antibody was also reported to inhibit granuloma formation in rabbits, suggesting central role of IL-8 in host defense against *M.tuberculosis* (Larsen *et al.*, 1995). Thus elevated plasma IL-8 level may have clinical value as a diagnostic marker for advancement to active tuberculosis in persons with latent TB.

This study also found increased plasma IL-6 level in MDR-TB patients before treatment compared to healthy controls which agrees with previous studies (Ellersten *et al.*, 2009; Tang *et al.*, 2013; Joshi *et al.*, 2015). This may be due to active inflammatory reaction as supported by increased plasma IL-8 and total protein found in MDR-TB patients before treatment in this present study. IL-6 produced by phagocytic cells exhibit both pro- and anti-inflammatory potential dependent on the cytokine environment. However, previous studies have demonstrated IL-6 induced regulation of inflammation in tuberculosis (Martinez *et al.*, 2013; Nagabhushanam *et al.*, 2003; Ladel *et al.*, 1997) via increased expression of RORγt

and FoxP3, leading to expansion of T_h17 and Treg cells as well as conversion into IL-17 producers (Afzali *et al.*, 2010). Therefore, increased plasma IL-6 in MDR-TB patients before treatment observed in this present study may be towards regulation of inflammation in these patients.

Percent LM is an *in vitro* method of assessing chemokine production and activity as well as delayed type hypersensitivity, in which LM inhibitory factor (LMIF) secretion by lymphocytes restricts the free movement of leukocytes, thus resulting in reduced %LM (Arinola, 2005). Lymphocytes of patients with active TB are expected to have been pre-sensitized following *M.tuberculosis* infection. Hence, *in vitro* stimulation with BCG (TB vaccine-attenuated strain of *M.bovis*) should result in production of LMIF leading to reduced %LM. Raised %LM in MDR-TB patients observed in this present study is indicative of failure of BCG vaccine to induce T-cell production of lymphokines *in vitro*. BCG vaccination has been reported to protect against disseminated forms of TB in children (Mangtani *et al,*. 2014). However, this is in contrast to the highly variable protection seen in adolescents and adults (Fine, 1995). Raised %LM observed in MDR-TB patients supports variable protection by BCG vaccination and buttresses the need for vaccines with more stable efficacy in adolescent and adult populations. It is also suggestive of differing etiology of drug-sensitive from drug-resistant PTB cases.

5.1.3.2 LEUCOCYTE INTRACELLULAR KILLING

The phagocyte respiratory burst is essential for containing infections including tuberculosis, as demonstrated in clinical reports of chronic granulomatous disease patients (Lau *et al.*, 1998). The role of oxygen radicals in anti-mycobacterial immunity was underscored by discovery of an association between TB susceptibility and mutation in the gene encoding the subunit $gp91^{phox}$ of NADPH oxidase 2 (NOX2) (Bustamante *et al.*, 2011). Coupling of the NADPH oxidase 2 (NOX2) complex at the phagolysosomal membrane yields superoxide ion (O_2^-), a highly potent antimicrobial agent, from molecular oxygen. Superoxide is converted to hydrogen peroxide, another potent antimicrobial agent, by superoxide dismutase (SOD).

This present study observed decreased plasma SOD activity in DS-TB patients before treatment compared with healthy. Decreased plasma SOD activity in DS-TB patients before treatment may be a direct modulatory effect of *M.tuberculosis* or due to the reduction of superoxide ions by *M.tuberculosis* secretory variants of SOD (SodA and SodC). This affects

the availability of substrate (superoxide ion) for host SOD conversion to hydrogen peroxide, which may truncate host respiratory burst. *M.tuberculosis* has been reported to produce two SODs, an iron-cofactored enzyme encoded by sodA and a copper-zinc cofactored enzyme encoded by SodC (Zhang *et al.*, 1991; Dussurget *et al.*, 2001; Piddington *et al.*, 2001). The iron-cofactored SOD – SodA, is produced by *M.tuberculosis* in large amounts and much of it is exported extracellularly (Harth and Horwitz, 1999). These enzymes protect *M.tuberculosis* from bactericidal superoxide ion and this is among the means by which *M.tuberculsosis* counteracts host immune response to ensure continued survival.

This present study also found decreased plasma H_2O_2 level in DS-TB patients before treatment compared with controls and MDR-TB patients before treatment. This observation may not be surprising as increased CAT and MPO activities were also observed in these patients. Therefore, the observed decrease in plasma H_2O_2 level could be due to increased utilization supported by increased CAT and MPO activities observed in this present study. In addition, a previous study demonstrated that H_2O_2 released by activated neutrophils serve as macrophage-activating factor through augmentation of tumor necrosis factor-α release (Sredni-Kenigsbuch *et al.*, 2000) while another study showed that H_2O_2 modulates the expression of leukocyte adhesion molecule and leukocyte endothelial adhesion (Sen and Roy, 2008). These activities are enhanced in active TB disease and may lead to increased H_2O_2 utilization thereby accounting for reduced H_2O_2 observed in this present study. Furthermore, *Mycobacterial* proteins; catalase-peroxidase (Kat G) and alkyl hydroperxidase reductase, confer resistance to H_2O_2 on *M.tuberculosis*. Activities of these bacterial proteins could also account for decreased H_2O_2 level observed in DS-TB patients before treatment in this study. However, decreased plasma H_2O_2 level in DS-TB patients observed in this study is in contrast with a previous study (Alli *et al.*, 2014) that showed increased plasma H_2O_2 in TB patients compared with healthy controls. This difference may be due to the inclusion of patients with HIV-TB co-infection among TB patients recruited for their study.

Catalase is a major scavenger of H_2O_2, playing an important role in regulation of the bactericidal oxidative burst during infection (Yuniasti, 2012). In this present study, plasma CAT activity increased in DSTB patients compared to controls and MDR-TB patients before treatment. This is contrary to previous reports of decreased catalase activity in TB patients before treatment with or without HIV co-infection (Reddy *et al.*, 2004; Dalvi *et al.*, 2012; Rajopadhye *et al.*, 2017). While increase in catalase activity seen in this present study could

be an attempt to control tissue damage resulting from the actions of free radicals, it may also confer protection on *M.tuberculosis* by scavenging potent bactericidal H_2O_2. The transcription factor PPARγ which has been shown to be a crucial contributor to the regulation of catalase expression (Glorieux *et al.*, 2015) was reported to be activated by the ligation of *M.tuberculosis* lipoarabinomannan in a phospholipase A2 and TLR-2 dependent manner (Rajaram *et al.*, 2010; Liu *et al.*, 2015). This study posits that increased host plasma CAT activity may be induced by *M.tuberculosis* to evade oxidative killing by H_2O_2.

Hypochlorous acid (HOCl) is highly microbicidal and its formation from H_2O_2 requires the enzyme myeloperoxidase (MPO). This present study observed an increase in plasma MPO activity in DS-TB patients before treatment compared with controls. Neutrophil extracellular traps (NETs) are a proposed mechanism of neutrophil antimicrobial activity that represents an important strategy to trap and kill microbes (Sorensen and Borregaard, 2016). Human neutrophil elastase (HNE) and myeloperoxidase (MPO) are reported to be essential for NET formation (Sorensen and Borregaard, 2016). Increased plasma MPO activity in DS-TB patients before treatment observed in this study may be a host immune defense mechanism towards production of microbicidal HOCl, and also supports the association of NET levels in human plasma with active TB disease (Schechter *et al.*, 2017). In contrast, MDR-TB patients had decreased plasma MPO activity before treatment compared to controls. This may be due to decreased plasma Fe level observed in MDR-TB patients before treatment compared to controls in this study. Myeloperoxidase is a heme-containing enzyme, hence it requires iron for its synthesis and function (Miatra *et al.*, 2013). This study proposes that the association of NET levels in human plasma with active TB previously reported by Schechter *et al.* (2017) will be dependent on iron status in these patients.

Interferon-γ-induced production of NO is recognized as a potent agent of *M.tuberculosis* immunity (Ernst, 2012). NO plays a vital role in intracellular killing of pathogens and vascular smooth muscle relaxation, and in addition, modulates gene expression via the agency of transcription factors, with the most important being nuclear factor-κB (NF-κB), which regulates transcription of pro-inflammatory cytokines (Antosova *et al.*, 2012). This present study observed decreased plasma NO level in MDR-TB and DS-TB patients before treatment compared with controls. Reduced NO observed in this study is in agreement with a previous report (Akiibinu *et al.*, 2011) but is in contrast with the reports of Kulkami and Madrasi (2008) as well as Dalvi *et al.* (2013). NO is produced by conversion of arginine, an

amino acid, into citrulline using inducible nitric oxide synthase (iNOS). Due to the vicious cycle of malnutrition and tuberculosis, there is an increased probability of arginine insufficiency in active TB disease patients. Hence, reduced NO observed in TB patients before treatment in this study may be due to arginine insufficiency. This is supported by report that arginine supplementation during anti-TB chemotherapy improved clinical outcome in TB patients (Schon *et al.*, 2003). Arginine supplementation as an adjuvant to standard anti-TB drug regimen in the management of TB patients is therefore recommended in TB patients in this environment.

5.1.4 CORRELATION OF MECHANISMS OF INNATE CELLULAR IMMUNITY

The NBT test measures intracellular ROI generation by phagocytes, hence it should be affected by ROI levels and factors that increase or decrease them. Positive correlations between %NBT and plasma H_2O_2 and NO levels in TB patients indicate a direct association between intracellular ROI and %NBT. Reactive oxygen species have been shown to act intrinsically within migrating cells and permissively in surrounding stationary cells to influence migration, (Hurd *et al.*, 2012) with hydrogen peroxide particularly suggested to act as a primary chemoattractant produced upon injury to attract immune cells to wounds (Niethammer *et al.*, 2009). This could imply that as a chemoattractant, hydrogen peroxide can also serve to restrict the movement of leukocytes within site of infection serving as a leukocyte migration inhibitory factor. This may explain the negative correlations between %LM and plasma H_2O_2 in DS-TB patients as well as the activity of H_2O_2 producing enzyme, SOD activity in DS-TB patients. In addition, it also explains the positive correlations were observed between %LM and the activity of H_2O_2 consuming enzyme, plasma CAT activity in MDR-TB patients.

This study also showed negative correlation between plasma H_2O_2 level and plasma level of IL-8, a primary chemoattractant. Given the chemoattractive function of H_2O_2 a positive correlation with IL-8 would have been expected to indicate a synergistic relationship. However, inverse correlation observed could indicate that the deployment of H_2O_2 for chemoattraction may be a compensatory mechanism in cases of lowered IL-8 levels. Apart from its function as a chemoattractant, IL-8 was reported to stimulate oxidative burst activity by way of a priming process involving either of the following secondary stimulus; increased expression and cycling of fMLP receptors (fMLP-R) (Metzner *et al.*, 1995), increased intracellular-free Ca^{2+} (Wozniak *et al.*, 1993) and increased phospholipase A_2 activation

(Daniels *et al.*, 1992). However, negative correlations between plasma IL-8 level and plasma H_2O_2 level could indicate an alteration of IL-8 induced oxidative burst which could be due to modulation of one or more secondary stimuli required for IL-8 induced oxidative burst in DS-TB patients. This may represent a mechanism exploited by *M.tuberculosis* to escape oxidative intracellular killing and requires further studies.

Positive correlation between plasma NO and %LM in MDR-TB and DS-TB patients respectively depicts an inverse relationship between NO and leukocytes attraction and retention at the site of infection in TB patients. Previous studies in mice associated NO with failure of neutrophil migration to inflammatory site (Benjamin *et al.*, 2002) via NO induced reduction of leukocyte-endothelial cell adhesion (Hickey and Kubes, 1997; Hickey, 2001).

IL-6 has been shown to induce pro-oxidant synthesis as an adaptive response in neutrophils and monocytes (Behrens and Dugan, 2008). Therefore, the negative correlations between between plasma CAT activity, plasma SOD activity and plasma IL-6 level in MDR-TB and DS-TB patients respectively may be indicative of promotion of oxidant production by IL-6 in TB patients. This was also supported by positive correlations between plasma IL-6 level and plasma NO level in MDR-TB patients, plasma IL-6 level and plasma H_2O_2 and NO levels in DS-TB patients.

5.1.5 CORRELATION OF MODULATORS WITH MECHANISMS OF INNATE CELLULAR IMMUNITY

Micronutrients influence immune responses through their interactions with several components of the immune system. This study found positive correlations between plasma Fe level with %NBT and plasma H_2O_2 level; plasma Cu level with %NBT and plasma H_2O_2 level in MDR-TB and DS-TB patients which indicates that Fe and Cu serve as pro-oxidants thereby promoting synthesis of reactive oxygen species. This is supported by positive correlation between plasma SOD activity and plasma Fe level in MDR-TB and DS-TB patients which may be explained by pro-oxidant action of Fe promotes ROS generation thereby leading to increased antioxidant enzyme (SOD) activity as a response by the body to prevent severe oxidative damage. Negative correlation between plasma TP and plasma H_2O_2 levels in MDR-TB, DS-TB patients and controls may be explained by the phenomenon of oxidative protein damage by reactive oxygen species including H_2O_2.

Negative correlation between plasma Zn level and %NBT may be indicative of antioxidant function of Zn in these patients. Negative correlations between plasma vitamin C level and plasma SOD activity in MDR-TB and DS-TB patients supports free radical scavenging activity of vitamin C. This study found negative correlation between plasma vitamin E level and plasma H_2O_2 level in MDR-TB and DS-TB patients which supports antioxidant action of vitamin E in these patients.

%LM correlated negatively with plasma Fe, Cu, Zn and vitamin A levels in MDR-TB and DS-TB patients, which indicates that these micronutrients may play a direct or indirect modulatory role in LMIF production by lymphocytes vis-à-vis retention of leukocytes at the site of infection. On the other hand, positive correlation between %LM and plasma vitamin C level shows that vitamin C promotes leukocyte motility.

Previous studies have demonstrated an association between micronutrients and cytokine production in health and disease (Mbugi *et al.*, 2010; Foster and Samman, 2012; Chen *et al.*, 2018). This study found negative correlations between plasma Fe level and plasma IL-6 level in MDR-TB, DS-TB patients and controls. This may be explained by IL-6 dependent activation of hepcidin transcription through STAT3 phosphorylation which could result in decreased total body iron as previously described (Nemeth *et al.*, 2004). This study also found negative correlations between plasma Cu as well as Zn levels with plasma IL-8 and IL-6 levels in MDR-TB and DS-TB patients. This is contrary to previous studies that demonstrated copper and zinc induced secretion of IL-8 and IL-6 by peripheral blood monocytes and endothelial cells (Scuderi, 1990; Bar-Or *et al.*, 2003). Though ascorbic acid induced down-regulation of IL-6 expression has been previously reported (Kong *et al.*, 2015), in this present study plasma vitamin C correlated positively with plasma IL-6 level in MDR-TB and DS-TB patients. These differences may be due to *M.tuberculosis* infection and may be a means by which *M.tuberculosis* escapes host immune response for continued survival within the host.

5.2 DURING SIX MONTHS OF ANTI-TB CHEMOTHERAPY

5.2.1 MECHANISMS OF INNATE CELLULAR IMMUNITY

Tuberculosis management involves the use of multiple anti-tuberculosis drugs to bring about bacterial clearance. Although the antibiotics used to treat TB are effective against actively dividing *M.tuberculosis,* this bacteria can persist in both dormant and actively dividing states

within the host, making treatment challenging. This leads to prolonged treatment duration and administration of drugs in two phases; the intensive phase (for rapidly growing *M.tuberculosis*) and the continuation phase (for slow growing *M.tuberculosis*). Previous studies demonstrated side effects of single and combinations of anti-tuberculosis drugs on various host immune functions (Tiemessen *et al.*, 2001; Tousif *et al.*, 2014; Chesdachai *et al.*, 2016) but there is dearth of information on the modulatory effect of anti-tuberculosis drug regimen on indices of phagocytosis in DS-TB and MDR-TB patients.

In this present study, %LM increased at 4 months of anti-TB treatment compared to before treatment. This represents decreased LMIF production by lymphocytes thereby affecting the control of leukocyte migration. Increased %LM vis-à-vis decreased production of LMIF observed in this study may be due to Isoniazid induced apoptosis of activated CD4+ T-cells previously reported by Tousif *et al.* (2014). It also supports the reports in previous studies that showed decreased neutrophil chemotaxis in TB patients receiving anti-TB treatment (Antonaci *et al.*, 1991; Rieger *et al.*, 1979).

The NBT dye reduction test is an *in vitro* measure of pathogen phagocytosis and ROI-generating activity of phagocytes (Biller-Takahashi *et al.*, 2013). Isolated leukocytes stimulated with an antigen produce ROIs, which reduce NBT to produce dark formazan deposit. Cells containing dark formazan deposit are involved in active ROI production via phagocytosis. Decreased %NBT at 2 months of treatment indicates a decrease in intracellular oxidative killing and supports decreased neutrophil killing of *Candida* in TB patients receiving anti-TB treatment reported previously (Antonaci *et al.*, 1991). This could indicate a possible increase in vulnerability to opportunistic infections in TB patients on anti-TB treatment.

This study found decreased plasma CAT activity whereas plasma MPO activity and NO level increased at 2 months and 4 months of anti-TB treatment compared to before treatment. Decreased plasma CAT activity and increased plasma NO level observed at 2 months and 4 months of anti-TB treatment may represent a reversal from what was observed in these patients before treatment compared with controls. Standard DS-TB treatment comprises 2 months intensive phase (rapid growing *M.tuberculosis*) and 4 months continuation phase (slow growing *M.tuberculosis*). In many cases, DS-TB patients that adhere to treatment usually achieve sputum smear and culture conversion after 2 months of intensive phase anti-

TB treatment. This reversal of changes observed in DS-TB patients at 2 months and 4 months of anti-TB treatment may be due to bacterial clearance or reduced bacterial load thereby ameliorating the immunomodulatory effects of the bacilli. At 6 months of anti-TB treatment, WBC lysate SOD and MPO activities decreased whereas plasma CAT activities, H_2O_2 and NO levels increased compared with before treatment. Increase in plasma CAT activity may be indicative of immune regulatory action following bacterial clearance or an effect of prolonged use of anti-TB drugs. On the other hand, increased plasma H_2O_2 and NO levels observed at 6 months of anti-TB treatment may represent a restoration of their levels to normal following bacterial clearance and completion of treatment.

Treatment of MDR-TB in Nigeria requires the use of second line drugs with a standardized treatment regimen. Patients undergo intensive phase for 6 – 8 months at designated MDR-TB facilities. In this study, MDR-TB patients were recruited before treatment and followed up during 6months of intensive phase anti-TB treatment. There were significant decreases in plasma IL-6 level at 2 months and 6 months of anti-TB treatment compared with before treatment. This decrease in plasma IL-6 level with anti-TB treatment is in consonance with the report of Djoba *et al.* (2009). *M.tuberculosis* has been reported to exploit IL-6 induction as mechanisms to inhibit IFN-γ as well as for the promotion of immune regulation (Pai *et al.*, 2003; Martinez *et al.*, 2013). Decreases in plasma IL-6 levels observed at 2 months and 4 months of anti-TB treatment in MDR-TB patients may be due to reduced bacterial load thereby leading to a shift from increased plasma IL-6 level observed before treatment compared to controls.

In contrast to IL-6, increased plasma IL-8 level was observed at 4 months of treatment compared to before treatment. Two IL-8 receptors, CXR1 and CXR2 are expressed on neutrophils, which function in the recruitment of leukocytes from circulation to sites of infection upon binding by IL-8 (Holmes *et al.*, 1991; Murphy and Tiffany, 1991). The expression of CXCR1 was reported to decrease with duration of anti-TB treatment (Tiemessen *et al.*, 2001). Increased plasma IL-8 level at 4 months of anti-TB treatment may be consequent to the decrease in CXCR1 receptor expression.

The observed decrease in %LM and increased %NBT at 6 months of anti-TB treatment compared with before treatment may be due to reduced load of active bacilli in these patients, as this present study observed sputum smear conversion from 2 – 4 months of anti-TB

treatment in these patients. Collectively, increase in plasma H_2O_2 levels at 4 and 6 months of anti-TB treatment compared with before treatment, increase in plasma CAT and MPO activities at 2, 4 and 6 months of anti-TB treatment compared with before treatment, increase in plasma NO level at 2 and 4 months of anti-TB treatment compared with before treatment are indicative of reversal of decreases in respiratory burst enzymes activities and oxidant levels observed in MDR-TB patients before treatment compared with controls. While this may be due to reduced bacterial load, it could also be as a result of the nutritional support, particularly in form of micronutrient supplements, given to these patients in the course of intensive phase anti-TB treatment. This shows the benefit of nutritional support in the form of multi-micronutrient supplementation on innate immune response, particularly phagocytosis and oxidative intracellular killing, in TB patients in addition to anti-TB treatment. Although previous studies showed improved outcomes in TB patients placed on anti-TB treatment with micronutrient supplementation (Range *et al.*, 2005; Villamor *et al.*, 2008; Lodha *et al.*, 2014; Campa *et al.*, 2017), none of them examined outcomes in relation to innate immune responses. Observation from this study further buttresses the need for integration of micronutrient supplementation into TB management schemes.

5.2.2 MECHANISMS OF INNATE CELLULAR IMMUNITY WITH ZINC SUPPLEMENTATION

Zinc is a potent mediator of host resistance to infection as it influences the innate and adaptive immune responses in many ways (Shankar and Prasad, 1998; Rink and Gabriel, 2000). The immune system is particularly sensitive to changes in zinc levels (Shankar and Prasad, 1998) and an imbalance in T_h1/T_h2 responses has been shown in zinc deficient individuals with T_h2 cytokines IL-4, IL-6 and IL-10 remaining unchanged while T_h1 cytokines IL-2 and IFN-γ decreased (Prasad, 2000). This shift was however corrected by zinc supplementation (Prasad, 2000). Previous zinc supplementation studies in TB patients have examined various outcomes, reporting no difference from placebo or improved treatment outcome with zinc supplementation (Range *et al.*, 2005; Lawson *et al.*, 2010; Pakasi *et al.*, 2010) but none examined the effect of zinc supplementation on intracellular killing (respiratory burst components and NO).

This present study found increased plasma SOD and MPO activities with decreased CAT activity at 2 months of anti-TB treatment with zinc supplementation compared with before treatment. Zinc and copper are cofactors of the enzyme SOD, hence increased SOD activity

observed may be due to increased availability of the nutrient in patients using zinc supplements. Increased MPO activity ensures increased production of hypochlorous acid, a very potent bactericidal component of the respiratory burst pathway. In combination with decreased activity of the H_2O_2 scavenging enzyme CAT, this is indicative of heightened anti-mycobacterial activity in TB patients on zinc supplements at 2 months of anti-TB treatment. Plasma MPO activity also increased at 4 months and 6 months of anti-TB treatment with zinc supplementation compared to before treatment. Hence, increased MPO activity may also be indicative of increased formation of neutrophil extracellular traps (NETs) as an extracellular measure to control *M.tuberculosis* expansion within the host. Plasma CAT activity decreased at 4 months, whereas plasma SOD activity, NO and H_2O_2 levels decreased at 6months of anti-TB treatment with zinc supplementation compared with before treatment. These decreases are indicative of regulation of immune response following the clearance of active *M.tuberculosis* evidenced by sputum smear conversion at 2 months of anti-TB treatment in these patients.

5.2.3 ANTI-TB TREATMENT OUTCOME MEASURES

Sputum conversion is considered an important interim indicator of the efficacy of anti-tuberculosis treatment, and it is used in combination with other clinical characteristics to determine tuberculosis treatment success (WHO, 2000). This study observed a 65.22% sputum culture conversion rate in MDR-TB patients at 2 months of anti-TB treatment with 26.09% converting at 3 months and 8.70% converting at 4 months of anti-TB treatment. This agrees with the study of Holtz *et al.* (2006) who reported sputum culture conversion in 77% of MDR-TB patients within 60 days of anti-TB treatment. The differences in time to conversion observed in these patients indicate varied responses to anti-TB treatment and may be due to differences in disease severity at onset of treatment or the number and combination of first-line drugs which the patients show resistance to. Furthermore, delay in accessing treatment in patients who failed first-line anti-TB treatment due to limited treatment facilities may also explain the differences in time to sputum conversion observed in these patients. This study observed a 100% sputum smear conversion rate in DS-TB patients at 2 months of anti-TB treatment. This differs from previous studies that reported 82% and 80% sputum smear conversion at 2 months of anti-TB treatment in Fiji (Prasad *et al.*, 2014) and Johannesburg (Budgell *et al.*, 2016) respectively. This difference may be due to exclusion of HIV infected patients from this present study.

Although sputum conversion serves as a hallmark for assessment of anti-TB treatment response, it is however cumbersome and time consuming. Hence there is the need for surrogate measures that are readily accessible and can be carried out easily for monitoring of response to anti-TB treatment. In this study, body weight, BMI, PBF, FMI and FFMI increased in MDR-TB patients who had achieved sputum culture conversion compared with patients who were still sputum culture positive at 2 months of anti-TB treatment. This may be due to nutritional recovery following reduction of bacterial load. Our findings of increased body weight and its associated anthropometric indices supports previous reports of weight gain as a useful predictor of TB treatment outcome (Vasantha *et al.*, 2009; Bernabe-Ortiz *et al.*, 2011; Gler *et al.*, 2013; Chung *et al.*, 2014).

Nutritional recovery in TB patients following reduction or clearance of bacterial load could help reverse impaired immune responses observed before treatment. This study observed increased plasma vitamin D level while plasma vitamin E level decreased in MDR-TB patients who had achieved sputum culture conversion compared with patients who were still sputum culture positive at 2 months of anti-TB treatment. Decreased plasma vitamin E level observed may be due to increased utilization of vitamin E for anti-oxidant scavenging. On the other hand, increased plasma vitamin D observed in this study differs from the report of Naik *et al.* (2017) that vitamin D deficiency worsened in PTB patients after treatment. The difference may be due to nutritional support given to patients in this present study. This shows that nutritional support for TB patients during anti-TB treatment could lead to early nutritional recovery following reduction or clearance of bacterial load.

This study also recorded a case mortality rate of 8.16% among MDR-TB patients during 6 months of anti-TB treatment. The deaths occurred within 8 weeks of anti-TB treatment. Case mortality observed in this study is less than 19% reported by Singla *et al.* (2009) in India and 33% reported by Schaaf and Marais (2011) in the Western Cape. The difference may be due to the period of follow up, as in this present study patients were only followed up during 6 months of intensive phase treatment. It could also be due to the exclusion of HIV infected patients from this present study. On the other hand, a case mortality rate of 1.67% was recorded in DS-TB patients on anti-TB treatment. This is less than 8% mortality previously reported (Budgell *et al.*, 2016). MDR-TB has been previously associated with increased risk of death during treatment when compared with DS-TB (Chung-Delgado *et al.*, 2015; Sun *et al.*, 2015) This could be due to more prolonged treatment duration, high cost of management

and more toxic anti-TB drugs used and their associated side effects. Further explanation can be premised on the delays in accessing treatment for patients who fail first-line anti-tuberculosis treatment prior to when second line treatment become available. At the facility where this study was carried out, patients wait on a queue for varied periods of time as the available spaces for second line treatment are limited. There is therefore, the need to increase access to second line treatment in this region.

CHAPTER SIX
CONCLUSION AND RECOMMENDATION

6.0 CONCLUSION

It can be concluded from this study that:

Under-nutrition remains a major concern in active TB and there is need for nutritional assessment of TB patients at diagnosis as well as nutritional support during anti-TB treatment.

Phagocytic mechanism and its plasma modulators (MPO, NO, H_2O_2) decreased in TB patients at baseline but were increased from 2 months of anti-TB chemotherapy.

Increase in plasma modulators of phagocytic mechanism (SOD, MPO, NO) were enhanced by combination of zinc supplementation with anti-TB chemotherapy.

Zinc supplementation with anti-TB chemotherapy elicits regulation of plasma modulators of phagocytic mechanism upon completion of anti-TB chemotherapy at 6 months.

6.1 CONTRIBUTION TO KNOWLEDGE

Phagocytic mechanism and its plasma modulators decreased in TB patients at baseline but were increased from 2 months of anti-TB chemotherapy.

Improvements in phagocytic mechanisms were notable from 2 months of anti-TB chemotherapy in both DS-TB and MDR-TB.

Increase in plasma modulators of phagocytic mechanism were enhanced by combination of zinc supplementation with anti-TB chemotherapy.

Zinc supplementation with anti-TB chemotherapy elicits regulation of plasma modulators of phagocytic mechanism upon completion of anti-TB chemotherapy at 6 months.

6.2 RECOMMENDATION

Nutritional assessment of newly diagnosed TB patients prior to treatment commencement.

Combination of zinc supplementation with anti-TB drugs

Further studies on the possible host genetic determinants of progression from latent TB to active TB and the spectrum of active disease states namely DS-TB, MDR-TB and XDR-TB.

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APPENDIX I

INSTITUTE FOR ADVANCED MEDICAL RESEARCH AND TRAINING (IAMRAT)
COLLEGE OF MEDICINE, UNIVERSITY OF IBADAN. IBADAN, NIGERIA.
Director: **Prof. A. Ogunniyi,** B.Sc(Hons), MBChB, FMCP, FWACP, FRCP (Edin), FRCP (Lond)
Tel: **08023038583, 08038094173**
E-mail: **aogunniyi@comui.edu.ng**

UI/UCH EC Registration Number: **NHREC/05/01/2008a**

NOTICE OF FULL APPROVAL AFTER FULL COMMITTEE REVIEW

Re: Effectors of Innate Cellular Immunity and Micronutrient Levels in Nigerians with Pulmonary Tuberculosis

UI/UCH Ethics Committee assigned number: UI/EC/13/0340

Name of Principal Investigator: **Victory F. Edem**

Address of Principal Investigator: Department of Chemical Pathology
University College Hospital, Ibadan

Date of receipt of valid application: 03/10/2013

Date of meeting when final determination on ethical approval was made: **20/03/2014**

This is to inform you that the research described in the submitted protocol, the consent forms, and other participant information materials have been reviewed and *given full approval by the UI/UCH Ethics Committee.*

This approval dates from 20/03/2014 to 19/03/2015. If there is delay in starting the research, please inform the UI/UCH Ethics Committee so that the dates of approval can be adjusted accordingly. Note that no participant accrual or activity related to this research may be conducted outside of these dates. *All informed consent forms used in this study must carry the* UI/UCH EC *assigned number and duration of* UI/UCH EC *approval of the study.* It is expected that you submit your annual report as well as an annual request for the project renewal to the UI/UCH EC early in order to obtain renewal of your approval to avoid disruption of your research.

The National Code for Health Research Ethics requires you to comply with all institutional guidelines, rules and regulations and with the tenets of the Code including ensuring that all adverse events are reported promptly to the UI/UCH EC. *No changes are permitted in the research without prior approval by the* UI/UCH EC *except in circumstances outlined in the Code. The* UI/UCH EC *reserves the right to conduct compliance visit to your research site without previous notification.*

UI/UCH EC
20 MAR 2014
APPROVED
COLLEGE OF MEDICINE, U.I.

Professor A. Ogunniyi
Director, IAMRAT
Chairman, UI/UCH Ethics Committee
E-mail: uiuchirc@yahoo.com

APPENDIX II

INSTITUTE FOR ADVANCED MEDICAL RESEARCH AND TRAINING (IAMRAT)
College of Medicine, University of Ibadan, Ibadan, Nigeria.

Director: **Prof. Catherine O. Falade,** *MBBS (Ib), M.Sc, FMCP, FWACP*
Tel: 0803 326 4593, 0802 360 9151
e-mail: cfalade@comui.edu.ng lillyfunke@yahoo.com

UI/UCH EC Registration Number: **NHREC/05/01/2008a**

Notice of Renewal of Approval

Re: Effectors of Innate Cellular Immunity and Micronutrient levels in Nigerians with Pulmonary Tuberculosis

UI/UCH Ethics Committee assigned number: UI/EC/13/0340

Name of Principal Investigator: **Victory F. Edem**

Address of Principal Investigator: Department of Chemical Pathology,
College of Medicine,
University of Ibadan, Ibadan

Date of receipt of valid application for renewal of approval: 27/10/2015

Status: **2nd Approval**

This is to inform you that the UI/UCH Ethics Committee has received your application for renewal of approval on the above titled research. The report indicates that a total of 15 patients with drugs sensitive pulmonary Tuberculosis have been recruited into the study. It also states that participants were followed-up throughout six months of anti-tuberculosis therapy. The report also showed that recruitment of study participants was delayed by industrial actions embarked upon by the resident doctors of the University College Hospital, Ibadan. The report does not indicate any adverse event.

The Committee notes the contents of the report and having found it satisfactory, hereby approves your request for renewal of approval for **One Year of Study Only.**

This renewed approval dates from 30/10/2015 to 29/10/2016. Note that no participant accrued or activity related to this research may be conducted outside of these dates. *All informed consent forms used in this study must carry the UI/UCH Ethics Committee assigned number and duration of UI/UCH EC approval of the study.* It is expected that you submit your annual report as well as an annual request for the project renewal to the UI/UCH EC early in order to obtain renewal of your approval and avoid disruption of your research.

The National Code for Health Research Ethics requires you to comply with all institutional guidelines, rules and regulations and with the tenets of the Code including ensuring that all adverse events are reported promptly to the UI/UCH EC. No changes are permitted in the research without prior approval by the UI/UCH EC except in circumstances outlined in the Code. The UI/UCH EC reserves the right to conduct compliance visit to your research site without previous notification.

UI/UCH EC APPROVED

Professor Catherine O. Falade
Director, IAMRAT
Chairperson, UI/UCH Ethics Committee
E-mail: uiuchec@gmail.com

APPENDIX III

QUESTIONNAIRE (NEWLY DIAGNOSED)

DEPARTMENT OF CHEMICAL PATHOLOGY

FACULTY OF BASIC MEDICAL SCIENCES

UNIVERSITY OF IBADAN

Good day Sir/Ma, this questionnaire is being administered to evaluate the cellular immune responsesto TB infection. Please respond honestly to the question below. The confidentiality of your response is guaranteed.

DATE ..... **I.DNUMBER.....**

INDEX NUMBER..... **PHONE NO.....**

SECTION A (DEMOGRAPHIC CHARACTERISTICS)

1. Age (yrs):.....
2. Sex: Male [ ] Female [ ]
3. State of Origin: .....
4. Place of residence: .....
5. Educational Status: No formal Education [ ] Primary [ ] Secondary [ ] Tertiary [ ]
6. Profession/ Occupation: .....
7. Description of work environment: indoor [ ] outdoor [ ] in a workshop [ ]

SECTION B (ANTHROPOMETRIC MEASUREMENTS)

8. Height.....
9. Upper Arm Circumference.....
10. Weight.....
11. BMI.....
12. Body fat.....
13. Body water.....
14. Waist Circumference.....
15. Hip Circumference.....

SECTION C (DIETARY HISTORY)

16. Vegetables and fruits? Daily [ ] weekly [ ] occasionally [ ]
17. Alcohol history? Yes [ ] No [ ]
18. If yes, Type..... Number of bottles per day.....
19. If stopped, when stopped? .....
20. Smoking history? Yes [ ] No [ ]
21. If yes, Type..... Number of sticks per day.....
22. If stopped, when stopped? .....

1

SECTION C (HEALTH HISTORY)

23. Do you have a history of TB in your family? Yes [ ] No [ ]
24. Do you have any history of friends with TB? Yes [ ] No [ ]
25. When were you diagnosed of TB? .....
26. Did you have BCG vaccination? Yes [ ] No [ ]
    If yes above, which year? .....
27. Any positive history of intravenous drug use? Yes [ ] No [ ]
28. Do you have any other disease apart from TB e.g. Diabetes, Hypertension, Malaria, etc?
    Yes [ ] No [ ]
    If yes state the disease.....
29. Family history of diabetes or hypertension? Yes [ ] No [ ]
    If yes, state relative.....

SECTION D (CLINICAL HISTORY)

Chronic cough > 2 weeks

Yes [ ] No [ ]

Sputum production

Yes [ ] No [ ]

Heamoptysis (coughing out of blood)

Yes [ ] No [ ]

Chest pain

Yes [ ] No [ ]

Night sweats

Yes [ ] No [ ]

Weight loss

Yes [ ] No [ ]

Breathlessness

Yes [ ] No [ ]

QUESTIONNAIRE (2 MONTHS FOLLOW-UP)

DEPARTMENT OF CHEMICAL PATHOLOGY

FACULTY OF BASIC MEDICAL SCIENCES

UNIVERSITY OF IBADAN

Good day Sir/Ma, this questionnaire is being administered to evaluate the cellular immune responses to TB infection. Please respond honestly to the question below. The confidentiality of your response is guaranteed.

DATE ..... **I.DNUMBER**.....

1. Height.....
2. Upper Arm Circumference.....
3. Weight.....
4. BMI.....
5. Body fat.....
6. Body water.....
7. Waist Circumference.....
8. Hip Circumference.....
9. Vegetables and fruits use from onset of TB therapy? Daily [ ] Weekly [ ] Occasionally [ ]
10. Use of supplements from onset of TB therapy? Yes [ ] No [ ]

    If yes above, state type_____

QUESTIONNAIRE (2 MONTHS FOLLOW-UP)

DEPARTMENT OF CHEMICAL PATHOLOGY

FACULTY OF BASIC MEDICAL SCIENCES

UNIVERSITY OF IBADAN

Good day Sir/Ma, this questionnaire is being administered to evaluate the cellular immune responses to TB infection. Please respond honestly to the question below. The confidentiality of your response is guaranteed.

DATE ..... **I.DNUMBER**.....

1. Height.....
2. Upper Arm Circumference.....
3. Weight.....
4. BMI.....
5. Body fat.....
6. Body water.....
7. Waist Circumference.....
8. Hip Circumference.....
9. Vegetables and fruits use from onset of TB therapy? Daily [ ] Weekly [ ] Occasionally [ ]
10. Use of supplements from onset of TB therapy? Yes [ ] No [ ]

    If yes above, state type_____

APPENDIX IV
Reagent preparations
Sputum Digestion-Decontamination
Reagents:

- N-acetyl-L-cysteine powder
- 6% NaOH

6g NaOH in 100ml distilled water

- 2.9% Na citrate

2.9g Na citrate in 100ml distilled water

- Phosphate buffer pH 6.8

7.1g Na_2HPO_4 and 6.8g KH_2PO_4 was dissolved in 200ml dd (distilled, deionized) water and made up to 1.5L

pH was modified to 6.8

Nitroblue tetrazolium dye reduction test (NBT)

Reagents

- 0.1% Nitroblue tetrazolium dye prepared by dissolving 0.1g Nitroblue tetrazolium dye in 100ml of distilled deionized water (ddH_2O)
- Bacterial endotoxin
- 0.3% Wright stain prepared by dissolving 0.3g Wright stain in 100ml of methanol with pH adjusted to 6.8.

Leukocyte Migration Inhibitory Factor (LMIF) test

Reagents

- 6% dextran prepared by dissolving 6g dextran in 100ml normal saline (0.9g NaCl dissolved in 100ml distilled water)
- Ringer solution

8.6g NaCl was dissolved in 200ml of ddH_2O

0.3g KCl was dissolved in 200ml of ddH_2O

0.33g $CaCl_2.2H_2O$ was dissolved in 200ml ddH_2O

The solutions of NaCl, KCl and $CaCl_2.2H_2O$ were mixed and made up to 1000ml with ddH_2O

- BCG vaccine
- Streptomycin

Total protein estimation

Reagents

- 0.2 M sodium hydroxide (NaOH)

  8g of NaOH was dissolved in ddH_2O and the solution made up to 1 litre
- Bovine Serum Albumin (BSA)

  20 mg of BSA was added to 2 ml ddH_2O to prepare 10 mg protein/ml stock solution
- Biuret reagent

  1.5g of $CuSO_4.5H_2O$, 5.5g of Na-K-tartarate and 4.5g of KI were dissolved in little quantity of the prepared 0.2M NaOH and later made up to 500 ml

Hydrogen peroxide (H_2O_2) estimation

Reagents

- 100 µmol/L xylenol orange

  0.0152g of xylenol orange was added to 200 mL of ddH_2O
- 250 µmol/L ammonium ferrous sulphate (AFS)

  0.0196g of ferrous sulphate was added to 200 mL of ddH_2O
- 100 mmol/L Sorbitol

  3.64g of sorbitol was added to 200 mL of ddH_2O
- 25 mmol/L H_2SO_4

  1 mL of 1M H_2SO_4 is made up to 40 mL with ddH_2O
- Phosphate buffer (0.1M, pH 7.4)

  4.96g of K_2HPO_4 and 9.73g of KH_2PO_4 was added to 200mL ddH_2O and the volume made up to 1000 mL. The pH was adjusted to 7.4
- 0.2 M H_2O_2

  11.50 mL of 30% (w/w) H_2O_2 was diluted with ddH_2O and the solution made up to 500 mL

Determination of catalase enzyme activity

Reagents

- 5% Dichromate solution ($K_2Cr_2O_7$)

  5g of $K_2Cr_2O_7$ was dissolved in 80 mL of ddH_2O and made up to 100 mL
- 0.2 M H_2O_2

11.50 mL of 30% (w/w) H_2O_2 was diluted with ddH_2O and the solution made up to 500 mL

- Dichromate/acetic acid solution

  This reagent was prepared by mixing 5% solution of $K_2Cr_2O_7$ with glacial acetic acid (1:3 v/v)

- Phospahte buffer

  3.58g of $Na_2HPO_4.12H_2O$ and 1.19g $NaH_2PO_4.2H_2O$ were dissolved in ddH_2O; the pH was adjusted to 7.0 and made up to 1 litre

- Assay mixture

  2 mL of H_2O_2 was mixed with 2.5 mL of phosphate buffer

Determination of superoxide dismutase (SOD) activity

Reagents

- 0.05 M Carbonate buffer (pH 10.2)

  3.58g of $Na_2CO_3.10H_2O$ and 1.05g of $NaHCO_3$ were dissolved in 200 mL of ddH_2O, the pH was adjusted to 10.2 and then made up to 250 mL with ddH_2O

- 0.3 mM Adrenaline

  0.01g of adrenaline (epinephrine) was dissolved in 200 mL of ddH_2O, It was prepared fresh on the day of the analysis

Determination of myeloperoxidase (MPO) activity

Reagents

- 0.1M potassium phosphate buffer (pH 7.0)

  0.53g of KH_2PO_4 and 1.06g of K_2HPO_4 were dissolved in ddH_2O, pH adjusted to 7.0 and made up to 100 mL

- 0.018 M Guaiacol

  22.3 mg of guaiacol was dissolved in 10 mL of ddH_2O and stored on ice. It was prepared fresh on the day of analysis

- Substrate

  0.1 mL of 30% H_2O_2 was diluted with ddH_2O and made up to 120 mL. The solution was stored on ice in the dark and was prepared fresh on the day of analysis

Nitric oxide (NO) estimation

Reagents

- 0.1% N-1-napthylethylenediamine dihydrochloride (NED)

  0.1g of NED was dissolved in ddH_2O and made up to 100 mL
- 5% Phosphoric acid (PA)

  5 mL of PA was added to 95 mL of ddH_2O
- 1% Sulfanilamide

  1 g of sulfanilamide was dissolved in 5% PA and made up to 100 mL with same
- 0.1M sodium nitrite

  0.85g of sodium nitrite was dissolved in ddH_2O and made up to 100 mL

APPENDIX V
PUBLISHED ARTICLES