Effects of Micronutrient Supplementation on Patients with Drug-Sensitive *Mycobacterium tuberculosis* and Genetic Identification of Multi-Drug-Resistant Strains in Ibadan, Nigeria

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Being a PhD Thesis Submitted to the Department of Biological Sciences
Faculty of Natural & Applied Sciences, Lead City University,
Ibadan, Oyo State, Nigeria

In Partial Fulfilment of the Requirements for the Award of Doctor of Philosophy Degree (PhD) in Molecular Biology and Genomics

2024

Certification

This is to certify that this research work is an original work carried out by Ekundayo Joseph OWOLABI with Matriculation Number LCU/PG/002022 under my supervision, in the Department of Biological Sciences, Faculty of Natural and Applied Sciences, Lead City University, Ibadan, Nigeria and that this has not been previously submitted.

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Dedication

This thesis is dedicated to my Maker and the Almighty God, family, wife, children and my late grandmother Deaconess Serah Owolabi.

Acknowledgement

My greatest gratitude and appreciation goes first and foremost to the Almighty God for his faithfulness throughout this work.

I appreciate the contribution of members of staff of Jericho Chest Hospital, Ibadan, Oniyanrin Health Centre, Ibadan, Alafia Hospital, Ibadan, Sabo Health Centre, Ibadan in the aspect of sputum samples collection. I am thankful to Jericho Chest Hospital Laboratory Staff for allowing me to use the Laboratory for molecular analysis of sputum samples. I also appreciate the scientist in Biorepository Clinical Laboratory, University College Hospital, Ibadan for the phylogenetic analysis of the sample. I am also grateful to Clinalancet Laboratory for the analyses of plasma zinc and selenium and Banby Specialist Hospital Akobo for allowing me space to store my samples and the use of their machines for analyses.

My special thanks go to my supervisor, Prof. Moses Akiibinu who sacrificed his time to go through this work severally, and for his understanding and encouragement during this study.
I specially appreciate the Head of Department, Dr. Felicia Adesina, the entire lecturers and the members of staff of the Biological Department, Lead City University, Ibadan

I also appreciate my parents, wife and children for their love and support from the beginning to the end of this study. The above mentioned person and institutions have assisted in the completion of this thesis, but the responsibility for any errors omissions is however mine alone.

Abstract

Drug-resistant tuberculosis has been implicated in about 13% of all antimicrobial resistance deaths all over the world. Limited studies have been able to report the genomic characterisation of the multi-drug resistant (MDR) strain of *M. tuberculosis* in Nigeria. Undernutrition is a factor implicated as a cause of immune deficiency in most tuberculosis (TB) patients. The effects of micronutrients as adjuvant therapy in the management of tuberculosis have not been well documented in Nigerian TB patients. This study was designed to investigate the possible influence of zinc, selenium and iron supplements on TB patients undergoing intensive anti-TB therapy and also compare the genomic sequence of MDR TB with drug-sensitive *Tubercle bacilli*. One hundred newly diagnosed patients with drug-sensitive (DS) *M. tuberculosis* patients were recruited after the screening of sputum at the molecular level. Ten MDR positive *M. tuberculosis* and twenty-five normal individuals (controls) who were mycobacterium negative screened by Genxpert at Jericho Chest Government Hospital, Ibadan also participated in this study. The 100 drug-sensitive positive *M. tuberculosis* patients were classified into 4; groups 1-3 had anti-TB drugs plus iron, selenium or zinc supplementation, while group 4 had anti-TB drugs only. Baseline and 2 months post-treatment weights were measured, and blood samples were collected. MDR TB and DS sputum samples were used for the culture and genomic characterisation of *M. tuberculosis*. Results showed that baseline weights were significantly ($p<0.05$) lower when compared with the controls. After two months, patients on iron and selenium supplementation showed a significant increase in weight compared to the baseline values. Baseline levels of ferritin, IgG, C3, CRP, and D-dimer were significantly ($p<0.05$) higher, while levels of Hb, iron, selenium, zinc, and albumin were significantly ($p<0.05$) lower compared with controls. However, patients on Fe, Se and Zn supplementation had significantly higher levels after 2 months of treatment. This study identified the *rpoB* gene and *Kat*G gene in MDR strains of *M. tuberculosis*. The phylogenetic analysis showed that some drug-sensitive and drug-resistant positive TB had clusters of both *M. tuberculosis* and *M. bovis*. It could be concluded that *M. bovis* shared similar clusters with *M. tuberculosis* probably due to a gene mutation that could accounts for drug resistance in MDR-TB. It may be necessary to consistently conduct a phylogenetic analysis of samples to identify which samples have a cluster common to MTB and *M. bovis*. This could help in reducing MDR-TB infection and possibly decrease the number of deaths. Consumption of micronutrients seems to have have effects on D-dimer, C-

reactive protein, complement factor-3, iron, ferritin and albumin, possibly by enhancing metabolic activities, reducing inflammation, preventing iron deficiency anaemia, reducing intravascular coagulation, and modulating immune response of patients to infection.

Keywords: DNA sequencing, Immune system, Micronutrients, Multi-drug resistance, Tuberculosis-infection,

Word Count: 429

Table of Contents

List of Acronyms

Abbreviation		Meaning
Alb	-	Albumin
Fe	-	Iron
Se	-	Selenium
Zn	-	Zinc
AFB	-	Acid fast bacilli
PTB	-	Pulmonary *Tuberculosis*
TB	-	*Tuberculosis*
MTB	-	*Mycobacterium tuberculosis*
C_3	-	Complement 3
CRP	-	C-Reactive Protein
IgG	-	Immunoglobin G
Hb	-	Haemoglobin
PT	-	Prothrombin Time
APTT	-	Activated Partial Thromboplastin time
Wbc	-	White blood cell
DNA	-	Deoxyribonucleic acid
PCR	-	Polymerase Chain Reaction
Rif	-	Rifampicin
NAAT	-	Nucleic acid amplification test
rPOB	-	Betasubunit of RiboNucleic Acid Polymerase
RNA	-	Ribo Nucleic Acid
MDR	-	Multi Doug Resistance

List of Tables

List of Figures

Chapter One

Introduction

1.1 Background to the Study

More than 150 years ago, in 1882, a German Scientist made the discovery of *Mycobacterium tuberculosis* (MTB), which paved the way for advancements in tuberculosis diagnosis and treatment. He defined it as a rod-shaped, non-motile, non-spore-forming, gram-positive, catalase-positive bacillus. Its dimensions are 2–10 micrometers in length and 0.2–0.6 micrometers in width[1].

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), is one of the most common causes of sickness and death in humans. It ranks as the second infectious killer globally after COVID-19 and the 13th largest cause of death overall. There are opportunistic infections in this class that have been known to result in serious diseases, especially in clinical settings. MTB is a cylindrical, spore-free aerobic bacteria. The staining ability of the bacterial wall indicates that the cell wall of MTB is composed of extended chains of mycolic acid[2,3]. Because of this unique structure, the wall has very little permeability to the majority of antibiotics. Once a bacterium has located a host, a specific substance called lipoarabinomannan helps it survive immune system attacks[4].

A prolonged cough, shortness of breath, chest pain, hemoptysis, and other disease symptoms such as anorexia, fever, and high temperatures in the evening, followed by night sweats, depression, weight loss, and weakness, are the telltale signs of tuberculosis[5]. Inhalation is the main mode of infection and the lungs offer MTB strains with an optimal environment to flourish[6, 7]. The lungs are typically affected by *Mycobacterium tuberculosis*, yet it can also cause problems in other body organs. Most infections are asymptomatic (latent TB), and ten percent of latent TB

disease progresses to active disease, which, in the absence of treatment, accounts for over fifty percent of deaths. Active tuberculosis manifests as a persistent cough with broken sputum, fever, weight loss, and night sweats[8,9].

Poor hygiene conditions in developing countries contribute to the annual loss of over 1.5 million lives from tuberculosis (TB), which is further exacerbated by poverty, malnutrition, and an inadequate diagnostic system[10]. Every year, approximately 9.5 million new cases of tuberculosis are reported worldwide[11]. Based on global tuberculosis statistics, an estimated 9.5 million new cases of tuberculosis are expected to occur, with approximately one-third of those cases going unreported owing to subpar diagnosis[12,13]. About 22 countries reported 80% of the cases of tuberculosis in 2019, with some cases remaining undiagnosed[14, 15].

Malnutrition (micronutrient deficiency) and immunodeficiency are risk factors. In addition to malnourishment, other lifestyle factors that are associated with tuberculosis include drugs, alcohol and tobacco use. Micronutrients like iron (Fe), copper (Cu), and zinc (Zn) are important players in MTB in a variety of biological processes. The development and defense of human tissues and organs depend on these components[16]. These elements are also believed to play a significant role in a number of infectious diseases. A few transition elements aid in the immune system's suppression of pathogens such as *Mycobacterium tuberculosis*, according to several studies. Individuals with long-term illnesses such as cardiovascular disease, TB, are susceptible to developing immunodeficiency syndrome and micronutrient deficiencies[17,18]. The sufficiency of micronutrients in the body affects how well the immune system responds to infection. A lack of certain micronutrients, including zinc, selenium, copper, iron, vitamin A, B6, and C, may increase a person's vulnerability to infectious diseases like MTB. Every immune system component needs the appropriate quantity of micronutrients in order to operate correctly. Skin

and mucous membranes are examples of physical barriers that mainly depend on micronutrients to maintain their structural and functional integrity. It is crucial to note that innate cell migration and the activity of antimicrobial proteins are influenced by micronutrients. Numerous vitamins and minerals are also crucial for the phagocytic and lethal functions of neutrophils and macrophages[19]. Deficiencies in vitamins and minerals can impact many aspects of the adaptive immune system, such as the humoral response (antibody-mediated) and cell-mediated immunity.[20]

A variety of infection outcomes, such as clearance, dormant TB, and active disease, are caused by the intricate evolution and relationship between MTB and the human immune system. There are many different cell types involved, such as recruited immune cells and local pulmonary cells, all of which show variability and plasticity in their capacity to eradicate or confine infection[10]. One of MTB's most sophisticated tactics is its ability to selectively infect the alveolar macrophage (AM), which is found in the lung and acts as a reservoir and effector for the Tubercle bacilli.

It is common knowledge that eating a diet low in nutrients increases a person's susceptibility to illness and its severity. The risk of malnutrition rises when anorexia is induced due to decreased nutrient intake, the emergence of malabsorption, or an increase in the nutritional needs of the body's metabolism. Thus, it's essential to keep each micronutrient at its ideal level. To ensure that one consumes all the vitamins and minerals required for good health, a well-balanced diet is essential. The recommended daily allowance (RDA) for micronutrients may be difficult for the general population and developed country populations to meet due to decreased intake, higher metabolic needs, and increased loss. Thus, research on the role of micronutrient supplementation

in the treatment of MTB patients has been based on this emphasis on the significance of micronutrients[21, 22].

As soon as an individual breathes in *Mycobaterium tuberculosis*, an attempt is made to mount a sufficient innate immune response to eliminate the bacteria (referred to as "early clearance"). Only 5–10% of infected immunocompromised individuals develop tuberculosis disease. These figures indicate that the majority of immunocompetent individuals exposed to tuberculosis generate a strong and sufficient immune response to clear the infection asymptomatically. This supports the theory that tuberculosis disease is caused by a compromised immune response, which can be therapeutically corrected with host-directed therapies (HDT) [23].

Mutations or parallel gene transfer facilitated by phages, plasmids, or transposon elements are two possible mechanisms by which bacteria acquire resistance to antibiotics. There is no evidence of horizontal drug resistance gene transfer in *Mycobacterium tuberculosis*; instead, resistance primarily results from chromosomal mutations brought on by antibiotic use-induced selection.[24] *Mycobacterium tuberculosis*'s development of drug resistance embodied the essence of Darwin's theory of evolution. Because antibiotic resistance gives emerging mutants under selective pressure a survival advantage, it becomes a dominant trait in populations of *Mycobacterium tuberculosis*. Long-term drug regimens, continuous exposure to drugs, combined with patients' non-adherence, and evolution have been forced to favor resistant mutants, which would not have otherwise become dominant in the population due to their lower fitness. *Mycobacterium tuberculosis* strains have been steadily evolving as a result of this process, which takes place during combination therapies, and they are now gradually becoming resistant to all current medications. Sublethal exposure to bactericidal antibiotics may have facilitated radical-induced mutagenesis in addition to survival and selection, which would have aided in the

development of multidrug resistance phenotypes in pathogenic bacteria, such as *Mycobacterium tuberculosis*. Prodrugs, like isoniazid and ethionamide, which are anti-tuberculosis medications, need to be activated by redox enzymes in the cytoplasm of mycobacteria in order to become cytotoxic. Reactive oxygen and radicals produced during the prodrug activation process have mycobactericidal effects. Reactive oxygen and radicals can be harmful if they don't destroy the mycobacterial cell because they can cause cellular mutagenesis and the emergence of drug resistance mutations[24, 25].

In light of the BCG vaccine's failure to reduce prevalence and mortality in adults, the last resort is chemotherapy using strong anti-tuberculosis drugs. Unfortunately, the emergence of drug-resistant tuberculosis, including extensively drug-resistant (XDR), totally drug-resistant (TDR), and multi-drug-resistant (MDR) tuberculosis, has complicated chemotherapy. When treating MDR-TB in particular, aminoglycosides are the second-line medication of choice after fluoroquinolones. The only available options for treating MDR-TB are aminoglycosides and fluoroquinolones, albeit they have some side effects and are less effective than first-line medications. Recently approved medications (bedaquiline and delamanid) have been used to treat MDR, XDR, and TDR tuberculosis when conventional treatments are not effective (with more side effects compared to first and second line drugs)[26].

The functionalities of immune cells have been associated with availability of micronutrients. Effects of micronutrients occus at various metabolic sites, such as the immune system's regulation and, more crucially, during the erythropoiesis process, which produces hemoglobin. Control over iron metabolism takes place both at the cellular level, where it ensures optimal biological functions, and at the level of the body, where it keeps the iron stock and distribution at appropriate levels[27]. Anemia is the last stage of many metabolic disorders that can be brought on

by an iron deficiency. On the other hand, an iron excess may raise the risk of active tuberculosis by three to five times[28]. A disorder of iron metabolism has been linked in some studies to patients who have active tuberculosis[18]. According to some research, the chronic inflammation brought on by the bacillus *Mycobacterium tuberculosis* may be the cause of the anemia linked to tuberculosis. Furthermore, hemolyticanemia may be caused by certain anti-tuberculosis medications[29].

Because iron is necessary for biological cells to properly metabolize their materials, it plays a crucial role in life's chemistry. The assimilation, storage, and utilization of iron are crucial processes that affect not only pathogenesis and pathobiology (growth, survival, virulence, and latency) but also the emergence of drug-resistant strains of *Mycobacterium tuberculosis* that are resistant to aminoglycosides[30].

All aerobic bacteria, with the exception of *lactobacilli* and *Borrelia burgdorferi*, require iron as a micronutrient. Because it transitions between the Fe^{3+} and Fe^{2+} states, it plays a crucial and important role in electron transport and other essential biological processes. It also participates in oxidoreduction reactions. Due to a process known as nutritional immunity, the mammalian host restricts the amount of free iron available to *Mycobacterium tuberculosis*, posing an additional iron limitation.

The presence of lactoferrin in extracellular fluids, polymorphonuclear leukocytes, and transferrin in the circulating plasma all contribute significantly to decreasing the amount of iron that the pathogen can access because of their strong affinity for Fe^{3+}. Iron storage proteins play a role in preserving iron homeostasis in *Mycobacterium tuberculosis*[31]. In *Mycobacterium tuberculosis*, bacterioferritin (Rv1876) and protein (Rv3841) are unique in that they manage iron storage in a manner akin to maintaining homeostasis. Mycobacteria are known to require a number of genes,

products, and interaction partners for high affinity iron acquisition. These include the production of siderophores, the uptake of ferric-siderophores, the production of iron storage proteins, and the uptake of heme. A regulatory protein called IdeR regulates the synthesis, storage, and function of iron uptake mechanisms. For *Mycobacterium tuberculosis*, heme (Heme) is the preferred iron source, and ferrochelatase (Rv1485) is the biosynthetic enzyme that completes its acquisition. The final stage of heme biosynthesis, where iron is interleaved to protoporfhyrin IX to create protoheame, is catalyzed by Rv1485. It is significant because it supplies heme, which may be *Mycobacterium tuberculosis*'s preferred iron source. It also acts as a cofactor for a number of metabolic enzymes, including catalase-peroxidase and the DosS/DosT two component system, whose active sites contain heme[31, 32].

High affinity systems are typically crucial for the maintenance of infection, virulence, and resistance in pathogenic microorganisms. Our understanding of mycobacterial iron acquisition and the interaction between elements of the iron systems will be improved by more precise definitions of the roles of both known genes and their products, ferritin (bfrB) and bacterioferritin (bfrA). These elements have accumulated intensities below iron-rich conditions and reduced intensities below iron-deprived conditions[32].

Mycobacterium tuberculosis suppresses iron acquisition and induces iron storage proteins in iron-rich environments, indicating the multiple roles that iron storage proteins play in iron homeostasis. Ferritin (bfrB) and bacterioferritin (bfrA) are the two iron storage proteins that *Mycobacterium tuberculosis* normally synthesises. While bfrA is unnecessary for successful adaptation to those stresses, bfrB is required to overcome iron limitation and defense against oxidative stress. Research on *Mycobacterium tuberculosis* lacking the bfrB gene, which codes for the iron storage protein ferritin AN, revealed an intracellular iron accumulation. They

demonstrated how resistance too many anti-TB medications, such as aminoglycosides and fluoroquinolones, was decreased by increasing iron concentration (absence of bacterioferritin and ferritin). They also proposed that ferritin and bacterioferritin may not only be required to maintain iron homeostasis but also to make *Mycobacterium tuberculosis* resistant to aminoglycosides. A study argued that by relieving Lsr2 repression in *Mycobacterium tuberculosis*, the iron-dependent regulator IdeR induces ferritin (bfrB) expression. Fe-S cluster-bound WhiB7 (Rv3197A) is a transcriptional regulatory protein that is predicted to be in the IdeR|Rv2711 regulon in addition to its association with resistance to aminoglycosides. According to recent research by study population machinery targets iron storage proteins to preserve iron homeostasis[33].

Previous expression proteomics studies found that clinical isolates of *Mycobacterium tuberculosis* resistant to aminoglycosides (kanamycin and amikacin) had overexpressed levels of bacterioferritin (Rv1876) and ferritin (Rv3841). According to molecular docking, the aminoglycoside medications AK and KM bind to the preserved ferritin and bacterioferritin domains of Rv1876 and Rv3841, respectively. Overexpressing these proteins may neutralize or modulate the effects of the drug and may play a role in the *Mycobacterium tuberculosis* resistance mechanisms to aminoglycosides. Despite the widespread recognition of the enzymes and associated pathways involved in iron metabolism in *Mycobacterium tuberculosis*, our knowledge of iron-dependent post-transcriptional and translational regulation, trafficking, iron transportation, and protein-protein interactions in mycobacteria remains insufficient. Recently, a study reported that recombinant ferritin's inducible overexpression in E. coli (BL21) causes a shift in the minimum inhibitory concentration (MIC) of AK and suggested that this may contribute to aminoglycoside resistance. As a result, protein-protein interactions in mycobacteria

are insufficient, and proteins involved in iron storage, assimilation, regulation, uptake, and their utilization can be a promising anti-mycobacterial target against the drug-resistant tuberculosis[34].

Similar to other mycobacteria, *Mycobacterium tuberculosis* generates siderophores, which are Fe^{3+}-specific high-affinity low-molecular-mass (1,000 Da) compounds used to chelate the metal ion from insoluble and protein-bound iron. Current developments in the transcriptional regulation of siderophore synthesis include the function of the iron-regulated histone-like protein HupB, updated models of desferri- and ferrisiderophore transport, and the significance of the iron storage protein BfrBar's role in storing excess iron. The low serum iron status and the expression of the iron-regulated protein HupB in tuberculosis patients are clinically significant, as they indicate the iron-limiting conditions that *Mycobacterium tuberculosis* faces. *Mycobacterium tuberculosis* needs iron to grow normally inside macrophages, where iron concentrations are low and range from 1 to 10ng ml. This is sustained despite the high metal ion flux within the macrophages as a result of erythrocyte destruction and iron internalization through particular cell surface receptors for hemoglobin-haptoglobin, lactoferrin, and transferrin. But the majority of the iron is moved to the bone marrow, where transferrin and lactoferrin bind any iron that is free. Due to its capacity to retain iron even in acidic pH environments, lactoferrin is crucial in preventing *Mycobacterium tuberculosis*, which is present in alveolar macrophages of pulmonary tuberculosis patients[35].

The *Mycobacterium tuberculosis*, which is found in the phagosome of macrophages and, by an unknown mechanism, inhibits the fusion of lysosomes and phagosomes, highlights the significance of iron acquisition in patient alveolar macrophages. The tuberculosis-containing *Mycobacterium tuberculosis* phagosome interacts with early endosomes, exhibits early endosomal traits, and does not acidify below pH 6.3–6.5. Because *Mycobacterium tuberculosis*

considers the phagosome to be a low-iron environment, iron-acquisition strategies are crucial when dealing with the host's iron-withholding behavior. At least 35 *Mycobacterium tuberculosis* genes are induced by iron limitation, along with mbtA–J, which encode enzymes involved in siderophore biosynthesis. BfrA, on the other hand, is repressed, and encodes a bacterioferritin that stores iron. The concept of iron restriction during immune control of *Mycobacterium tuberculosis* replication is supported by the observation that several weeks after infection, mbtB mRNA levels increase and bfrB mRNA levels decrease[36].

Undernutrition raises the risk of tuberculosis and vice versa. Undernutrition and tuberculosis can have a particularly harmful impact on young children and expectant mothers. Pre-eclampsia and other pregnancy disorders may be more complications in women with tuberculosis. TB also raises the risk of low birth weight, premature delivery, and neonatal mortality. Some patients have been reported to have low circulating concentrations of micronutrients, including iron, zinc, and selenium, as well as vitamins A, E, and D. However, levels typically return to normal following two months of appropriate TB treatment[37].

According to the United Nations Multiple Micronutrient Preparation, all pregnant and lactating women with active tuberculosis should receive multiple micronutrient supplements that contain iron and folic acid along with other vitamins and minerals (UNICEF, WHO, UNU report) to complement their maternal micronutrient needs[37]. In settings where calcium intake is low, calcium supplementation as part of antenatal care is recommended in pregnant women with active TB for the prevention of pre-eclampsia, particularly among those pregnant women at higher risk of developing hypertension[38].

1.2 Statement of the Problem

The bacteria that causes tuberculosis (TB) is called *Mycobacterium tuberculosis* (MTB). A major global health issue, tuberculosis has existed since the Bronze Age (c. 3000–5000 BCE). TB and nutrition are closely linked in a number of ways. Undernourished people are more prone to develop tuberculosis. The pathogenicity of tuberculosis can result in anorexia and undernutrition due to increased metabolic demands. Dietary deficiencies may cause the immune system to become suppressed, which can cause the illness to worsen or take longer to cure. A healthy diet is the cornerstone of the World Health Organization's recommendations for tuberculosis patients' nutrition and support. Although, the pathophysiology of tuberculosis is not well-established, previous studies have demonstrated the critical role vitamin A plays in the immune system's reaction to infection. Based on studies done on humans and animals, Mellanby and Green first proposed vitamin A as a "anti-infective" agent in the 1920s. Additionally, it has been demonstrated that selenium is a crucial micronutrient that enhances immunity and well-being when a person is ill. Additionally significant as an antioxidant, selenium is required for the activity of the immune system-supporting enzyme glutathione peroxidase (GPx). This aids in the body's removal of mycobacterium. Patients with MTB produce reactive oxygen species (ROS) quickly, and because of GPx dysfunction, their lipid peroxidation value is elevated. Zinc has also been connected to many other bodily functions, such as apoptosis, DNA replication and damage repair, oxidative stress responses, immune responses, and more.

Researchers are becoming more interested in the role that micronutrients play in the relationship between diet and tuberculosis. Certain studies assert that deficiencies in different micronutrients cause immunologic issues in people that result in specific illnesses. Research suggests that vitamin supplementation during the course of a disease may improve the prognosis.

1.3 Justification of the Study

Despite effective tuberculosis chemotherapy, the disease still remains a global menace. Understanding the factors that influence disease severity is therefore very crucial. Morbidity and mortality of tuberculosis infections are still high particularly in patients with malnutrition. More so, treatment outcome depends on a well-functioning cell-mediated immune system. The genetic characterization in MDR-TB could aid the understanding of the genetic mutations that are associated with MDR-TB which can be used to develop diagnostic tests. Also, genetic characterization of MDR-TB could help to track the spread, and to identify areas where MDR-TB is most prevalent.

1.4 Aim and Objectives of the Study

To determine the effect of micronutrient supplementation on patients with drug sensitive *M. tuberculosis*, and to genetically identify strain of multi-drug resistant *M. tuberculosis*.

The specific objectives of the study were to:

i. Determine the influence, effectiveness of iron, selenium and zinc supplementations in the treatment of patients with pulmonary *tuberculosis* (PTB) infection.

ii. Compare if treatment with anti-*tuberculosis* drugs with micronutrients as adjuvant have any potential benefit to treatment with anti-*tuberculosis* drugs only.

iii. Assess the effect of PTB infection on some markers of coagulopathy, and if iron supplementation could prevent iron deficiency anaemia (IDA)

iv. Evaluate the use of body weight as an index of nutrition in relationship between *tuberculosis* and nutritional status and if micronutrients could improve weight gain in TB infection.'

v. Determine the genetic variation in MDR and DS-TB.

1.5 Significance of the Study

Tuberculosis is an infectious disease, one of the top ten causes of death worldwide, killing almost two million people each year. Malnutrition can cause secondary immunodeficiency, increase susceptibility to *M. tuberculosis* infection and also cause metabolic disorders leading to wasting disease. The genetic characterization of MDR-TB could help to identify the genetic mutations in MDR-TB, which can be targeted in the development of drugs for combating TB infection. Therefore, this study could reveal the status of Fe, Se and Zn and suggest the ameliorating effects of the elements in the management of tuberculosis.

1.6 Scope of the Study

This study focused on assessing the potential benefit of iron, selenium and zinc supplementation as adjuvants in the treatment of patients with pulmonary *tuberculosis* infection. The study included genetic characterization of MDR and DS Tubercle bacilli. Also to compare the weights of patients with active pulmonary *tuberculosis* infection with the apparently healthy control.

1.7 Limitation of the Study

This study have some limitations;

- Non-availability of some reagents in Nigeria

  (i) Costs of material/equipment

  (ii) Team members cost.

  (iii) Cost of foreign exchange

  (iv) Non-compliance of some patients

  (v) Ethical issues that prevented the treatment of MDR-TB patients with micronutrients.

1.8 Operational Definition of Terms

PCR: means polymerase chain reaction, it is a test to detect genetic material from a specific organism, such as bacteria to diagnose infections, certain diseases and genetic changes. It works by finding the DNA or RNA of a pathogen.

DNA: it is the genetic material that contains instructions and information for all living things.

RNA: it is another type of genetic material. It contains information that has been copied from DNA and is involved in making proteins.

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Chapter Two

Literature Review

2.1 History of *Mycobacterium tuberculosis*

Mycobacterium tuberculosis belongs to the mycobacteriaceae family in the order Actinomycetales. *Mycobacterium tuberculosis* (MTB) is the infectious agent that causes tuberculosis (TB), a communicable disease. When stained with ziehl-Neelsen stain, the *Mycobacterium tuberculosis*—an obligate aerobe that is non-motile and non-sporeforming—appear small and rod-shaped. It is unique among all mycobacteria in that it can combine with arylmethane dyes (carbolfuchsin, auramine, and rhodamine) to form stable mycolate complexes. *Mycobacterium tuberculosis* is airborne in 98% of cases, when a person suffering from pulmonary disease coughs[1]. *Mycobacterium tuberculosis* bacilli enter the alveoli through inhaled droplet nuclei and are subsequently consumed by alveolar macrophages. When granuloma forms in an individual with sufficient immunity, the bacilli become latent. In some, *Mycobacterium tuberculosis* resists the alveolar macrophages' attempts to destroy it and instead spends weeks replicating inside the macrophages[2].

On the other hand, tuberculosis infection can develop into tuberculosis disease in certain people. The bacteria can spread hematogenously to other locations, such as the lung apices, vertebrae, peritoneum, meninges, liver, spleen, lymph nodes, and genitourinary tract, as they grow. They are often transported into regional lymph nodes by alveolar macrophages. At this point, the majority of patients are asymptomatic and often lack radiologic evidence of the disease. However, at this point, they develop cell-mediated immunity and test positive for tuberculosis (TB) infection using interferon gamma (IFN-γ) release assays (IGRAs) and the tuberculin skin

test. Majority of people experience this as the end of the pathogenesis; they continue to be asymptomatic and are diagnosed as having tuberculosis infection[3].

Healthy adults infected with *Mycobacterium tuberculosis* have a 5 to 100 percent probability of developing TB disease within their lifetime, and the majority of those who do so develop disease within the first 1 to 2 years after infection. Adults and children are more susceptible to infection to disease progression when they have conditions or treatments that weaken cell-mediated immunity, such as HIV infection, diabetes mellitus, low nutritional status, or tumor-necrosis factor alpha inhibitors[4].

The organisms typically move from the initial lung focus in young children to the mediastinal and regional hilar lymph nodes, which then swell in cases of severe inflammation. The so-called "collapse-consolidation" lung lesion is often caused by a distal atelectasis or parenchymal infection, which is often the result of the lymph nodes compressing or eroding into the bronchi. Nonetheless, intra-thoracic lymphadenopathy, with or without subsequent parenchymal disease, is the hallmark of childhood tuberculosis. Because there are typically few tubercle bacilli involved in this process, childhood TB is frequently referred to as paucibacillary. Since the organism is rarely found directly in bodily fluids or tissues, less than 40% of childhood TB cases in most case series can be microbiologically confirmed[4]. The remaining 60% of cases are diagnosed based on signs and symptoms, radiography, infection tests, and epidemiology, all of which are used in the knowledge that the child has recently been in contact with a case of communicable tuberculosis. However, cavitary lung lesions or in-depth infiltrates, which are indicative of adult-type disease and contain a large number of organisms detectable by multiple methods, are typically present in adolescents with pulmonary disease[5].

One of the many manifestations of tuberculosis is when it targets the bone, resulting in skeletal abnormalities. People with bone TB who passed away more than 4,000 years ago can almost certainly be identified because hard tissues like bone can be preserved for thousands of years. The prevalence of tubercular deformities in ancient Egyptian skeletons indicates that the disease was widespread in that society. Similar malformed bones have been found in Neolithic sites in Denmark, Italy, and the Middle East, suggesting that tuberculosis was present throughout the world as long as 4,000 years ago. *Mycobacterium tuberculosis*, the causative agent of tuberculosis, is thought to have originated in soil, where certain species of actimomycetes evolved to measure themselves. This theory is based on extensive research conducted recently on the genus *Mycobacterium* and other actimomycetes. A *Mycobacterium* pathogen from domesticated livestock to humans would have been able to spread due to the domestication of cattle, which is estimated to have happened between 10,000 and 25,000 years ago. As a result of the bacterium's adaptation to a new host, it would have developed into the closely related *Mycobacterium tuberculosis*. It has been specifically proposed that *Mycobacterium bovis*, which in cattle induces a disease resembling tuberculosis, was the putative evolutionary progenitor of *Mycobacterium tuberculosis*[6]. In light of new information, this hypothesis is now regarded as questionable because it was developed prior to the genomes of the complex *Mycobacterium tuberculosis* strains, which include the animal and human pathogens *Mycobacterium canetti*, *Mycobacterium microti*, and *Mycobacterium africanum,* as well as the *Mycobacterium tuberculosis* strain and *Mycobacterium bovis*, being characterized by DNA sequencing and related techniques. More than 99.9% of the DNA sequences of the members of the *Mycobacterium tuberculosis* complicated have been found to be similar in studies. Nevertheless, it is possible to distinguish between these closely related bacteria thanks to the rare synonymous

single-nucleotide polymorphisms (sSNPs)[7]. According to sSNP analyses, *Mycobacterium bovis* evolved at the same strong evidence for the independent evolution of both *Mycobacterium tuberculosis* and *Mycobacterium bovis* from another precursor species, possibly related to *Mycobacterium canetti*, has been found through time as *Mycobacterium tuberculosis* and a study of the distribution of deletions and insertions in the genomes of the *Mycobacterium tuberculosis* complicated[8].

Hippocrates (5th century B.C.) described patients with consumption (the Greek term is phthisis), i.e., wasting away associated with chest pain and coughing, often with blood in the sputum. Assyrian clay tablets describe patients coughing blood in the seventh century B.C. The frequency of reports of patients exhibiting symptoms similar to tuberculosis by this point suggests that the illness was well established. It's believed that the migration of Indo-European cattle herders, who were infected with tubercle bacillus through exposure to infected cattle, may have brought TB to these areas. It has also been suggested that during Indo-European migrations into Europe and Asia, numerous human phenotypic traits, such as lactose tolerance, that are linked to cattle rearing and selection for the capacity to use milk, as well as the consequent exposure to *Mycobacterium tuberculosis*, spread the disease[9].

Europe became the epicenter for multiple tuberculosis epidemics starting in the sixteenth and seventeenth centuries due to its rapid population growth in the second millennium A.D. and the development of massive urban centers. During the first part of the 1800s, TB reached its peak in Europe, killing an estimated 25% of the continent's population. During this time, a study conducted in a hospital in Paris revealed that this disease had claimed the lives of 250 out of 696 cadavers that were examined[10]. The best-known example of improved housing and sanitation in the latter half of the 19th century is the urban renewal of Paris in the 1850s, which was started

and overseen by Baron Georges Haussmann. Naturally, political as well as public health concerns drove this enormous project, as Louis Napoleon's troops were better able to control the increasingly radicalized working class along the wide, straight boulevards of the rebuilt Right Bank[11]. The 19th-century decline in tuberculosis incidence has also been attributed to natural selection favoring individuals who are resistant to the disease, although the rate of decline has been too quick for these changes to account for[12].

TB death rates in major cities like Boston and New York were 6 to 7 per 1,000 people in 1800, and they dropped to 4 per 1,000 people in 1860 to 1870. Despite the fact that the disease's mortality rate never approached that of Europe, European immigrants had brought it to the New World. These decreasing death rates were probably also influenced by public health initiatives[13].

Throughout the 20th century, the developed world saw a steady decline in the morbidity and mortality rates of tuberculosis (TB) due to improved public health practices, widespread use of the *Mycobacterium bovis* BCG vaccine, and the development of antibiotics in the 1950s. In the middle of the 1980s, the declining trend came to an end, and the number of new cases began to rise. The rise in AIDS and its ability to destroy the cell-mediated immune response in co-infected individuals were the main causes of this, along with rising rates of homelessness and poverty in the developed world. This "miniepidemic" of new TB cases in Europe and the US has only been stopped by massive financial and human resource investments, primarily through closely monitored antibiotic delivery[14].

Nonetheless, the following data indicates that tuberculosis is still a problem in the developing world. Less than 10 cases of tuberculosis (TB) are reported in North America, 100–300 cases in Asia and Western Russia, and more than 300 cases in Southern and Central Africa. Eight million people contract TB annually, and one person dies from the disease every fifteen seconds, or more

than two million annually. Up to 60% of those who have the illness will pass away if they don't get therapy[15]. Most of these cases are found in the Third World, which is indicative of the region's poverty, lack of hygienic living conditions, and inadequate access to healthcare[16,17]. The emergence of multidrug resistance in nations such as South Africa, India, and the former Soviet Union, where antibiotics are available but of lower quality or are not used long enough to control the disease in accordance with recommended regimens, exacerbates this global crisis[18].

Over the ages, medical professionals and researchers have documented tuberculosis (TB) in all of its manifestations and worked to comprehend the disease's genesis in order to improve diagnosis, treatment, and prophylaxis. Hippocrates believed that the illness was mostly hereditary, but Aristotle (4th century B.C.) and Galen (2nd century A.D.), two of the greatest Roman physicians, emphasized the illness's contagious nature. This opposing theory of the disease's origins reappeared in the second half of the 17th century when Italian physicians continued to believe that tuberculosis was communicable, carrying on Galen's ideas and influencing nations around the Mediterranean basin. On the other hand, medical professionals and experts in the North advocated for constitutional or inherited reasons for this illness. It was thought that the Southern theory of contagion was not thoroughly established scientifically and did not explain why some people in urban settings did not get TB even in areas where the disease was highly prevalent[19]. This belief reflected the empiricism of medical authorities of the time, such as Paracelsus of Switzerland. The 19th century saw the height of this philosophical divide, which is best summarized as the well-known nature versus nurture debate. A French military physician named Jean-Antoine Villemin claimed in 1865 that he had successfully infected laboratory rabbits with tuberculous tissue from a deceased person. The French medical establishment, particularly Herman Pidoux, attacked this report right away. He vehemently

argued that more "modern" and social solutions were needed to address the TB epidemic, which he and others believed was caused by outside factors such as malnourishment, inadequate sanitation, and overindulgence. Many of Pidoux's claims were refuted by the report written by Robert Koch 17 years later, which unequivocally demonstrated that tuberculosis was, in fact, caused by a bacterium[20]. Though the revolutionary syndicalist movement in France used tuberculosis (TB) as an example of a disease brought on by malnourishment and overwork in their fight for an eight-hour workday, belief in the societal causes of TB persisted into the early 20th century. Proponents of this viewpoint attempted, in the present, to refute Koch's definitive experiments by citing arguments akin to those of 17th-century Northern European physicians as well as Pidoux and associates[21].

The apparent contradiction in explaining the etiology of tuberculosis was resolved, beginning with the research of Edward Trudeau in the late 19th and early 20th centuries. He demonstrated that TB could be induced in rabbits using a purified culture of virulent M. tuberculosis in a well-known experiment that, by today's standards, might be regarded as statistically limited. However, the conditions in which the animals were kept had a significant impact on how the disease progressed[22]. In the experiment, five rabbits with *Mycobacterium tuberculosis* were housed in a small, dimly lit cage that was overcrowded and fed little. Of these, one developed a serious case of the illness and four passed away from TB within a three-month period. One rabbit died within a month of infection when five similarly infected animals were allowed to live outside on a small island with extra food, but the other four survived after six months without showing any symptoms of the illness. The five tidy rabbits in the management series, who were kept in a dim, packed cage with little food and no light, became malnourished and obviously unhappy, but they did not contract TB[23]. The treatment of tuberculosis (TB) with fresh air and plenty of food,

which was the cornerstone of the TB sanitarium movement started by European physicians in the mid-1800s and which Trudeau also employed in his Saranac Lake TB treatment center that opened in 1884, was given scientific validity by this straightforward experiment. An interesting and educational review describes the history of TB research and treatment at the Trudeau Institute.[24]

So, although a bacterium causes tuberculosis (TB), environmental factors also have a significant impact—a point that Rene Dubos succinctly reiterated half a century ago[25]. According to Dubos, treating TB with only medicinal measures would not be sufficient. Regretfully, the occurrences during the latter part of the 20th century have demonstrated his prophetic abilities. The 1940s saw the discovery of streptomycin by Schatz and Waksman, which marked the beginning of the antibiotic era. The introduction of different antibiotics such as isoniazid, rifampin, and pyrazinamide after its use to treat tuberculosis has not resulted in the disease's eradication[26]. Similarly, there has been no decrease in the incidence of tuberculosis (TB) in recent years, despite the widespread use of BCG, an attenuated vaccine strain created by successive passage of a virulent *Mycobacterium bovis* strain by Calmette and Guerin in Paris in the 1920s[27]. It is evident that the fight against tuberculosis (TB) requires new medications and vaccines. The strategies covered in this review are intended to aid in this search. But it's always important to keep in mind Dubos' admonitions, which emphasized the social aspect of TB[28].

2.1.1 Immunology of TB

A significant portion of the human population's lives and health are directly impacted by the understanding of immunity to *Mycobacterium tuberculosis*, making it an important scientific challenge. Even with the availability of several antituberculosis medications for more than 50 years, tuberculosis (TB) continues to be a leading cause of morbidity and mortality, taking the

lives of over 1.5 million annually—nearly twice as many as HIV infection (refer to the World Health Organization's TB data). Understanding immunity to *Mycobacterium tuberculosis* is scientifically challenging due to the fact that most humans and experimental animals develop seemingly appropriate immune responses following infection, but these responses are not consistently effective in eliminating the bacteria. Rather, these reactions lead *M. tuberculosis* to enter a latent, clinically silent infection state that allows the bacteria to reactivate. The limits of immunity to *M. tuberculosis* and the mechanisms by which the bacteria impose these limitations are poorly understood, despite the fact that studies in humans and experimental animals have revealed general outlines of the mechanisms of protective immunity[29].

Practically speaking, the lack of knowledge about immunity to *Mycobacterium tuberculosis* hinders the development of TB vaccines that are more effective than *Mycobacterium bovis* bacillus Calmette–Guérin (BCG), which was created in the early 1900s and has been given to more than 3.5 billion people. The conceptual framework that underpins many studies may be oversimplified, which could be a limitation in understanding immunity to *M. tuberculosis.* Comparing the effects of bacterial and host variants on immunity to *M. tuberculosis* is made possible by end points like mortality, bacterial burdens, and tissue pathology in experimental animal models, which include zebrafish, mice, guinea pigs, rabbits, cattle, and non-human primates. A clear path for the development and discovery of an effective tuberculosis vaccine has not yet been established, despite the fact that these models have produced a great deal of information on the mechanisms of pathogenesis and innate and adaptive immunity[29]. Similar to this, research on the immune system's reaction to *M. tuberculosis* typically contrasts the immune system's functioning in healthy people who are latently infected with that of people who have active tuberculosis. These studies are valuable, but they haven't yet revealed any distinct

correlates or mechanisms of effective immunity against human *M. tuberculosis* infection[30]. The present study provided a framework for comprehending and researching tuberculosis immunity in humans and animal models. The underlying premise of this framework is that the human immune response to *M. tuberculosis* occurs in multiple stages, some of which are mimicked by current animal models, but not all of them. Furthermore, this framework may be used to design and assess human studies, with the hope that defining immune responses at different stages will lead to a better understanding of the mechanisms and correlates of immunity. i.e. The various stages of *M. tuberculosis* infection can be broadly regarded as forming a "immunological life cycle," even though the disease does not experience the dramatic morphological changes in fixed time frames typical of eukaryotic parasites during their life cycles. Several developments offer opportunities and highlight the need to comprehend the benefits and limitations of immunity to *Mycobacterium tuberculosis*. These include the development of new technologies (like cytometry by time of flight) and the identification of new markers and mediators of human immune responses, as well as the increasing dedication of funding agencies, regulatory bodies, and pharmaceutical companies to the development of TB vaccines. Recent advancements in systems biology approaches to analyze human responses to specific vaccines are also included in this[31]. This could help develop and assess TB vaccines as well as other novel strategies for boosting immunity against *M. tuberculosis*, hopefully lowering the incidence of this infectious disease[33,34].

Stage 1: Innate Immune Responses

Innate immune cells. *M. tuberculosis* is transmitted by aerosol, and largely, if not exclusively, inhabits professional phagocytic cells in the lungs, including macrophages, neutrophils, monocytes and dendritic cells (DCs) [35,36]. In mice, the early innate immune response to

M. tuberculosis is characterized by the progressive accumulation of neutrophils, inflammatory monocytes, interstitial macrophages and DCs in the lungs. As these cells are recruited, they become infected by the expanding population of mycobacteria and establish early gran- ulomas. In other infectious diseases, the recruitment of phagocytic cells restricts and even eliminates invading pathogens, whereas the recruitment of phagocytes to sites of mycobacterial infection actually benefits the pathogen during the early stages of infection, by providing additional cellular niches for bacterial population expansion[37].

Considerable evidence indicates that *M. tuberculosis* and other pathogenic mycobacteria, such as *Mycobacterium marinum*, have evolved multiple mechanisms to manipulate their cellular niches for their own advantage. First, pathogenic mycobacteria modulate the trafficking and maturation of the phagosomes in which they reside, allowing them to evade lysosomal mechanisms of restriction, killing and degradation. Second, mycobacteria use several virulence mechanisms to optimize their spread from cell to cell. For example, the ESX1 type VII secretion system — the absence of which attenuates the strain of *M. bovis* used in the BCG vaccine — promotes the necrotic death of infected cells and the recruitment of macrophages[38,39,40]. This allows the intracellular bacteria to be released from the cell for uptake by the freshly recruited adjacent phagocytes, resulting in subsequent intracellular growth and bacterial population expansion[37]. Third, *M. tuberculosis* possesses multiple mechanisms for inhibiting host cell apoptosis; among other benefits to the bacteria, such inhibition allows for the prolonged survival of infected cells and for a larger number of bacteria to accumulate in a given cell before they are released by cell death[41,42,43]. Although *M. tuberculosis* clearly possesses distinct mechanisms to regulate apoptotic and necrotic cell death, it remains to be determined how these mechanisms are regulated and how they are manifested during various stages of infection. During the innate

immune stage of *M. tuberculosis* infection, there appears to be little restriction of bacterial growth in the lungs, although this is a highly dynamic stage of infection. The expanding bacterial population spreads from cell to cell and increases the range of cell subsets that it infects to include DCs, which can subsequently initiate adaptive immune responses.

Mechanisms of Innate Immunity in TB

The innate immune stage is characterized by the recognition of *M. tuberculosis* components by multiple pattern recognition receptors. Of the Toll-like receptors (TLRs), TLR2 has the largest number of identified mycobacterial agonists, including lipoproteins (as many as 99 of them), phosphatidylinositol manners and lipomannan[43]. In addition, TLR9 senses mycobacterial DNA and contributes to the production of cytokines by macrophages and DCs in *M. tuberculosis*-infected mice[44]. Although deletion of Tlr2 and Tlr9, singly or in combination, does not have a marked effect on the control of *M. tuberculosis* in mice, deletion of the gene encoding the shared TLR adaptor molecule MYD88 results in a rapidly lethal infection[45]. This is probably due to defective signalling in response to interleukin-1α (IL-1α) and IL-1β, as such signalling also depends on MYD88. Additional recognition of *M. tuberculosis* is mediated by specific members of the C-type lectin receptor (CLR) family, including DC-SIGN[46]. Deletion of any one of these CLR genes has little or no effect on the course of infection, whereas deletion of the gene encoding the shared CLR adaptor molecule CARD 9 is associated with accelerated mortality and excessive neutrophilic lung inflammation[47]. Of the cytosolic pattern-recognition receptors, nucleotide-binding oligomerization domain protein 2 (NOD2) and NOD-, LRR- and pyrin domain containing 3 (NLRP3) recognize the peptidoglycan subunit N-glycolyl muramyl dipeptide and one or more ESX1-secreted substrates (such as ESAT6), respectively[48,49,50,51]. Therefore, stimulation of these pattern recognition receptors, individually or collectively, induces

the expression of pro-inflammatory cytokines, selected chemokines and cellular adhesion receptors that contribute to local and systemic immune cell mobilization and activation[52,53]. However, the initial effects of these responses appear to provide additional cellular niches that favour bacterial growth. Nonetheless, they also provide the basis for the subsequent initiation of cellular adaptive immune responses by driving the recruitment and maturation of DCs. Despite the multiple molecular and cellular innate immune events that occur during stage 1 of infection (either primary or secondary infection), accelerating the availability of antigen-specific CD4+ effector T cells — through the adoptive transfer of these cells — has no effect on the survival or growth of *M. tuberculosis* during the first 7 days of infection[53]. This suggests that the bacteria are in one or more niches where they either are not recognized by CD4+ T cells or are resistant to any anti-mycobacterial action of these cells. This finding indicates that characterizing the status and location of the bacteria during this first stage of infection and immunity is of paramount importance in designing CD4+ T cell-directed TB vaccines that block infection. The innate immune stage of TB is clearly a dynamic one, although the current weight of evidence indicates that it is a stage of infection in which the pathogen dominates, and innate immune responses have little immediate antibacterial effect. Therefore, the significance of the innate immune stage may be its role in establishing an environment that allows an adaptive T cell response to follow. Consequently, understanding the variation of innate immune responses in individuals with differential outcomes of *M. tuberculosis* infection is likely to provide valuable insight into how best to design and choose vectors and adjuvants (which direct innate immune responses) for optimal TB vaccine development[54].

Stage 2: Immunological Equilibrium

Delayed Initiation of Adaptive Immunity. A prominent characteristic of adaptive immune responses to *M. tuberculosis* is a long delay in onset. Based on currently available data from tuberculosis skin tests, measurable adaptive immune responses emerge in humans approximately 42 days after *M. tuberculosis* exposure and infection[55]. Interestingly, a similar delay is observed in hepatitis C virus infection, which is another persistent infection of humans[56,57]. A delayed onset (after 11–14 days) of *M. tuberculosis* antigen-specific T cell responses is also observed in mice following aerosol infection[58,59]. In mice, the activation of *M. tuberculosis* antigen-specific CD4+ T cells occurs earliest in lymph nodes that drain the lungs[58,59]. And requires the transport of live bacteria from the lungs to the draining lymph nodes by myeloid dendritic cells[60]. After aerosol infection of mice, this transport takes 8–10 days (compared with the ~20 hours required for the transport of influenza virus), implying that this delayed transport is the rate-limiting step in initiating adaptive immune responses to *M. tuberculosis*. It is currently unclear why this step is so prolonged, although there is evidence that *M. tuberculosis* infection of myeloid DCs inhibits their migration in response to ligands for CC-chemokine receptor 7 (CCR7)[61,62]. Furthermore, *M. tuberculosis*-induced inhibition of neutrophil apoptosis plays a role in the delayed kinetics of adaptive immune response induction[63]. Mice infected by aerosol with a pro-apoptotic mutant (ΔnuoG) strain of M. tuberculosis show increased DC-mediated bacterial transport to lymph nodes and accelerated naive CD4+ T cell activation; this effect is counteracted by selective neutrophil depletion[63]. Once the bacteria are moved to the draining lymph nodes, they multiply, differentiate, and traffick effector CD4+ T cells to the lungs with kinetics resembling those seen with soluble protein antigens[60]. These cells then present the antigens to naive CD4+ T cells. However, *M. tuberculosis* antigen-specific regulatory T cells also develop during the course of infection and contribute to the delayed priming of CD4+ and CD8+ T cells in the lung-draining

lymph nodes[64]. The onset of adaptive immune responses in TB results in the arrest of the progressive growth of the bacterial population and may result in transient disease symptoms, including fever and an unusual skin rash termed erythema nodosum[55]. After the onset of adaptive immunity, most humans become asymptomatic, do not shed bacteria and are considered to have latent TB infection. It is important to note that the size of the bacterial burden in human latent TB infection is unknown, owing to the current lack of available methods to determine it. The progressive growth of the bacteria in immunocompetent mice infected with virulent strains of *M. tuberculosis* is also arrested concurrently with the accumulation of effector CD4+ and CD8+ T cells in the lungs, and these cells maintain a plateau population size of approximately 106 bacteria until the mice die 12–20 months later [65]. These data indicate that, although adaptive immune responses in mice are sufficient to arrest the growth of *M. tuberculosis*, their ability to eliminate *M. tuberculosis* is limited. Multiple mechanisms probably contribute to the limited ability of adaptive immune responses to kill *M. tuberculosis*. Such mechanisms include: impaired MHC class II-mediated antigen presentation[66,67]; induction of the anti-inflammatory mediator lipoxin A4 (REF. 17); restriction by regulatory T cells[68] down regulation of bacterial antigen gene expression and, therefore, failure to induce antigen-specific CD4+ T cells[69,70] and resistance to the macrophage-activating effects of interferon-γ (IFNγ)[71,72]. It is noteworthy that, although the size of the bacterial population remains stable, a subpopulation of bacteria continues to replicate during this chronic, clinically silent stage of infection in mice[73]. Moreover, a recent study in non-human primates revealed that *M. Tuberculosis* also accumulates mutations during latency[74]. Taken together, these data provide convincing evidence that latent TB is not simply a state of bacterial stasis, but a state of dynamic bacterial and immunological equilibrium.

Immunological Mechanisms that Contribute to Equilibrium

Adaptive immunity to *M. tuberculosis* in humans, mice, cattle and non-human primates depends on CD4+ T cells; additional contributions of CD8+ T cells are well established in mice and non-human primates[75,76,77]. In addition to responses by classical MHC class I- or class II-restricted αβ T cells that recognize bacterial peptide epitopes, responses by other T cell subsets are observed. Such cells include CD1-restricted, mycobacterial lipid-specific T cells (which are predominantly CD4+), HLA-E-restricted CD8+ T cells and mucosa-associated innate-like T cells[78,79,80]. Although these other T cell subsets are under active investigation, their roles in immunity to TB have not yet been determined[81,82]. Among the mediators of immunity to *M. tuberculosis*, tumour necrosis factor (TNF) and IFNγ are the best described in humans, owing to the use of TNF-blocking therapeutic agents and the characterization of mutations in the IFNγ receptor gene[83,84]. Additional molecules that contribute to the immune control of *M. tuberculosis* in mice, but that have not yet been shown to be significant in humans, include IL-17, cytolytic T cell-expressed perforin and the IFNγ-induced molecules nitric oxide synthase 2 (NOS_2)[85,86]. Furthermore, several mediators have been characterized for their specific roles in the human immune response to *M. tuberculosis*. Granulysin is a cytolytic T cell granule protein that has direct anti-mycobacterial activity *in vitro*, although its role in controlling *M. tuberculosis* in vivo remains unknown. Vitamin D also has broad functions in vitro that contribute to immune-mediated control of *M. tuberculosis*; for example, it is an essential cofactor for the IFNγ-mediated induction of the antimycobacterial peptide cathelicidin[87,88]. Furthermore, vitamin D levels in humans are closely associated with susceptibility to active TB[89]. Despite extensive investigation, a clear, reproducible correlate of human immunity to *M. tuberculosis* infection has not yet been identified. There are several potential reasons for this. First, our knowledge of the full repertoire of T cell subsets and molecular mediators of protective immunity is still emerging, implying that

one or more crucial determinants have not yet been examined. Second, it seems increasingly likely that no single parameter will mediate or correlate with protective immunity in tuberculosis, implying that increasing use of systems biology, bioinformatics and biostatistics will be needed to formulate optimal models and test them in expanded studies. Third, it is possible that using healthy subjects with latent TB infection as the 'gold standard' of protective immunity may lead to erroneous conclusions, as latent TB does not equate with sterile immunity, and latent TB progresses to reactivation TB in a substantial fraction of individuals. Therefore, there is clearly a great need for methods to reliably identify distinct states of infection and the corresponding immune responses after exposure to *M. tuberculosis*[30].

The Bacterial Contribution to Equilibrium

Strong evidence exists that the mycobacteria are also active contributors to the immunological equilibrium state in latent TB. First, a well-characterized bacterial regulon that is controlled by DosR–DosS — a two-component signal transduction system in mycobacteria — is induced by several stimuli thought to prevail during latent TB, including local hypoxia[90, 91], nitric oxide[92] and carbon monoxide[93,94]. This 'dormancy' regulon controls the expression of genes that allow the bacteria to use alternative energy sources, especially lipids, and genes encoding factors that are selectively recognized by T cells from humans with latent TB (but not active TB)[95,96]. The expression of this gene network — as well as of other genes involved in using alternative energy sources (such as genes encoding isocitrate lyases[97]) — implies that *M. tuberculosis* has evolved specific mechanisms to adopt a state of latency, and that latency is not merely the suppressive effect of the host immune response on bacterial replication. Furthermore, five proteins that are encoded by *M. tuberculosis* bear resemblance to the well-studied secreted protein known as Micrococcus luteus resuscitation-promoting factor (Rpf), which has the capacity to "resuscitate"

bacteria from a nutrient-starved dormant state[98]. Bacteria with a weakened dormant state are produced when one or more of the *M. tuberculosis* Rpf genes are deleted, suggesting that these genes could be involved in the transition from latency to reactivation[99,100]. Lastly, *M. tuberculosis* encodes 88 pairs of toxin-antitoxin genes, the balance of whose expression controls a variety of phenomena, such as the bacteria's ability to replicate or stay stationary[101]. Thus, *M. tuberculosis* possesses at least three systems (the dormancy regular, resuscitation promoting factors and toxin–antitoxin gene pairs) that regulate its metabolic and growth state. Further investigation is likely to provide insights into the host and bacterial mechanisms that regulate these systems and that determine whether the bacteria remain in an equilibrium state with the host or resume growth and reactivate to cause active TB disease.

Stage 3: Reactivation of TB

Reactivation of latent TB reflects progression to active, symptomatic disease, which is usually characterized by the shedding of *M. tuberculosis* in respiratory secretions, especially during coughing. Reactivation TB must be distinguished from re-infection with a second strain of bacteria, which can occur even in immunocompetent individuals[102]. However, most cases of TB in adults are attributable to reactivation, except in geographical regions with an extremely high prevalence of TB. One study clearly showed, through the genotyping of strains, that reactivation TB can occur decades after initial infection[103]. Reactivation TB is widely attributed to 'weakened' immunity, although only a minority of cases are attributable to well-characterized defects in immunity.

Established Mechanisms Underlying TB-reactivation

In humans, only two mechanisms have been identified that explain reactivation TB both of which have become relevant only in the recent past. The first mechanism involves the

quantitative and qualitative CD4+ T cell defects that occur in people infected with HIV[75]. In addition to the extensive depletion of CD4+ T cells, there is strong experimental evidence from human studies to suggest that, before this profound CD4+ T cell depletion, HIV targets and depletes M. tuberculosis antigen-specific CD4+ T cells at a greater frequency than CD4+ T cells specific for other antigens[104,105]. This finding may account for the increased risk of active TB early after HIV infection, before there is a measurable depletion of circulating CD4+ T cells[75]. In addition, the depletion of CD4+ T cells by simian immunodeficiency virus (SIV) in non-human primates causes the reactivation and progression of TB[78], and depletion of CD4+ T cells during the chronic stage of M. tuberculosis infection in mice allows for the resumption of net bacterial growth in the lungs[69]. Despite the abundant evidence that deficiencies of CD4+ T cells cause reactivation of *M. tuberculosis*, the precise mechanisms that these cells use to establish and maintain immune control of *M. tuberculosis* in the latent state remain to be identified. The second well-characterized mechanism that is clearly associated with reactivation TB is the therapeutic neutralization of TNF[104], especially by monoclonal antibodies[106]. Despite the strength of the association, the effects of TNF blockade that account for reactivation TB are not fully characterized, but they include: decreased macrophage-mediated anti-mycobacterial activity and the subsequent death of macrophages the induction of a higher frequency of regulatory T cells and the depletion of a subset of CD45RA+ effector memory CD8+ T cells that contain granulysin and have been shown to contribute to *M. tuberculosis* killing in vitro[107,108]. Together, the increased frequency of TB in people infected with HIV or treated with TNF-blocking agents establish CD4+ T cells and TNF as two of the major elements that mediate protective immunity in TB and that prevent reactivation, although the underlying mechanisms are incompletely understood[109].

Established Associations with other Medical Conditions

In addition to the mechanisms known to promote TB reactivation, other medical conditions have been found to be associated with an increased risk of reactivation, although the underlying mechanisms are not well understood. These conditions include diabetes mellitus, the increasing prevalence of which in developing countries is leading to the convergence of its geographical distribution with that of TB to increase the severity of the TB epidemic[110]. There is recent intriguing evidence that mice with diabetes have a longer delay in the onset of adaptive immune responses to *M. tuberculosis* than mice without diabetes, owing to delayed trafficking of DCs from the lungs to the lymph nodes[111]. However, the mechanism underlying this delay has not yet been determined, and little is known regarding the mechanisms by which diabetes predisposes to TB in humans. Treatment with glucocorticoids is also a well-known risk factor for reactivation TB although the pleiotropic primary and secondary effects of glucocorticoids on immunity and inflammation make it difficult to determine which have the most potent impact on the reactivation of *M. tuberculosis*. Furthermore, a thin body habitus (with or without malnutrition) has long been linked to TB reactivation[112,113]. This relationship might be partially explained by the effects of leptin, which is best characterized for its regulation of energy expenditure and appetite, as the circulating levels of leptin are low in thin and malnourished people[114]. Leptin also modulates the development and function of T helper 1 (TH1) cells, suggesting a mechanism for the enhanced susceptibility to TB in thin people. Indeed, leptin-unresponsive mice poorly control *M. tuberculosis* infection[115]. Other conditions that have been epidemiologically linked to an increased likelihood of *M. tuberculosis* reactivation include silicosis, haematological malignancies, cancer chemotherapy, uraemia, gastrectomy and advanced age, but none of these have been studied with respect to their effects on specific immune mechanisms. Although the

aforementioned mechanisms and associations are notable, they account for a small minority of cases of TB reactivation. This suggests that the widely held model that 'weakened' or waning immunity accounts for TB reactivation requires reconsideration. In particular, advances in basic immunology suggest several alternative models that warrant attention[116]. The hypothetical models described below were selected because they can be tested, and because they are plausible. However, it is highly likely that additional mechanisms and models exist and are worthy of investigation[117].

Possible Mechanism: T-cell Exhaustion.

One increasingly well-characterized mechanism of failed immunity in chronic infections is T cell exhaustion, in which pathogen specific T cells are present but express inhibitory receptors that prevent their proliferation and their ability to mediate effector functions[118]. T cell exhaustion is best described in chronic viral infections, such as lymphocytic choriomeningitis virus (LCMV) infection in mice, and hepatitis C virus and HIV infection in humans[118]. So far, it is not clear whether T cell exhaustion occurs in TB, although a recent study revealed an inverse relationship between the numbers of polyfunctional *M. tuberculosis* antigen-specific CD4+ T cells in the blood and the apparent bacterial burden in the lungs[119]. This finding is consistent with CD4+ T cell exhaustion and warrants further investigation. By contrast, a recent study in mice revealed that programmed cell death protein 1 (PD1) — an inhibitory receptor expressed by exhausted T cells — is expressed on *M. tuberculosis* antigen-specific CD4+ T cells in the lungs, but that these cells retain the ability to proliferate and can differentiate into cytokine-producing effector CD4+ T cells[120]. Moreover, PD1-deficient mice — which can clear infection with an otherwise persistent strain of LCMV — succumb to overwhelming pulmonary inflammation when infected with *M. tuberculosis*[121]; this effect of PD1 deficiency is attenuated by the depletion of CD4+

T cells. Together, these data indicate that pathways that operate in exhausted CD8+ T cells in chronic viral infections have different functions in CD4+ T cells in TB. In addition, these data suggest that a complex pathogen containing multiple antigens, such as *M. tuberculosis*, may use mechanisms other than T cell exhaustion to prevent its elimination.

Possible Mechanism: Altered Antigen Expression.

Unlike viruses, which have a programmed pattern of gene expression, *M. tuberculosis* and other bacteria and parasites respond to signals from their environment to regulate their gene expression. In addition to allowing bacterial survival and growth under diverse conditions, this ability to regulate gene expression contributes to the alteration of antigen gene expression profiles at distinct stages of infection, allowing the bacteria to evade recognition by T cells specific for certain antigens. In particular, the expression of at least two antigens that are immune dominant in humans and mice — ESAT6 and Ag85B — is down regulated after the appearance of CD4+ and CD8+ T cells in the lungs of infected mice[122,123] In the case of Ag85B, which is contained in several of the lead candidate TB vaccines, down regulation of gene expression contributes to a marked reduction in the frequency of activated Ag85B-specific effector CD4+ T cells during the chronic stage of infection, and this contributes to the persistence of bacteria in the lungs[69]. It is likely that *M. tuberculosis* responds similarly to environmental cues in humans; whether this results in reduced activation of effector T cells and contributes to TB reactivation remains to be determined. However, the magnitude of the reduction in gene expression is more marked for Ag85B (and the closely related antigen Ag85A) than for ESAT6[123], and the expression of genes encoding other antigens (such as HspX and Rv2660c) is maintained or increased during chronic infection. This indicates that, although the profile of antigen expression may change during infection, a distinct repertoire of antigens and T cells may contribute to the maintenance of host–

pathogen equilibrium during latency. Given the implications of these results for choosing antigens for new TB vaccines, the characterization of antigen gene expression during distinct stages of infection should be a high priority. Indeed, a protein subunit TB vaccine incorporating a 'latency' antigen that is expressed predominantly during the chronic stage of infection has shown a greater efficacy than the same vaccine containing antigens expressed exclusively

Possible Mechanism: Altered Cell Trafficking.

Maintaining an efficacious immune response at the site of *M. tuberculosis* infection is likely to require the continuous recruitment of effector immune cells, although little is known about the kinetics of cell turnover in granulomas. If cell trafficking to granulomas needs to be maintained for decades to maintain local immunity in latent TB, it stands to reason that defective cell trafficking, even if slight or intermittent, could allow for TB reactivation. In mice, transgenic overexpression of CC-chemokine ligand 2 (CCL2; also known as MCP1) or the absence of CCR2 decreases the recruitment of monocytes and DCs to the site of *M. tuberculosis* infection and is associated with poorer immune control of infection[124]. By contrast, CXC-chemokine receptor 3 (CXCR3)-deficient mice are more resistant to infection and can control chronic *M. tuberculosis* infection in the lungs more effectively than wild-type mice[125,126]. In humans, several polymorphisms in genes encoding chemokines and chemokine receptors — such as functional variants of CCL2, CCL3L1 and CCR5 — have been associated with active *M. tuberculosis* infection. Because the effects of these polymorphisms have been described in adults, it is likely that their association is with reactivation TB, suggesting that maintaining optimal recruitment of specific myeloid and lymphoid subsets is required for durable control of *M. tuberculosis*, and that suboptimal cell trafficking may permit reactivation[127,128].

Can the Bacteria be the Primary Drivers of Reactivation?

As noted above, *M. tuberculosis* has specific programmes for initiating a state of dormancy in response to certain environmental signals (some of which are imposed by adaptive immune responses), and this state manifests as clinical latency. In turn, *M. tuberculosis* also has specific programmes for recovering from dormancy, suggesting that the bacteria may assume a primary role in some cases of reactivation TB that are not explained by immune defects or deficiencies[129].

Spontaneous Deactivation.

One variation of reactivation that occurs in a substantial proportion of actively infected humans is the spontaneous resolution or deactivation of the infection, that is, progression from active to inactive disease without anti-tuberculosis chemotherapy. Inactive TB differs from latent TB in that in the former there are often abnormalities detected on chest X-rays, whereas such findings are absent in latent TB. Although spontaneous resolution of active TB to inactive TB is reported to have occurred in as many as 50% of individuals in the pre-chemotherapy era, its mechanisms are not understood. A recent study of long-term survivors of untreated TB revealed that approximately 70% of these individuals had CD4+ effector memory T cell responses to *M. tuberculosis* antigens, suggesting that they were persistently infected[130]. By contrast, a substantial fraction of the remaining individuals had CD4+ central memory T cell responses, consistent with clearance of infection[113]. These observations further highlight the spectrum of host– pathogen interactions in TB, and suggest that future studies of immune responses in humans with active TB should be designed to account for the possibility that a substantial fraction of the subjects might have immune responses that allow them to regain control of their infection. Overall, reactivation TB is poorly understood, especially from the perspective of the mechanisms that allow progression from latency to reactivation. As studies to understand this important phenomenon in the context of 'weakened' immunity have revealed only two clearly

established mechanisms (CD4+ T cell depletion and TNF blockade), and these apply to a minority of cases of reactivation TB, there is considerable opportunity for the discovery of additional mechanisms that account for a greater number of cases. As one especially promising example, transcriptional profiling of peripheral blood cells from humans with latent and active TB has revealed the previously unsuspected association of active TB with a type I IFN signature and with the expression of neutrophil-specific genes[131]. In this study, a range of transcriptional signatures was observed among individuals, and some of these may be attributable to distinct stages of the immunological life cycle of TB. It is likely that additional prospective analyses — particularly of people recently exposed to active TB cases and presumably newly infected — will clarify the roles of the type I IFN and neutrophil signatures in the pathogenesis of and immunity to TB[132].

Stage 4: Transmission

An obligate step in all infectious diseases is transmission to new hosts. In the case of TB, this occurs through the airborne route, in which bacteria are expelled (usually by coughing) from an individual with active disease and then inhaled by susceptible hosts. As in many other infectious diseases, the transmission of TB is not uniform, and certain individuals cause far more secondary cases than do others[133,134]. In particular, individuals with a form of TB termed cavitary TB are especially infectious[135]. Cavitary TB is the consequence of lung tissue destruction and the formation of macroscopic open spaces that contain numerous *M. tuberculosis* bacilli[136] and connect to large airways, which facilitates efficient expectoration of the bacteria.

Evidence that Immune Responses Promote Transmission.

Several lines of evidence indicate that — in addition to their widely known roles in protecting an infected individual from rapidly lethal TB — human T cell responses contribute to the lung

tissue destruction underlying cavitary TB, and thereby may contribute to host-to-host TB transmission. In particular, multiple studies have revealed that individuals with TB who are co-infected with HIV have a lower frequency of cavitary TB, and a recent systematic review revealed a linear correlation between the number of circulating CD4+ T cells and the frequency of cavitary TB[75]. Indeed, this study showed that the likelihood of cavitary TB was fourfold higher in subjects who had more than 200 CD4+ T cells per µl of blood than in individuals with fewer than 200 CD4+ T cells per µl of blood. In addition, HIV-infected people transmit TB less efficiently than do HIV-uninfected people[75]. This is in contrast to observations of other infections, such as influenza virus infections, in which immune deficient people shed higher levels of virus and for longer periods of time than those with intact immunity[137]. It is unclear whether the effect of CD4+ T cells on the promotion of cavitary TB is direct or indirect, and the mechanisms by which CD4+ T cells contribute to lung tissue damage and cavitary TB are not well characterized. Although the collagen-degrading metalloproteinase MMP1 has been implicated as a mediator[137], its relationship to the contributions of CD4+ T cells has not yet been established. Precedents provided by studies of tissue damage in T cell-dependent autoimmune diseases may guide studies to determine whether specific functions of effector CD4+ T cells are involved, and whether the antigen specificity of CD4+ T cells is distinct in humans with cavitary TB compared with those with non-cavitary TB. A recent study of genetic diversity in human T cell epitopes of *M. tuberculosis* contributed evidence consistent with a role for T cell responses in TB transmission[138]. The study of nearly 500 experimentally verified human T cell epitopes in a collection of geographically and genetically diverse strains of *M. tuberculosis* (the ancestors of which had diverged at least 15,000 years ago) revealed that the known human T cell epitopes of *M. tuberculosis* are the most conserved elements of the *M. tuberculosis* genome[138]. These results

are consistent with a model in which human T cell responses, although providing (partial) protection to individual infected hosts, provide a net evolutionary benefit to *M. tuberculosis*. T cell responses probably mediate this effect by contributing to inflammatory tissue damage and lung cavitation, which promotes the transmission of the bacteria to new hosts.

Non-human Models of TB-transmission are needed

Although the results in humans demonstrate an association between CD4+ T cells, cavitary TB and TB transmission, the discovery of the underlying direct and indirect mechanisms is likely to require studies in a nonhuman animal model. However, a small-animal model that can be used to study TB transmission has yet to be developed. Although mice, guinea pigs and rabbits all provide models for certain stages of TB infection and immunity, none of these animals transmit TB efficiently. By contrast, bovine strains and other animal adapted strains (such as Mycobacterium microti in voles) in the *M. tuberculosis* complex and closely-related species (such as M. marinum in fish) are transmitted in the wild, and these strains represent opportunities for the study of determinants of transmission in naturally co-evolved host–pathogen pairs. For the optimal design and execution of such studies, as well as of studies on the effects of vaccination and other immunological interventions in preventing and promoting TB transmission, an investment in generating and validating immunological and genetic tools for use in these animals is necessary[139,140].

A deeper comprehension of the protective and pathogenic immune responses in tuberculosis is necessary to develop effective vaccines against the disease, which poses certain challenges. First, using surrogate end points to assess the effectiveness of the TB vaccine is insufficient because there are currently no well-defined correlates of protective immunity, particularly in humans.

Second, even though systematic study and selection of vaccine antigens has led to the development of promising candidate vaccines, the efficacy of these vaccines in preventing TB in a human population remains to be determined[141,142]. Third, TB may be unique in its exploitation of immune responses to promote transmission. A much better understanding of the mechanisms and targets of pathogenic immune responses in TB is needed to minimize the likelihood of vaccine-induced pathogenic immune responses and the risk of serious adverse effects of new TB vaccines. Thus, although vaccine development for TB needs to proceed as rapidly as possible, these efforts need to be matched by a more extensive understanding of beneficial and detrimental immune responses to natural infection. These studies will benefit from consideration of the stage of the TB immunological life cycle that is represented in the subjects, as distinct immune responses may have different roles at distinct stages of the life cycle. For example, studies in mice indicate that IL-17 is beneficial to the host during early *M. tuberculosis* infection, whereas it appears to be detrimental, especially in high concentrations, later in infection[143,144]. Likewise, neutrophils promote the development of adaptive immune responses to *M. tuberculosis* early after infection whereas they can be detrimental in later stages[145,146]. These examples in a simplified system illustrate how the failure to consider the stage of infection and the immunological life cycle would lead to conflicting conclusions regarding the roles of IL-17 and neutrophils in mice with tuberculosis. Considering the immunological life cycle stage in studies of human subjects with TB may allow for better distinction of beneficial and detrimental innate and adaptive immune responses and mechanisms. This Review proposes and presents evidence that immunity to *M. tuberculosis* develops and proceeds in a manner consistent with four distinct stages of a life cycle, in which the state of the bacteria and the nature of immune responses exhibit specific features. In some cases, the distinctions between the stages of the immunological

life cycle are clear, as exemplified by the stage limited to innate immune responses and the stage of immunological equilibrium after adaptive immune responses develop and latency is established. However, much more knowledge is needed to fully understand the differences that occur in T cell phenotypes and functions, and in the targets of T cell responses, between the stage of immunological equilibrium and the reactivation and transmission stages. Although our understanding of the mechanisms and targets of immunity to *M. tuberculosis* has advanced considerably, a higher resolution understanding is needed, and some of this will require the development of new experimental models. The overall goal of this Review is to provide a conceptual framework for prioritizing, designing and interpreting the results of future studies, in order to derive the maximum benefit from efforts to decrease the global burden of tuberculosis[147].

2.1.2: Latent *Tuberculosis* Infection (LTBI)

Persons with LTBI have *Mycobacterium tuberculosis* inactive due to the activities of granuloma cells. However, they don't have TB disease and can't transfer the infection to others. Within weeks after infection, the immune system is usually able to halt the multiplication of the tubercle bacilli, preventing further progression[148]. LTBI could also be detected by utilizing the tuberculin skin test (TST) or an interferon-gamma release assay (IGRA). It will take two to eight weeks once the initial TB infection for the body's system to be ready to react to tuberculin and for the infection to be detected by the TST or IGRA. Even though people with latent *tuberculosis* infection may not develop TB disease as it remains inactive but in person with low immune system, malnutrition, HIV infection, diabetes, the bacteria may become active, multiply and cause *tuberculosis* disease[148].

2.1.3 *Tuberculosis* Disease

In some individuals, the tubercle bacilli overcome the immune system and multiply, resulting in progression from LTBI to TB disease.

Without treatment, approximately 5% of persons who have been infected with *Mycobacterium tuberculosis* can develop disease within the initial year or two immediately after infection, and another five-hitter will develop disease sometime later in life. Thus, if without treatment, approximately 10% of persons with normal immune systems who are infected with *Mycobacterium tuberculosis* will develop TB disease at some point in their lives[149].

2.1.4 Sites of Tuberculosis Disease

TB disease will occur in pulmonary and extrapulmonary sites.

Pulmonary: Pulmonary TB is a *tuberculosis* infection that affect the lungs, this can cause various symptoms like chest pain, weight loss, breathlessness, coughing which could be very severe, pulmonary *tuberculosis* is a life threatening disease which is very contagious and very easily spread through the air. Risk factors are people who are exposed to the infection, people who are born in an endemic area[150,151].

Diagnosis of pulmonary *tuberculosis* is by direct smear examination of sputum using Zeehl Neelsen stain, sputum culture and the use of molecular techniques (Polymerase chain reaction) this method is very quick, sensitive and specific as it can categorised into drug sensitive and drug resistant *tuberculosis*[152].

Extra Pulmonary: Extra pulmonary TB disease happens in places apart from the lungs, including the larynx, the lymph nodes, the pleura, the brain, the kidneys, or the bones and joints. In HIV-infected persons, extra pulmonary TB disease is commonly followed by pulmonary TB.

Persons with extra pulmonary TB disease sometimes don't seem to be infectious unless they could be

(1). Pulmonary disease additionally to extrapulmonary disease;

(2). extra pulmonary disease placed within the oral cavity or the larynx; or

(3). Extrapulmonary disease that features an open symptom or lesion during which the concentration of organisms is high, especially if drainage from the abscess or lesion is extensive, or if drainage fluid is aerosolized[153].

Miliary Tuberculosis: Miliary TB happens when tubercle bacilli enter the bloodstream and circulate to any or all components of the body, where they grow and cause disease in multiple sites. This condition is rare but serious. "Miliary" refers to the radiograph appearance of millet seeds scattered throughout the respiratory organ. It is most typical in infants and youngsters younger than five years of age, and in severely immunocompromised persons. Miliary TB could also be detected in an individual organ, including the brain; in several organs; or throughout the whole body. The condition is characterised by an oversized quantity of TB bacilli, although it may easily be missed, and is fatal if untreated. Up to twenty fifth of patients with military TB might have meningeal involvement[154].

Central Nervous System: When TB is established within the tissue surrounding the brain or spinal cord, it is called tuberculous meningitis. This form of *tuberculosis* is commonly seen at the bottom of the brain on imaging studies. Symptoms include headache, decreased level of consciousness, and neck stiffness. The period of sickness before diagnosing is variable and relates partly to the presence or absence of different sites of involvement. In several cases, patients with meningitis have abnormalities on a chest radiograph consistent with old or current TB, and often have miliary TB[155,156].

There are factors that can be responsible for developing active *tuberculosis*, any factor that can weakened the immune system or cause damage to the lung can increase the risk of developing *tuberculosis*, such factors as HIV infection, malnutrition prolong use of steroid, Diabetes mellitus, smoking of tobacco, poor ventilation, proximity with infectious source and long duration of exposure to TB bacteria1[157].

2.1.5 Immune Response to *Mycobacterium tuberculosis*

Pathogens have evolved elaborate defence and survival strategies for their persistence during infection and replication in a host organism[158]. Likewise, infected organisms have developed equally elaborate methods for his or her protection and therefore the institution of effective antibacterial defence.

Dendritic cells (DCs) and macrophages constitute the first line of defence against TB and retain complementary roles in the clearance of the infective bacteria[159]. DCs internalize the infective bacteria and present their peptides to T cells, inflicting their activation and initiating the onset of the adaptative immune reaction[160]- macrophages clear the offensive pathogens by internalising and exposing them to the acidic, hydrolytically active environment of the phagosome, eventually triggering a signalling cascade that culminates in the fusion of the lysosome to the phagosome[161]. Macrophages, together with epithelioid cells and multinucleated giant cells (also known as Langhans' giant cells) and T lymphocytes are also the principal cellular constituent of granulomas[162].

The institution of *Mycobacterium tuberculosis* infection needs a strict management over the production of pro-inflammatory and anti-inflammatory cytokines. TNF-α, IFN-γ, and IL-1β are particularly important for enhancing the shielding function of the granuloma, whereas IL-10 is one of the main negative regulators of the inflammatory response[163]. TNFα could be a pro-

inflammatory cytokine that promotes granuloma formation, while IFN-γ promotes antigen presentation and the recruitment of CD4+ T lymphocytes and/or cytotoxic T lymphocytes, thereby mediating mycobacterial killing. IL-1β is a pro-inflammatory cytokine which is mainly produced by monocytes, macrophages, and DCs and is involved in the host's immune response to MTB. IL-1β was shown to mediate signals through the IL-1 receptor (IL-1R) in response to mycobacterial infection[164,165]. Notably, IL-1β is targeted by a MTB secreted protein (Zmp1, Rv0198c) that negatively modulates IL-1β activity, resulting in the quenching of the inflammatory response[166].

In contrast to the pro-inflammatory cytokines listed above, the cytokine IL-10 has anti-inflammatory properties and is produced by macrophages and T-cells upon infection with MTB. IL-10 deactivates macrophage function by downregulating TNFα, which in turn reduces the production of IFNγ by T-cells and thus aids in MTB survival[167].

All these cytokines are secreted and controlled by macrophages and DCs upon detection of specific pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs) which sense MTB signature molecules. The PRRs are responsible for initiating the innate as well as the adaptive immune response to MTB[168].

2.2 Cellular Metabolism of TB

Dendritic cells (DCs) or monocyte-derived macrophages infected with MTB move to the thoracic draining lymph nodes, where they offer antigens to naive T-cells, causing effector T cells to travel to the lungs. MTB, however, thwarts this process by delaying it for up to three weeks after infection. B-cells also play a role in TB immunity by generating cytokines that control macrophage and T-cell function as well as supplying MTB-specific antibodies to induce opsonization. When complete sterilization fails, the extracellular inflammatory milieu and

recruited immune cells form a granuloma, with infected macrophages at the center, surrounded by layers of uninfected macrophages of various phenotypes, and finally T-cells with some follicular B cells and fibroblasts to limit infection and prevent subsequent spread. Although the induction of inflammatory mediators within granulomas is necessary for preventing MTB dissemination, excessive pro-inflammatory responses result in reduced disease tolerance, including granuloma necrosis, increased lung parenchymal damage, lung cavitation, and transmission, which leads to the onset of active disease . TNF inhibition improved outcomes in immune-compromised patients with advanced inflammatory mycobacterial illness, including *tuberculosis*. Furthermore, investigations in both animal and human models of tuberculosis have shown that inflammatory signaling is highly structured within the granuloma, with pro-inflammatory signaling predominating in the core and anti-inflammatory signaling predominating in the periphery. The granuloma's function in modulating both disease tolerance and host resistance to MTB is determined by this spatial compartmentalization of pro- and anti-inflammatory signals. As a result, the best host response could be a combination of inflammatory and anti-inflammatory signals that regulates inflammation within and surrounding the granuloma and reduces the frequency of active disease.

The tubercle bacilli invaded-macrophage undergoes intracellular transformation to generate reactive nitrogen intermediate (RNI) and reactive oxygen species. The purpose of the free radical generation is to keep the tubercle bacilli in a latent form through the synergistic effect of interferon-γ and the reactive nitrogen intermediate (RNI). Several authors reported that animals lacking inducible nitric oxide synthase were found to be more susceptible to *Mycobacterium tuberculosis* and Leishmania major infections. Cellular immune deficiency and decreased NO synthesis (through malnutrition and other factors) enhance the progression of the latent *tuberculosis* to active pulmonary tuberculosis.

Active pulmonary *tuberculosis* (PTB) is defined by a higher rate of macrophage activation and oxygen consumption by activated macrophages than normal. In the presence of NADPH oxidase, enhanced oxygen absorption is transformed to superoxide anion and other free radicals. Oxidative stress occurs when the free radical load surpasses the detoxifying capacity of the endogenous antioxidant defenses. Fragmentation of proteins and peroxidation of lipids are some of the effects of oxidative stress in disease, as are malfunctioning of cell membranes and enzymes, degradation of cell membrane function, decreased fluidity, inactivation of membrane-bound receptors, and increased ion permeability. One of the metabolic results of macrophage activation in TB patient is neopterin. It's a pro-inflammatory immunological state signal as well as a cellular activation marker that's secreted into the bloodstream. Neopterin levels have been found to be higher in a variety of disorders, including *tuberculosis* and cancer, according to several studies. [169]

TB and weight:

Globally, tuberculosis (TB) continues to be a major cause of death and a major burden on health[1]. One risk factor for the development of tuberculosis is malnutrition[2]. Reduced T-cell proliferation and compromised cell-mediated immunity are the results of malnutrition, which also increases susceptibility to infection[3,4,5]. It has been demonstrated that adults in the USA who have low body mass index (BMI), little subcutaneous fat, or decreased skeletal muscle mass are more likely to develop tuberculosis (TB) than people who have normal nutritional status[6]. Sixth, TB is also characterized by weight loss. In a Los Angeles County study of TB patients, 40.6% had anorexia and 44.5% had weight loss[7]. Because of the loss of both fat mass and fat-free mass, tuberculosis causes a decrease in BMI in patients and One known risk factor for death during the first four weeks of tuberculosis treatment is the presence of moderate-to-severe malnutrition[8,9]. During TB treatment, there is a nutritional recovery and weight gain. Ten, nine Weight gain may

be a sign of a treatment response, but this is still up for debate[10]. Scientist discovered that treatment-related weight gain was unaffected by relapse or a microbiological reaction, another study discovered that, after controlling for confounders, weight changes over time are predictive of treatment outcomes[11,12].

It has also been demonstrated that underweight patients at diagnosis who do not gain five percent of their body weight during the two-month intensive phase of tuberculosis therapy are at higher risk of relapsing[13]. Inadequate nutritional intake may complicate the effect of tuberculosis treatment on weight. Patients in Tanzania, for instance, saw improvements in weight during TB treatment, but these benefits were only sustained while the patients remained in hospitals, most likely as a result of improved dietary intake during hospitalization[14].

2.3 Multi Drug Resistance (MDR)

The frequency of microbial infections has sharply increased over the past few decades. The emergence of resistance among the various strains of microorganisms has been directly attributed to the ongoing use of antimicrobial medication in treating infections. Multidrug resistance (MDR) is the incapacity or resistance of an organism to the antimicrobial drugs that are administered, even though those drugs have completely different molecular targets and are structurally unrelated to one another[170]. The World Health Organization claims that these resistant microorganisms—such as viruses, bacteria, fungi, and parasites—can fend off the effects of antimicrobial medications, which results in inadequate treatment that allows infections to persist and spread. While multidrug resistance (MDR) may have arisen naturally, there has been a significant increase in the number of immune compromised conditions, such as HIV infection, diabetes, organ transplant recipients, and Patients with severe burns are more susceptible to hospital-acquired infections, which fuels the spread of multidrug resistant bacteria. Research

from the World Health Organization has revealed extremely high rates of resistance in bacteria to antibiotics like cephalosporin and fluoroquinolones; Klebsiella pneumoniae against cephalosporin and carbapenems; Staphylococcus aureus against methicillin; Streptococcus pneumoniae against penicillin; Nontyphoidal Salmonella against fluoroquinolones; Shigella species against fluoroquinolones; Neisseria gonorrhoeae against cephalosporin; and *Mycobacterium tuberculosis* against rifampicin, isoniazid, and fluoroquinolone, which cause common infections and a high percentage of hospital-acquired infections. Chronic fungal infections can be treated with a restricted range of antifungal medications. Isolates of Candida spp., Aspergillus spp., Cryptococcus neoformans, Trichosporon beigelii, Scopulariopsis spp., and Pseudallescheria boydii exhibit resistance to medications such as polyene macrolides (amphotericin B), azole derivatives (ketoconazole, fluconazole, itraconazole, and voriconazole), DNA and RNA synthesis inhibitors (flucytosine), and 1,3-glucan synthase inhibitors (echinocandins). Extended drug use and <"art-13">. Antiviral resistance has become a concern in patients with disorders as a result. Immunosuppressed transplant recipients and cancer patients infected with the cytomegalovirus (CMV), herpes simplex virus (HSV), varicella-zoster virus (VZV)[171,172]. Hepatitis B virus (HBV), influenza A virus, hepatitis C virus (HCV), or human immunodeficiency virus (HIV)[173]. Various drugs, including pentavalent antimonials, artemisinin, miltefosine, paromomycin, atovaquone, sulfadiazine, and amphotericin B, have been tested for parasitic multidrug resistance in isolates of Plasmodia, Leishmania, Entamoeba, Trichomonas vaginalis, schistosomes, and Toxoplasma gondii[174,175]. The malaria parasite Plasmodium falciparum is one of the most prominent examples of a disease susceptible to multidrug resistance. In many tropical and subtropical nations, amoebiasis—a serious threat to public

health—is also brought on by Entamoeba spp., another protozoan parasite[176,177]. Schistosomiasis is also thought to pose a similar threat to global health as malaria and other chronic illnesses[178].

2.3.1 Causes of Multidrug Resistance

The production of pharmaceuticals releases large amounts of antibiotics into the environment, as does the treatment of wastewater. Antibacterial agents found in soaps and other products can also contribute to the development of antibiotic resistance. Ingestion of contaminated water, contact with air emanating from animal housing or released during animal transportation, and interaction with infected meat processors or farm workers[179].

Antibiotic resistance is increasingly likely as a result of viral diseases such as the common cold, even with the overuse of antibiotics in unnecessary and inappropriate situations[180].

2.3.2 Consequences of Multidrug Resistance

2.3.2.1 Health Consequence

Infectious diseases are especially dangerous for children. They are very likely to suffer long-term harm or pass away if drug resistance slows down or stops effective treatment. Poor countries often lack access to more effective drugs, and the most deadly childhood illnesses—dysentery, pneumonia, and other respiratory infections—often no longer respond to standard treatment[181].

Even with tremendous efforts, it is still too simple to locate instances of failure in global health. The two most common infectious disease killers of children under five are respiratory infections and diarrheal illnesses. On the other hand, child fatalities from respiratory infections remain high and are expected to decline only very slowly, despite a significant decline in diarrheal mortality in recent years[182]. The percentage of children under five receiving antibiotic treatment for upper respiratory tract infections increased from 42% to 71% worldwide between 1998 and, but only

35% of those children received care in accordance with clinical guidelines during that time[183]. Because of the unacceptable levels of resistance to older drugs, countries have changed their standard treatment guidelines for malaria repeatedly. However, African households with young children are far more likely to use older drugs than new, effective ones. These links between inappropriate drug use and poor health outcomes suggest that drug resistance is a factor, not a cause, and that progress in reducing childhood diseases will be difficult to achieve unless drug efficaciousness is increased and new medications are closely monitored to extend their therapeutic value[184].

The fact that the ability to treat other diseases is impacted by drug resistance to a particular kind of treatment for one disease is even more concerning. The duration of antibiotics is a major source of concern. The global supply of new antibiotics is almost completely depleted, and two thirds of all antibiotics are sold without a prescription. Antibiotics are, in fact, the cornerstone of medical care; they are just as necessary for treating illnesses as they are for safe surgery, reproductive health, and other medical requirements. Doxycycline, for instance, is used to treat a variety of bacterial and parasitic illnesses. As a result, there is less therapeutic efficacy overall and more selection pressure for resistance to various infectious agents[185]. The sharp increase in the proportion of individuals co-infected with multidisease-causing pathogens, particularly tuberculosis and HIV/AIDS, highlights the necessity of tracking treatment effectiveness across illnesses[186].

Several examples illustrate the complexities of drug interactions across diseases.

- There is evidence of increased carriage of cotrimoxazole-resistant strains of S. pneumoniae in children after they have been treated with Sulfadoxine-Pyrimethamine (SP) for malaria[187].

- Heavy use of chloroquine to treat malaria appears to select for ciprofloxacin resistance in Escherichia coli[188].
- Rifampin use for TB treatment severely limits treatment options for HIV/AIDS by lowering anti-HIV protease inhibitor concentrations (through cytochrome oxidase inhibition)[189].

2.3.2.2 Economic Consequences

Drug-resistant disease types cost the patient and the healthcare system money and resources. The cost of second- and third-line medications for the treatment of resistant diseases is significantly higher. For instance, second-line antiretrovirals are primarily funded by the Health Ministry in Brazil[190]. In addition, more medical staff, hospital beds, test kits, and other supplies are required to treat diseases with resistance. Additionally, patients who have infections that are resistant to treatment stay in the hospital and receive treatment for much longer.

Treating diseases that are resistant comes with significant opportunity costs. Curing one patient with extensively drug-resistant tuberculosis (XDR-TB) comes at the same cost as curing 200 patients with susceptible TB. For economic reasons, society has every incentive to find means of containing, slowing down, and eliminating resistance.

Ultimately, donors are being compelled to reallocate funds in order to cover the cost of more costly medications, but their financial efforts are failing to reach even a small percentage of those who develop resistant forms of disease and will likely run out as the number of patient's increases[191].

2.3.3 Complications Associated with MDR

Antimicrobial resistance has a major influence on the efficacy of antimicrobial agents and is linked to high death rates and medical expenses. MDR causes barriers to disease control by increasing the likelihood that resistant pathogens will spread, which lowers treatment efficacy and prolongs the patient's infection period. Due to the pathogens' development of resistance to commercially available drugs, which has led to the use of Multidrug resistance (MDR), which results in more expensive therapies, has also raised treatment costs. The success rate of modern medical procedures like cancer chemotherapy and organ transplantation has made a significant contribution to MDR development. Variations in the resistance profiles of plant and bacterial pathogens, as well as the standard of public hygiene, significantly affect how effective antimicrobial agents are. Growth in international trade and tourism increases the potential for MDR to spread globally and decreases the import and export of various goods that have an impact on the economies of developing nations[192].

2.3.4 Classification of MDR

The high levels of resistance that have developed in various microbial strains are recommended by their survival, even when appropriate doses of medication are administered for a specific duration of time. In addition to antimicrobial resistance, other factors contributing to this clinical failure include immunological suppression, reduced drug bioavailability, or accelerated drug metabolism. The ability of bacteria to persist in the face of conventional or standard treatments

highlights the various forms of antimicrobial drug resistance, which is a growing medical setback. There are two types of MDR resistance: primary and secondary.

Primary Resistance: It happens when the organism has never encountered the drug of interest during a specific host.

Secondary Resistance: Also known as "acquired resistance," this term is used to describe the resistance that only arises in an organism after an exposure to the drug[193]. It may further be classified as follows.

a) **Intrinsic resistance**: it refers to the insensitivity of all microorganisms of a single species to certain common first-line drugs, which are used to treat diseases based on the clinical evidence of the patient. It is additionally called multidrug resistance (MDR), for example, *Mycobacterium tuberculosis* to rifampicin and isoniazid[194].

b) **Extensive resistance**: It defines the ability of organisms to withstand the inhibitory effects of at least one or two most effective antimicrobial drugs. Also termed as XDR, this seemed to arise in patients after they have undergone a treatment with first line drugs, for example, XDR-TB resistance against fluoroquinolone[195].

c) **Clinical Resistance**. In addition to the above listed types, clinical resistance is defined by the situation in which the infecting organism is inhibited by a concentration of an antimicrobial agent that is associated with a high likelihood of therapeutic failure or reappearance of infections within an organism due to impaired host immune function. In other words, the pathogen is inhibited by an antimicrobial concentration that is higher than could be safely achieved with normal dosing [196; 197].

2.3.5 Molecular Basis of MDR-TB

Anti-TB resistance in M. tuberculosis is thought to be conferred by spontaneous chromosomally borne mutations that occur at a predictable rate. Sometimes, drug resistance does not translate into resistance to a different drug. There are typically 107–109 bacilli in a tuberculosis cavity. Assuming that mutations leading to isoniazid resistance arise in approximately 1 in 106 bacterial replications, and that mutations resulting in rifampicin resistance occur in approximately 1 in 108 replications, the likelihood of spontaneous mutations causing resistance to both antibacterial drugs and rifampicin would be 106x108 = 1 in 1014. Since even patients with extensive cavitatory pulmonary tuberculosis do not contain this quantity of bacilli, it is extremely unlikely that spontaneous dual isoniazid and rifampicin resistance will arise[198]. Therefore, the scientific underpinning of antituberculosis chemotherapy is the actual finding that mutations are unlinked. Individual drug target gene perturbations are the main cause of multiple drug resistance in tuberculosis[199].

In research published from India, a number of novel mutations were also found in the rpoB (rifampicin), katG and the ribosomal binding site of inhA (isoniazid), gyr A and gyr B (ofloxacin), and rpsL and rrs (streptomycin) genes in addition to the previously known mutations. 43 out of 44 resistant isolates had 53 mutations of 18 different types, 17 point mutations, and 1 deletion. Two novel mutations outside the Rifampicin Resistance-Determining Region (RRDR) and three new alleles and novel mutations within the RRDR were reported[200]. These findings indicate that although some mutations are common, indicating the magnitude of the polymorphisms at these loci, others are uncommon, suggesting diversity in the multidrug-resistant population. Additionally, it was noted that rifampicin resistance was discovered to be a significant marker for examining multi-drug resistance in clinical isolates of *M. tuberculosis*[201].

2.3.6 Types of MDR-TB

Drug resistance comes in two flavors: primary and acquired. Primary drug resistance is defined by the World Health Organization as drug resistance in a patient who has never before received anti-tubercular treatment. Another way to define acquired drug resistance is as drug resistance that develops in a patient after they have previously undergone chemotherapy. Initial drug resistance is the state in which one is unsure of whether the resistance is primary or acquired as a result of a concealed history of prior treatment or ignorance of prior treatment. The sum of primary and acquired resistance is the definition of combined resistance[202].

HIV infection and MDR-TB: It has been discovered that breaks in MDR-TB among HIV-positive patients in the USA are caused by the HIV infection's amplifying and accelerating effects as well as the delayed detection and diagnosis of tuberculosis. In 1995, Shafer et al. used Restriction Fragment Length Polymorphism (RFLP) to study temporal trends and transmission patterns in New York City[203,204]. They discovered that MDR-TB cases were clustered, especially among those living with HIV who disproportionately experienced drug-resistant illness. HIV-positive individuals were found to have a significantly higher likelihood of having contracted MDR-TB recently, according to a follow-up survey of 167 tuberculosis cases seen at five New York hospitals in 1992 and 1993. In fact, RFLP revealed that 79% of the drug-resistant cases were clustered, suggesting a clear indication of recent transmission[205]. There is also a correlation between HIV/AIDS and MDR-TB for the reasons listed below.

- Increase in the number of cases of tuberculosis due to HIV/AIDS will give rise to an increase in the number of cases with primary drug resistance
- Overloading of the tuberculosis treatment services because of the expected increase in the number of cases will give rise to more cases with acquired drug resistance

- Immune compromised status of HIV patients may lead to decreased efficacy of antituberculosis treatment regimens and thereby increasing the chance of acquired drug resistance
- The malabsorption of antituberculosis drugs has been shown to occur with high frequency among persons with AIDS, presumably because of various HIV-caused, parasitic or other enteropathies

This may result in wildly different drug concentrations and, even with strict adherence to the recommended regimen, developed resistance. Recent research, however, has shown that, in contrast to western literature, the prevalence of MDR-TB in HIV seropositive patients is extremely low in South East Asian countries like India, where HIV seropositivity is highly prevalent. If appropriate action is not taken, there could soon be a sharp increase in MDR-TB cases among HIV patients, even though the correlation between MDR-TB and HIV infection is not very strong in these nations[206,207].

2.3.7 Diagnosis of MDR-TB

For an accurate diagnosis of MDR or XDR TB, DST is essential. Employing phenotypic (or culture-based) methods is the conventional approach to this. From patient sputum, M. tuberculosis is isolated, and its growth in the presence of anti-TB medications is subsequently monitored. Weeks to months may pass when using culture-based techniques. They are mostly unavailable in environments with limited resources because they are costly and challenging to learn.

The diagnosis of MDR-TB has been transformed by genotypic (or molecular) techniques. To identify the genetic mutations known to confer drug resistance, these approaches typically

employ polymerase chain reaction techniques. Notable commercial systems include the INNO-LiPA Rif. TB line probe assay (Inno-genetics Inc., Belgium), the GenoType MTBDRplus and MTBDRsl assays (Hain Lifescience GmbH, Germany), and the GeneXpert System (Xpert MTB/RIF, Cepheid, USA). Other systems are in development as well. Molecular techniques for DST provide results far more quickly than culture-based techniques. Certain commercially available systems require little training and are nearly entirely automated. For these reasons, in environments with limited resources, these systems are becoming more and more the preferred DST method.

Drug resistance cannot be diagnosed using a "gold standard." Molecular testing has the potential to identify low-level resistance- conferring mutations that are clinically significant but are not picked up by culture-based testing. Furthermore, not all mutations known to confer drug resistance can be detected by molecular testing. Even so, physicians are free to choose to routinely cross-check the findings of molecular DST with culture-based DST if the clinical picture so dictates. More than 90% of strains of MDR-TB are also resistant to isoniazid in most countries, so a positive molecular test for rifampicin resistance can specifically be regarded as diagnostic for MDR-TB.

With the increasing availability of molecular DST, a public health strategy of "universal DST," i.e., testing all patients with active TB disease for drug resistance at the onset of therapy, is certainly feasible in the near future. This would be a life-saving and economical approach for any nation where the proportion of new patients with MDR-TB is higher than 1%, according to a WHO analysis[208].

Currently, patients who have risk factors for MDR-TB are given priority for testing because DST is not generally available in many nations. When MDR-TB treatment fails to produce the desired

results, as demonstrated by bacteriological evidence (e.g., consistently positive sputum smears following four months of consistent treatment with a first-line DOTS regimen), empirical treatment may be considered. If a delay in DST is anticipated, experimental treatment for MDR-TB patients' household contacts should also begin[209].

Testing for XDR-TB is recommended for all patients with an MDR-TB diagnosis. This involves checking for resistance to at least one fluoroquinolone as well as the three second-line injectable medications (kanamycin, amikacin, and capreomycin). Conversely, second-line DST-capable laboratories are even less common than first-line DST-capable ones. Patients who have shown no improvement after an injectable and fluoroquinolone-based MDR-TB treatment regimen, as well as those in close proximity to someone who has been diagnosed with XDR-TB or whose treatment with a regimen involving second-line drugs is failing or has failed, should be given priority for second-line DST[210].

The gyrA gene, which confers resistance to fluoroquinolones, and the rrs gene, which confers resistance to injectable drugs, are the two genes that are tested for mutations using the GenoType MTBDRsl assays (Hain Life-science GmbH, Germany). Although it cannot consistently rule out XDR-TB in cases where no genetic mutations are found, this assay can be regarded as a "rule-in" test for second-line drug resistance. This assay's sensitivity and specificity are poorly defined, so culture-based DST ought to be utilized as a confirmatory test[211].

2.4 Role of Iron in MDR-TB

2.4.1 The Importance of Iron to Myobacteria

For many metabolic enzymes, iron serves as a structural and catalytic cofactor, making it an essential nutrient for mycobacteria. Iron is utilized by mycobacteria as a cofactor of enzymes that are involved in the biogenesis of amino acids and nucleic acids, such as those with pyrimidine

synthesis and ribonucleotide reducase activities. It is also utilized by proteins that are involved in electron transport, the tricarboxylic acid cycle, superoxide dismutase, and 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase. More specifically, the genome of *Mycobacterium tuberculosis* encodes at least 40 distinct enzymes for which iron is an obligatory cofactor[212].

The labile iron pool, which is made up of very low levels of ferrous soluble, chelatable iron found in the cytoplasm of cells in mycobacterial host mammals, is present in these cells. Due to its ability to produce reactive oxygen species, the labile iron pool may be hazardous. Because of this, the majority of iron is stored in complexes with iron-binding proteins. The concentration of total serum iron in blood is 10–50 M, while the concentration of free iron is roughly between 10–18 M and 10–12 M. The majority of iron in blood is bound to lactoferrin or transferrin glycoprotein. As a result of internalizing iron via particular cell surface receptors for transferrin, lactoferrin, and hemoglobin-haptoglobin, and recycling iron from senescent erythrocytes, macrophages are known for their high iron flux. This makes macrophages an ideal environment for mycobacteria to acquire iron[213].

Microenvironments known as granulomas are where mycobacteria resist malnutrition, including iron deficiency. On the other hand, long-term survival in the absence of growth is possible for virulent mycobacteria. The development of granulomas in response to *Mycobacterium tuberculosis* infection is diverse and is primarily typified by necrotic caseous granulomas in advanced tuberculosis infection, cavitary granulomas, and solid cellular granulomas[214]. According to transcriptional analysis, solid cellular granulomas express a lot of iron-uptake genes, including hemoglobin receptor, heme binding proteins, and transferrin receptor 1, which is encoded by TFRC; high ferritin and hemeoxygenase expression in cavity granulomas indicates a permissive iron environment. Furthermore, extracellular iron, hemoglobin, and heme

sequesters like transferrin, haptoglobin, and hemopexin, as well as lactoferrin, lipocalin, and calprotectin, are expressed in necrotic and cavity granulomas, indicating an iron deficiency in the host [215].

M. tuberculosis halts replication in environments of prolonged iron starvation, such as granulomas, but maintains metabolic activity with an intact cell envelope, high expression of iron acquisition mbt genes, decreased heme and iron-protein synthesis, and suppressed oxidative phosphorylation[216], among other characteristics. Like all effective pathogens, mycobacteria have evolved complex defenses to transport and store iron, compete with host iron-scavenger proteins for iron acquisition, and obtain iron from intracellular iron pools, lactoferrin, and extracellular transferrin[217].

2.4.2 Siderophores: The Mycobacteria Iron Scavengers

Mycobacteria produce siderophores, which are tiny iron-binding molecules, when they live in low-iron environments. These molecules scavenge metal ions from host insoluble and protein-bound iron because of their strong affinity for iron. Siderophores from non-pathogenic and pathogenic mycobacteria can be separated into two categories. Exochelins are hydrophilic peptidicsiderophores that are extracellular and primarily used by non-pathogenic mycobacteria like *M. smegmatis* and *M. neoaurum.* Another pathogenic *Mycobacterium* that uses siderophoreexochelin for iron acquisition is *M. leprae*[218].

Mycobactins are derived from salicylic acid and include the mycobactin and carboxymycobactinforms of siderophores isolated from pathogenic mycobacteria, such as *M. tuberculosis, M. bovis, M. bovis BCG, M. africanum and M. microti. Mycobactin*is lipophilic and envelope-associated while carboxymycobactin is an extracellular hydrophilic molecule[219; 220]. Desferricarboxymycobactin competes with host iron-binding proteins for iron; it chelates Fe3+

iron bound to host transferrinafter phagosome fusion with early endosomes, as well as from lactoferrin and ferritin. In early endosomalphagosomes, mycobacteria communicate with the endocytic iron uptake system of the host macrophage and take advantage of this source of iron[221,222] · Complexes of iron-siderophores The Msp (Mycobacterium smegmatisporin) family porins, a multisubunit transport system of the mycobacterial outer membrane, are the route by which ferricarboxymycobactins are transported. Ferricarboxymycobactin then delivers iron to the inner membrane-bound iron-regulated transporters A and B (IrtAB) or to the cell-wall associated mycobactin. Iron from internalized ferric-siderophore complexes is reduced to Fe2+ and then released through the action of IrtAB, an ATP-binding cassette transporter synthesized under iron-limited conditions[223]. Desferricarboxymycobactin is exported and recycled through the inner membrane by the MmpS4/MmpL4-MmpS5/MmpL5 transporter complex, which is formed with Mmps, the membrane proteins of mycobacteria. Desferricarboxymycobactin recycling is essential to the survival of bacteria[224]. It is true that *M. tuberculosis* is poisoned by these molecules, which accumulate in mycobacteria due to genetic disruption of the recycling process[225].

The nonribosomal peptide synthetase enzyme system produces mycobactins, and it needs a number of enzymes that are encoded in the two gene clusters mbtA-J and mbtK-N in *M. tuberculosis*[226]. The growth of *Mycobacterium tuberculosis* in mice and macrophages is inhibited by genetic disruption of siderophores expression, indicating the critical role iron acquisition plays in mycobacteria virulence[227]. Secretion systems enable the efficient transport of biomolecules known as virulence factors, which are essential for the pathogenicity of bacteria. Mycobacteria have unique secretion systems known as ESX or Type VII systems, which are necessary for protein transport across the lipid-rich outer membrane barrier. The genesencoding

the ESX-1 system have been identified in the genome of the virulent *M. tuberculosis* strain H37Rv, but these genes are absent from the genome of M. bovis BCG, which corresponds to the genomic region of difference 1 (RD1)[117]. The ESX-3 system is crucial for iron acquisition in *M. tuberculosis and M. smegmatis* contributing to growth and virulence. The esx-3 mutants display severe growth defects in the presence of low concentrations of iron, which can be rescued by the addition of iron or heme[228]. In fact, mycobacterial esx-3 mutant strains produce and accumulate large amounts of mycobactinsiderophores in response to iron deficiency, but they are unable to absorb iron and develop poorly. Mycobactin addition has been demonstrated to rescue the esx-3 mutant growth defect in *M. tuberculosis*[229]. Subsequent research revealed that the esx-3-encoded secreted PE5-PPE4 proteins are essential for iron acquisition, and that the virulence phenotype is correlated with the secretion of the EsxG-EsxH complex, which hinders phagosomal maturation [230–231].

2.4.3 Management of DS-TB and MDR - TB

Resistance to the antimicrobial medication used to treat tuberculosis develops in the bacteria that cause the illness. When tuberculosis does not respond to at least isoniazid and vitamipicin, the two most potent anti-TB medications, it is said to be multidrug resistant.[232"]

At least four second-in-command anti-TB medications, including an injectable medication, and pyrazinamide should be included in the intensive phase of MDR-TB treatment. Isoniazid and vitampicin are essential for the treatment of drug-sensitive patients[233].

2.4.4 Serum C - reactive protein (CRP)

During the examination or treatment of active *tuberculosis*, the status of serum C-reactive protein (CRP) concentration is usually useful. CRP values are influenced by a variety of disease states (e.g., site of disease), host parameters (age, gender, and ethnicity), and mycobacterial features

(strain type). Furthermore, because CRP is a marker of the acute innate immune response, such information could reveal changes in this response based on host and mycobacterial features. CRP promotes phagocytosis by attaching to ligands such as phosphocholine on dead or dying cells and bacteria, activating complement C1q and the classical complement pathway, and activating complement C1q and the classical complement pathway[234]. Synthesis of CRP is largely created in the liver (though some tissue creation occurs) in response to Il6 (plus Il-1 and TNF) secretion, with macrophages serving as the primary source. It has a long genetic history and is largely preserved. Because of its short half-life (approximately 19 hours), serum CRP levels reflect the rate of generation and so can be used as a biomarker for disease activity. Several studies have looked at the utility of CRP testing in TB, with findings suggesting TB has a lower median CRP than bacterial pneumonia and that adding CRP testing to clinical assessment can help manage HIV positive patients in high TB prevalence settings. Diverse TB strains are thought to have co-evolved with distinct human populations, and different MTB lineages can elicit different inflammatory responses in human macrophages in vitro.

In addition, different ethnic groups have documented varied inflammatory responses in active *tuberculosis*. The extent to which these potential effects have an impact on assessments of inflammatory response seen in clinical practice is unknown, as the distribution of CRP results in active TB and how clinical characteristics may influence this has not been investigated in a large patient group[235].

2.4.5 Oxidative Stress

Many disorders, including PTB, have been linked to oxidative stress (OS) caused by an imbalance between reactive oxygen species (ROS) and antioxidant defense mechanisms favoring the former. Increased production of reactive oxygen and nitrogen species (ROS and RNS), as

well as a reduction in antioxidant defense systems, can change this ratio. Changes in cell membranes and other components such as proteins, lipids, lipoproteins, and deoxyribonucleic acid are caused by oxidative stress. Infection with *Mycobacterium tuberculosis* has been shown to cause oxidative stress by increasing the production of reactive oxygen species (ROS) by mononuclear and polymorph nuclear phagocytes[236].

As a result, *Mycobacterium tuberculosis* can initiate and facilitate oxidative damage to macromolecules and cellular structures like DNA, proteins, and lipids. PTB patients have been observed to have a depleted antioxidant state, which could expose the host to oxidative tissue damage. PTB is also linked to an imbalance in the oxidant-antioxidant system, which is necessary for normal lung function. An increase in oxidants combined with a reduction in antioxidant defense could result in lung dysfunction and tissue damage[237].

2.4.6 Complement 3 (C3)

Complement protein C3 is a central molecule in the complement system (an integral participant in the innate mechanisms of immunity) whose activation is essential for all the important functions performed by this system. C3 supports the activation of all the three pathways of complement activation i.e. the classical, alternative, and lectin pathways. Among the complement proteins, it is probably the most versatile and multifunctional molecule identified to date, having evolved structural features that allow it to interact in a specific manner with at least 25 different proteins[238, 239].

Prior studies have established that *Mycobacterium tuberculosis* is phagocytosed by the macrophage complement receptors (CR) CR1, CR3, and CR4 and the mannose receptor. *Mycobacterium tuberculosis* phagocytosis is enhanced in the presence of low concentrations of

non immune serum as a result of binding of complement protein C3 to the surface of the bacteria and increased ligation of CR, although non opsonic binding of *Mycobacterium tuberculosis* to CR3 is also important in phagocytosis[240].

In contrast to blood monocytes, human alveolar macrophages are reported to have more CR4 than CR1 or CR3[241]. Consistent with these data, antibodies to CR4 blocked phagocytosis of *Mycobacterium tuberculosis* to a greater extent in human alveolar macrophages than did antibodies to CR1 and CR3, whereas the opposite was true for phagocytosis by blood monocytes[242]. It is likely that specific proteins of the complement system as well as their proteolytic forms that are bound to *Mycobacterium tuberculosis* may affect the subsequent fate of the organism by directing the pathogen to particular receptors and altering phagocytosis.

Proteolytic cleavage forms of the complement proteins are generated upon activation of the complement cascade. Three activation pathways have been described: the classical pathway, the alternative pathway, and the lectin pathway. Each of these pathways is initiated by specific stimuli and serves distinct but not exclusive roles in innate immunity[243]. Although CR and the complement system have been studied in some detail, less is known about the mechanisms of complement protein binding to *Mycobacterium tuberculosis* or its role in the initial pathogenesis of the disease. The first encounter of *Mycobacterium tuberculosis* with its human host is in the airspaces of the lung, where interactions between the bacteria and airway surface components may affect the initial pathogenesis of the disease. Alveolar cells (macrophages and epithelial cells) produce several proteins of the classical and alternative pathways of complement[244], and complement proteins have been detected in the bronchoalveolar lavage (BAL) fluid from several different mammalian species including humans[245]. Prior studies indicate that functional

complement activity is present in the lung, although C3 activation is reduced when compared to the level of activity in serum, possibly due to the presence of an inhibitor[246].

Additionally, classical pathway activity is more readily detected than alternative pathway activity, possibly due to very low levels of alternative pathway components in lavage fluid[247].

2.4.7 Matrix Metalloproteinase

MMPs (matrix metalloproteinases) are a class of proteolytic enzymes with a variety of physiologic functions that have been linked to the pathophysiology of PTB. Because matrix metalloproteinases may degrade all fibrillary components of the extracellular matrix, they have been linked to the pathophysiology of lung tissue injury and cavitation. They aid in the transfer of *Mycobacterium tuberculosis* from the lung parenchyma to the airways, making transmission easier. MMP levels are linked to sputum smear status, radiographic evidence of disease extent, and cavitary illness, among other indicators of TB disease severity. MMPs are also known to have an anti-inflammatory effect. Tissue inhibitors of matrix metalloproteinases (TIMPs) are endogenous MMPs17 regulators that have also been identified as key biomarkers of TB disease severity. MMPs and TIMPs are both indicators for extra pulmonary *tuberculosis*[248].

In people with active *tuberculosis*, several MMPs are elevated in their blood, sputum, and bronchoalveolar lavage. MMP levels have also been linked to lung pathology, with plasma concentrations of numerous MMPs linked to radiologic abnormalities. MMPs are also recognized biomarkers of disease severity and therapy responses in patients with TB and diabetes[249].

2.4.8 Interleukin-12

Interleukin (IL)-12 is a multifunctional cytokine that regulates cell-mediated immune responses by causing naive CD4+ T cells to differentiate into type 1 helper T cells (Th1) that produce interferon-g.

When compared to healthy tuberculin-reactors (HTR), patients with active *tuberculosis* (TB) generally have poor protective response to mycobacterial antigens (Ag) and low production of the Th1 cytokine gamma interferon (IFN-g). Interleukin-12 (IL-12) and interleukin-18 (IL-18) are regarded to be the major mediators skewing the immune response toward a Th1 cytokine profile at the moment. IL-12 is a 70-kDa heterodimeric cytokine made up of covalently linked p35 and p40 chains. It was first discovered to aid in the development of cytolytic T-lymphocytes and the increase of natural killer (NK) cell function. It is required for T cells to produce IFN-g, and mice lacking IL-12 are more susceptible to *Mycobacterium tuberculosis* infection. IFN-g-inducing factor (IL-18) is a newly discovered cytokine. It increases IFN-g and granulocyte-macrophage colony-stimulating factor production in Th1 cells while inhibiting IL-10 synthesis. The action of IL-18 in activating NK cells and polarizing T cells toward Th1 cell activity is similar to that of IL-12. Furthermore, IL-12 and IL-18 have a synergistic effect on the generation of IFN-g by anti-CD3-activated T cells. Furthermore, a recent study found that IL-12 and IL-18 have a synergistic impact in boosting *Mycobacterium leprae-specific* Th1 responses[250].

IL-18, on the other hand, has a different effect in the regulation of gene expression in NK and T cells and may act as a potent co-inducer of Th1 or Th2 cytokines. *Tuberculosis* and the immune response to infection are currently of significant interest, as these proteins are particularly important candidates for the formation of protective immunity as well as clinical signs and consequences of the disease[251].

2.4.9 Function of the IL-12 cytokine family

The biological functions of IL-12 are pivotal in both innate resistance and adaptive immunity through activation of the JAKSTAT signaling pathway. It induces IFN-g production from CD4+ T cells, natural killer (NK) cells, and NKT cells in the early phases of the immune response and induces the differentiation of CD4+ T cells into Th1 effectors, relevant in protection against bacterial infection. Based on Mendelian vulnerability to mycobacterial and other infectious diseases in children with genetic abnormalities of the IL-12/23–IFN-g circuit, a protective role for the Th1 response induced by IL-12/IL-23 has been proposed. 21IL-12 also triggers a flip in immunoglobulin isotypes by acting on B cells both directly and indirectly via T-cell-derived IFN-g, resulting in increased production of IgG2a antibodies and suppression of IgE and IgG1 synthesis[252]. Findings show that IL-12 may play a role in CD8+ T cell development as a third signal. Another study shows that IL-12 has a crucial role in the reactivation and survival of memory CD4+ T cells. Furthermore, the involvement of IL-12p40 as an antagonist of IL-12p70 biological activity has been identified as a result of its binding to the b1 subunit of the common IL-12 receptor. Due to the low affinity of the human p40 homodimer for the IL-12 receptor, this process may only work in mice cells and not in human cells. In addition, IL-12p40 suppresses IL-23-mediated activities[253].

The IL-12p40 cytokine, on the other hand, has been shown to have agonist action in beginning the immune response. It has been shown that IL-12p40 can operate as a chemotactic molecule for macrophages in this regard. IL-12p40 is also essential for dendritic cell migration after M. tuberculosis infection, according to research. More recent findings have shown that this biological role of IL-12p40 is associated with enhanced synthesis of the chemokines CCL1 and

CCL17, implying that one of the primary mechanisms by which IL-12 enhances cellular activation is by increasing chemokine production[254].

2.4.10 Role of the IL-12 cytokine family in the control of cellular immunity against mycobacterial infection

The importance of the IL-12 cytokine family in tuberculosis immunity has been demonstrated by the fact that the absence of IL-12p40 or IL-12Rb1 has a significant impact on TB incidence. In this respect, IL-12p40 has been shown to have a role as a mycobacterial challenge inducer of dendritic cell migration in the periphery. These findings are backed up by a study that found that dendritic cells that express IL-12p40 are more effective at migrating to the draining lymph node and triggering T-cell responses than dendritic cells that don't[255].

Furthermore, a research study demonstrated that delivering rIL-12p80 to IL-12p40-deficient mice during infection with the attenuated *Mycobacterium bovis* BCG vaccine restores delayed type hypersensitivity and antibacterial responses to the levels seen in IL-12 deficient mice during infection with the attenuated *Mycobacterium bovis* BCG vaccine[256]. Recently, a researcher reported that IL-23 compensates for the lack of IL-12p70 and that this IL-12 cytokine family member is required for the IL-17 response during TB. The fact that the presence of IL-12 is required for the preservation of pulmonary Th1 effectors function in chronic TB backs up this observation[257]. Clearance of *Mycobacterium tuberculosis* infection is induced by IFN-g-producing T-cells, according to previous research. Furthermore, animals with this mycobacterial infection showed antigen-specific immune responses, but they were not as effective in controlling the infection as wild-type mice were *Mycobacterium bovis* BCG also activates the IL-12/IFN-g axis via the NF-kB essential modulator (NEMO) gene[258]. More recently, a study

found that NEMO/NF-kB-mediated induction of IL-12 plays an important role in immune protection against mycobacteria[259].

2.4.11 Interferon-Gamma Release

IFN-γ release assays (IGRAs) are used to diagnose *tuberculosis* (TB), but not to monitor therapy response. IFN-γ levels in EPTB patients did not vary over time or in response to baseline culture or medication resistance status. When previously primed effector T-cells are re-exposed to *Mycobacterium tuberculosis* -specific antigens, they create interferon-gamma (IFN-γ), and measuring this host response to IFN-γ is extensively employed to diagnosis latent *tuberculosis* infection (LTBI) [260].

The value of monitoring IFN-γ level changes over time in the treatment of active *tuberculosis* and several research have looked at its relationship with treatment response. IFN-γ levels have been shown to decrease following therapy in some studies, while others have showed an increase or no change depending on the response to treatment. In response to treatment, Grange et al, 1984 predicted that IFN-γ levels would decrease over time. They tested this hypothesis in a prospective natural history study (NCT01071603) at the Henan Provincial Chest Hospital in Zhengzhou, Henan, China, whose primary goal was to improve TB diagnosis and testing by determining the number and proportion of patients admitted with suspected TB who actually had TB. In tuberculosis, the albumin inflammatory response, which is generally assessed by acute phase reactants and other indicators was described.

Acute phase reaction refers to changes in serum protein profile and cellular immune response caused by infection, inflammation, or trauma, and includes symptoms such as fever, fatigue, and general malaise. CRP, haptoglobin, alpha-1-acid glycoprotein (AAGP), caeruloplasmin,

fibrinogen, and alpha-1-antitrypsin (AAT) are all acute phase proteins. T-lymphocytes, macrophages, neutrophils, and antibodies are all involved in *tuberculosis* immunity. Grange et al, in 1984, reveal that acute phase proteins in pulmonary *tuberculosis* have yielded mixed results, except for transferrin, AAT, -2-macroglobulin, AAGP, CRP, caeruloplasmin, and haptoglobin were shown to be considerably enhanced in tuberculosis. Others discovered that in PTB patients, CRP, caeruloplasmin, haptoglobin, and alpha-1-acid glycoprotein levels were lowered in response to treatment. The levels of - 2-macroglobulin (A2MG), transferrin (TRF), and haptoglobin (HPT), total protein (TP), and albumin (ALB) in newly diagnosed PTB patients and those on chemotheraphy were determined in this study[261].

2.5 Resident Alveolar Macrophages (AM)

2.5.1 The AM response to MTB

It is now widely accepted that AM, like many tissue resident macrophages, is derived from embryologically distinct progenitors (yolk sac) that can proliferate locally rather than blood-borne monocytes. Due to the large amounts of darkly staining particulate matter indicative of their key phagocytic function of surveying the external environment and maintaining homeostasis, AMs were first dubbed "Dust-cells". These characteristics, however, make these cells vulnerable to MTB infection because they express a wide range of phagocytic scavenger receptors (SRs), which MTB uses to enter phagocytes. MTB infection thrives in the lung's immuno-friendly environment, which is aided by both AM and AEC-derived TGF and the Vitamin A metabolite all-trans Retinoic Acid, which drives anti-inflammatory and regulatory T-cell activation. The lipoprotein mixture that modulates lung compliance, pulmonary surfactant, has an effect on the AM phenotype as well[262]. This combination of lung characteristics promotes disease tolerance, and AMs have a profile that allows them to maintain a stable lung immuno-

tolerant environment. AMs, on the other hand, have a large number of surface and endosomal TLRs that detect mycobacterial epitopes and trigger pro-inflammatory responses. MTB has perfected techniques to evade and suppress this, the most important of which is to prevent phago-lysosomal fusion, which allows it to avoid destruction and establish an intracellular niche for survival. Although AMs' responsiveness appears to be dictated by their expression of surface receptors and interactions with the immuno-tolerant pulmonary environment, researchers are now looking into their metabolic activity, which could explain why they fail to effectively contain MTB during the early stages of infection[263].

2.5.2 Macrophage Activation

The activation of macrophages is a key aspect of TB. The macrophage invaded by Tubercle bacilli undergoes a change from a resting to hyper-activated state, with enhanced oxygen uptake, enlargement, increased number of organelles (mitochondria, lysosomes, and ribosomes), total protein synthesis, and phagocytosis rate and speed. Interferon gamma, inflammatory mediators, and exogenous compounds like polyinosinic-cytidylic acid all aid the transition from resting to hyper-activated macrophage. The ability of hyper-activated macrophages to eliminate intracellular microorganisms reflects these functional alterations. When an active macrophage's phagocytosis fails, hydrogen peroxide (H2O2) is released[264].

According to broad in-vitro classifications used to identify opposing functional programs, macrophage biology has traditionally held that macrophages exist on two metabolic spectrums. Even in the presence of oxygen, classically activated macrophages, which are generated in vitro by treating naive human blood or mouse bone marrow derived macrophages (hMDM or BMDM). While M1 and M2 represent two ends of the cellular metabolism continuum, they are helpful

benchmarks for defining the changes in macrophage metabolism and phenotype found during *Mycobacterium tuberculosis* infection[265].

2.6 T-Cell Responses in *Tuberculosis*

2.6.1 T-cells and MTB Containment

A significant T-cell response can be found 6-8 weeks after infection during a nascent MTB illness. This immune response does not appear to be linked to infection clearance, according to epidemiological findings. Rather, in clinical practice, this response is utilized as a proxy for continued infection, with tuberculin skin-test (TST) positive individuals receiving antibiotic treatment because they are suspected of harboring live MTB. The size of infected contacts' TST responses is indistinguishable from infected contacts without disease among the minority of infected contacts (5-10%) who advance to active pulmonary TB disease, indicating that this immune response does not correlate with pathogen killing. In fact, studies that have enumerated interferon-gamma generating cells that respond to MTB specific antigens have found that a higher T-cell response, rather than protection, corresponds with illness. In disseminated types of TB, such as miliary TB and meningeal TB, a much lower proportion of patients with TB illness have a meaningful TST response. Extra pulmonary TB is more common in CD4 T-cell-deficient HIV-positive patients who have been exposed to MTB, which is consistent with this. As a result, whereas epidemiologic research suggests a link between T-cell responses and infection containment, there is no evidence that these responses are linked to MTB elimination[266, 267].

MTB has co-evolved with humans to establish an evolutionary trade-off that rarely sacrifices host fitness in the interest of survival. Rapid mutations in a pathogen's genome (for example, the influenza virus) are usually used as an evolutionary tactic to surpass host immunity. MTB genomes, on the other hand, have a modest mutation rate, and human T-cell epitopes of MTB

show no more sequence variation than genes essential for pathogen survival, implying that MTB genomes are not being selected for evasion of T-cell recognition. T-cells have been implicated in the transmission of tuberculosis by assisting in the establishment of cavitary lung disease. Furthermore, necrotic granulomas cause a mouse strain (C3HeB/FeJ) that develops powerful T-cell responses to succumb to experimental MTB infection. In addition to the research mentioned above, boosting T-cell responses above the level of natural immunity development may not provide greater protection. Despite the fact that MVA85A (recombinant vaccinia Ankara-expressing Ag85A), a TB vaccine candidate, induces increased T-cell mediated immunity, there was no detectable protection against TB illness in a phase IIb infant clinical trial, Similarly, a major randomized adult clinical trial comparing BCG revaccination with H4:IC31 (a potential subunit vaccine that contains both Ag85B and TB10.4 MTB antigens) found that the BCG group received significantly better protection. However, genetic investigations have revealed that prominent MTB T-cell antigens (e.g., ESAT-6, CFP-10, MPT64, MPT70, MPT83) are absent from BCG strains used in clinical trials, which is consistent with the clinical observation that there was no correlation between the proportion of subjects who converted their TST and subsequent protection against TB in BCG trials. Furthermore, while it was proven that BCG elicited enhanced T-cell responses in human studies, there was no evidence that these responses were linked to protection[268].

Although adjuvants' potential to stimulate effector T and B cell development is the basis for their use in non-live vaccines (e.g., M72 in tuberculosis), the direct impact of adjuvants on innate immune cells via pattern recognition receptors (PRR) and innate immunity has been overlooked thus far. Adjuvant containing a variety of PRR agonists have boosted the effectiveness of various human vaccinations dramatically. Immunologists have long investigated host-resistance to

infections, which focuses on pathogen identification and killing. Tolerance of infection, as well as management of tissue damage, appears to be important for host defense against infections. While disease tolerance is widely understood in plants, its importance in mammalian host defense has only lately been recognized. It was discovered (unpublished data) that cyclophilin D, a mitochondrial protein, is a crucial molecular mediator of T-cell-mediated TB host-tolerance[269,270].

2.6.2 Contribution of T-cells to Disease Tolerance

The discovery of mutations in the IL-12/IFN/STAT1 axis that cause disseminated mycobacterial infections, known as Mendelian Susceptibility to Mycobacterial Disease (MSMD), as well as the susceptibility of T-cell-deficient hosts to mycobacterial infections, established the dogma that IFN-producing T-cells play a critical role in host resistance to *tuberculosis* (TB). T-cells/IFN-γ, on the other hand, do not appear to protect against MTB directly, but rather restrict infection via modulation of the inflammatory response. During the chronic phase of MTB infection, IFN-γ has been found to decrease pulmonary neutrophilic inflammation, preventing lung tissue damage. Increased lung pathology and mortality occur from high neutrophil numbers, which cause a robust inflammatory response. Importantly, neutrophil depletion increased the survival of IFNR -/- animals. In mice, NHPs, and humans, the role of neutrophils in immune-pathology during MTB infection has been well documented[271].

These findings suggest that the IFN pathway is more important in the modulation of inflammatory signals and disease tolerance than in the regulation of host resistance. In addition, dysregulated T-cell responses appear to be harmful to the host by causing overt immunological disease. Constant exposure of T-cells to antigens and inflammatory cytokines during persistent viral infection has been well shown to result in T-cell fatigue, a phenomenon known as "T-cell

exhaustion". Programmed cell death is one of the well-defined processes in T-cell exhaustion (PD1). T-cell proliferation and cytokine production are inhibited by the interaction between PD1 and its ligands PDL-1 and PDL-2, which are expressed on antigen-experienced T-cells. Thus, it was anticipated that inhibiting PD1 signaling would protect against persistent MTB infection by "reviving" T-cell-mediated immunity. Genetically disrupting PD1 is signaling or using neutralizing antibodies to disrupt PD1 signaling greatly improved T-cell-mediated immunity to MTB infection, this was accompanied with an increase in bacterial infection. While disrupting PD1 signaling either genetically or through neutralizing antibodies improved T-cell-mediated immunity to MTB infection, it was linked with increased bacterial growth, extensive pulmonary immune-pathology, and decreased survival. As a result, the regulatory mechanisms that control the development and contraction of T-cell responses become a key predictor of TB infection outcome. While the surface expression of several of these markers on T-cells (e.g., PD1 or KLRG) appears to be important in determining their functional role during infection, the intrinsic immune-regulatory mechanisms of T-cells remain poorly understood[272, 273].

2.6.3 T-cell Immune-Metabolism

Mitochondria quickly convert from catabolism to anabolism to fulfill the metabolic demands of active cells, supplying biosynthetic intermediates that are particularly crucial for lymphocyte activity. Nave T-cells have a low metabolic rate, which is defined by poor food intake and production. The energetically efficient mechanisms OXPHOS and fatty acid oxidation (FAO) provide ATP to these cells. TCR activation, on the other hand, triggers a massive metabolic reprogramming to provide the extra energy required for T-cell proliferation, differentiation, and cytokine generation. Activated T-cells boost food absorption and switch from OXPHOS and FAO to aerobic glycolysis to ensure appropriate metabolic resources are available. While

inefficient in terms of energy, glycolysis allows cells to quickly create ATP and other biosynthetic precursors required for cell growth and proliferation. The "Warburg Effect" refers to the switch from primarily OXPHOS to aerobic glycolysis notwithstanding the presence of ample oxygen. The inflammatory and anti-inflammatory roles of immune cells are also strongly linked to metabolic shifts from OXPHOS to glycolysis or vice versa[274].

Mitochondria also play a part in the cell death program, which is as critical. Cyclophilin D (CypD) is a conserved protein found in the mitochondrial matrix and belongs to the cyclophilin protein family. CypD regulates the mitochondrial permeability transition pore (MPTP), which permits the flow of solutes and water from the cytoplasm into the mitochondria, and hence plays a vital role in necrosis. Macrophage necrosis is thought to be a way for MTB to leave the body[275]. An *in vitro* study revealed that pharmacological inhibition of CypD in human macrophages resulted in the suppression of necrosis and the reduction of MTB proliferation. This finding was subsequently expanded to zebrafish and mouse *tuberculosis* models, where genetic blocking of CypD avoided macrophage necrosis and improved their anti-mycobacterial capacity[276]. It was first postulated that CypD-deficient mice (CypD -/-) are resistant to MTB infection because of the role of CypD in macrophage immunity to MTB infection. Surprisingly, despite identical amounts of pathogens in both groups, CypD -/-mice were far more susceptible to MTB infection than control mice. It was also discovered that this susceptibility was linked to an increased T-cell response, which encouraged lung immune-pathology regardless of host resistance. The CypD modulates T-cell metabolism and illness tolerance in *tuberculosis* (TB)[277]. Similarly, the C3HeB/FeJ mouse strain, which has a strong T-cell response to MTB infection, succumbs to necrotic granulomas and dies fast. Although it is unknown why the functional role of CypD differs in macrophages and T-cells, we believe that because T-cells are intrinsically designed to

proliferate, the functional role of CypD in these cells is wired to govern metabolism and proliferation rather than cell death. This shows that T-cells are a double-edged sword: while they are required to initiate granuloma formation during the early stages of MTB infection and prevent disease spread, they also play an important role in MTB transmission by promoting granuloma necrosis during the active phase of the disease. The function and location of these effector immune cells in TB are important factors of host resistance and disease tolerance[278, 279].

2.7 Micronutrients

Micronutrients are materials and vitamins that the body needs in a small quantity. They are very important component of diet needed for cellular and molecular activity, they play a very important role in the maintenance, metabolism of body tissue and imuume functions, insufficient amount of micronutrients may give rise for opportunistic infection. Therefore, their sufficient level in human in very critical for body development.

Tuberculosis and micronutrients are connected in a compound relationship. Tuberculosis may cause a reduction in the body absorption of nutrients or under nutrient through increased in nutrient metabolism[1].

2.7.1 Zinc

Zinc is an essential micro nutrient which cannot be produced or stored in the body. It can only be gotten through constant diet that contain Zinc. Zinc supplementation can be obtained from whole grains, milk products, cashews, almonds baked beans, oysters, chickpeas and poultry. Some cereals are also fortified with zinc for children, fruits like guavas, avocados, peaches and apricots are very rich in zinc[280].

Zinc has been proven to be critical for skin and mucosal cell structural and functional integrity. Zinc, along with vitamin C and iron, may aid in the synthesis of interferon's (IFNs), which help

to stop viral replication. T lymphocytes require zinc for their development, differentiation, and activation, while iron, copper, and selenium are required for their differentiation and proliferation[281].

Zinc has also been demonstrated to improve the phagocytic activity of peritoneal macrophages against Escherichia coli and Staphylococcus aureus[84].

The deficiency of zinc causes impaired cell-mediated immunity and compromise neutrophil functions, its deficiency can also increase human susceptibility to tuberculosis infection.

The benefit of zinc in human may effectively reduce inflammation and increase the immune health in man, it also reduces the risk of age related disease, increasing the healing of wounds, zinc is also known to increase skin condition[282].

2.7.2 Selenium

Selenium is a component of various proteins and enzymes which help to make DNA and also protect cell against damage and infection. These components of enzymes are protein is called selenoproteins. Selenium is a mineral that is found in soil, naturally found in water and some foods. It is an important factor and body metabolism process, it increases the effects of anti-oxidant in human body. Crab, fish, poultry and wheat are good success of selenium[283].

Selenium is required for the function of seleno-proteins, which operate as redox regulators and cellular antioxidants, and are thus critical for leukocyte and NK cell function. It has been demonstrated that supplementing people with selenium (50 or 100 g per day for 15 weeks) boosts IFN- production whereas vitamin A inhibits it[284].

Selenium is a necessary micronutrient for development and a range of physiological activities, including immunological responses. The immune system relies on proper dietary selenium intake, and selenium's biological effects are mostly mediated via its incorporation into seleno-proteins[285].

2.7.3 Copper

Copper is an essential trace element for growth and development of human and organism. It is an important food component that has a role in a number of physiological processes in the human body. Copper is required for newborn growth, host defense mechanisms, bone strength, mature red and white blood cells, iron transport, cholesterol, glucose metabolism, and myocardial infarction. Drugs with potentials to enhance clotting factors may influence copper metabolism. Metallothioneins, cytochrome oxidase, lysyl oxidase, superoxide dismutase, ceruloplasmin, and metallothioneins are all copper-containing enzymes that operate in redox processes[286]. While copper has several biological benefits, too much of it is toxic and may delay the progression of *Mycobacterium tuberculosis*. Organs in meat such as liver are highly nutritious in copper, nuts, lobster, leafy green vegetable, sashew, Avocado are all very rich in copper. Insufficient copper in the body may result to fatigue, weakness, weak and brittle bones, pale skin, premature gray hair and vision loss[287].

M. tuberculosis is transmitted from person-to-person via aerosolized droplets created by coughing or sneezing, and only naturally infects humans. Although one might assume that *M. tuberculosis* does not have to adapt to widely variable external conditions like other microbes in the environment, the human body provides numerous challenges to which *M. tuberculosis* must adjust. These include toxic chemicals (reactive oxygen and nitrogen species), changes in temperature (*e.g.* from the inside of a human macrophage to an aerosol droplet in transit to the next host; fever), and accessibility to nutrients. Over the years the concept of "nutritional immunity" has become increasingly appreciated; several studies have determined that an infected host can sequester essential metals to prevent microbial pathogenesis[319]. For example, Fe sequestration by mammalian host proteins during infections has been appreciated for decades.

More recently, it has been determined that calprotectin, which is present in abundant amounts in neutrophils, is recruited to sites of infection to sequester Zn and Mn during *Staphylococcus* and *Salmonella* infections[288,289]. Strikingly, several studies almost simultaneously determined that the toxicity of excess Cu plays a critical role in suppressing *M. tuberculosis* infections and that different pathways contribute to Cu resistance in *M. tuberculosis*[290,291,292]. Thus, in contrast to Fe, Mn, and Zn, Cu appears to co-localize with bacteria to limit their growth[293,294].

The first evidence that Cu may play an important function during tuberculosis came from transcriptional analysis of *M. tuberculosis* grown in mice *versus* in culture. Several genes encoding putative cation transporters, which are integral cytoplasmic membrane proteins, are more highly expressed in bacteria isolated from mice than from broth culture and are found in an *in vivo* expressed genomic island[295]. One of the genes in this locus was eventually determined to encode a Cu-sensing transcription factor known as CsoR for copper-sensitive operon repressor[296]. CsoR is the founding member of a family of Cu-sensing transcriptional repressors in numerous Gram-positive and acid-fast bacterial species, and has a novel distinctive helical structure. In *M. tuberculosis*, CsoR binds to operator sequences as a dimer, with each monomer coordinating a +1 Cu ion (Cu^{+}). Later studies determined that CsoR regulates a single promoter, *csoRp*, which controls the expression of a four-gene operon. Metallation of CsoR leads to a derepression of this operon, resulting in its expression in the presence of Cu. Included in this operon is *ctpV* (cation transport protein V), one of the cation transporter genes identified in the *in vivo* expressed genomic island[297,299]. Several years earlier, *ctpV* was identified as a gene that is up-regulated in cultured human macrophages, further suggesting that it may be important during infection[300]. Ultimately, *ctpV* was deleted and

disrupted from *M. tuberculosis* and shown to have a Cu-sensitive phenotype *in vitro* and an attenuated growth phenotype in a guinea pig infection model. Interestingly, a *ctpV* mutant does not have a phenotype in a mouse model of infection; however, tuberculosis infections in mice do not always represent all aspects the human disease[301].

Based on the finding that a Cu-sensing regulator was induced in mice, Talaat and co-workers hypothesized that Cu may induce the expression of other genes important for the pathogenesis of *M. tuberculosis*[302]. Numerous genes are strongly induced when *M. tuberculosis* is treated with Cu sulfate, including several transcription factors and many genes of unknown function[303].

At about the same time Talaat's group concluded that Cu played a role during tuberculosis infections, one of the first bacterial metallothioneins was discovered in *M. tuberculosis*[304,305]. Metallothioneins are generally small, cysteine-rich proteins that bind metal ions and protect cells from metal overload. Nathan and colleagues determined that expression of *mymT* (*Mycobacterium* metallothionein) in the non-pathogenic saprophyte *Mycobacterium smegmatis* confers resistance to a compound called ebselen (2-phenyl-1,2-bensioselenazol-3(2*H*)-one). Because Ebselen was known to interact with metallothioneins, this led to the hypothesis that MymT was a metallothionein. Indeed, the Nathan laboratory determined that MymT binds up to six Cu^{+} ions and protects *M. tuberculosis* from Cu toxicity *in vitro*[306,307]. Although deletion of *mymT* results in increased Cu sensitivity *in vitro*, a *mymT* mutant is as virulent as wild type *M. tuberculosis* in mice[308].

Mycobacteria have an unusual and non-canonical outer membrane referred to as the mycobacterial outer membrane ("MOM"). Although this feature might categorize *Mycobacterium* as a Gram-negative genus, mycobacteria do not colorize properly using Gram-staining methods and are thus referred to as "acid-fast" organisms[309]. Despite

lacking a traditional Gram-negative outer membrane, the Niederweis group[310] determined that some mycobacteria possess outer membrane channel-forming proteins including porins. Among the putative MOM proteins, the Niederweis group identified MctB (mycobacterial copper transport protein B), which is required for Cu resistance. Although it was initially believed to be a MOM protein, a more recent study suggests that it is a cytoplasmic membrane protein[311]. The link between MctB function and Cu resistance was a fortuitous discovery; deletion of *mctB* in *M. smegmatis* results in a strong growth defect when the bacteria are inoculated onto a specialized mycobacterial medium called Middlebrook 7H10 ("7H10") agar, but not when they are inoculated onto Luria-Bertani (LB) agar. Niederweis and colleagues ultimately determined that it was the presence of Cu in 7H10 agar that limited the growth of the *mctB* mutant. A deletion mutation in *mctB* of *M. tuberculosis*, a species that does not grow on LB agar, also results in a growth defect on 7H10 agar. More importantly, the *mctB* mutant is less fit for growth in mice and guinea pigs. Similar to the *ctpV* data, the attenuated phenotype of the *mctB* mutant is more dramatic in guinea pigs than in mice. Supplementation of the animals' drinking water with Cu, however, decreases the bacterial burden of the *mctB* mutant, whereas the parental *M. tuberculosis* strain is not dramatically affected for growth under these conditions.

A researcher determined that MctB is important for maintaining normal Cu levels in the cytosol of mycobacteria[312]. Interestingly, the authors of this study also determined that *M. tuberculosis* has about 100 times less Cu in the cytosol than *M. smegmatis*. It is possible that this reflects proteomic differences between the bacteria; *M. smegmatis* has a genome that is about twice the size of the *M. tuberculosis* genome, and may thus encode more Cu-binding proteins or other Cu-chelating molecules[312].

More recently, a group of scientist determined that porins are important for Cu homeostasis in *M. smegmatis*[313]. Although porin genes have not yet been identified in *M. tuberculosis*, there is some evidence to suggest this pathogen uses one or more porins for Cu uptake. Another writer showed that incubation of *M. tuberculosis* with spermine, which can block porin-mediated transport, protects bacteria in media with otherwise toxic Cu levels, suggesting that one or more porins exist in *M. tuberculosis* that allow Cu into this bacterium[313].

In yet another independent but coincidental study, my laboratory identified a third Cu homeostasis system called the regulated in copper repressor (RicR) regulon[314]. In a microarray analysis of three *M. tuberculosis* H37Rv strains defective for proteasomal protein degradation, my colleagues and I identified several genes that are repressed for expression when compared with expression in a wild type strain. Proteasome-dependent protein degradation is essential for the pathogenesis of *M. tuberculosis*, and it is likely that proteolysis is linked to several pathways responsible for virulence. Ultimately, my colleagues and I determined that several of the genes repressed in proteasome-defective *M. tuberculosis* are also highly expressed in the presence of Cu[315]. One gene, RicR (Rv0190), is a homologue of *M. tuberculosis* CsoR. RicR regulates the expression of genes from six different promoters, including its own, distributed throughout the *M. tuberculosis* genome. Under low Cu conditions, RicR represses the expression of the genes encoding a multicopper oxidase (*mmcO*), two putative membrane proteins (*lpqS* and Rv2963), *Mycobacterium* metallothionein (*mymT*), and two putative open reading frames called *socAB*. All of these genes include a palindromic sequence near the −10 region of their promoters that is required for RicR binding and repression in the absence of Cu[315].

RicR is conserved in many Gram-positive bacterial species and, as mentioned previously, highly similar to CsoR. Interestingly, RicR is more similar to CsoR orthologues in other bacteria than *M.*

tuberculosis CsoR. The crystal structure of the RicR orthologue in *Streptomyces lividans* (CsoR) revealed a dimer of tetramers configuration[316]. Based on this, my colleagues and I presume that RicR binds to DNA as a dimer of tetramers in *M. tuberculosis*, with each monomer capable of binding a Cu^{+} ion, leading to their release from DNA.

The RicR regulon genes, with the notable exception of *ricR* itself, are almost all exclusively found in pathogenic mycobacteria, suggesting that the RicR Cu response is important during infections. Almost nothing is known about *lpqS*, a putative lipoprotein gene, or Rv2963, a putative permease gene. Genetic disruption of *lpqS* or Rv2963 alone does not lead to Cu sensitivity, and their roles in pathogenesis are unclear; transposon mutations in either gene result in hypervirulence. However, this phenotype could not be rescued by restoring a wild type copy of the respective genes into the mutants. Thus, it remains to be determined what the functions of these putative membrane proteins are in *M. tuberculosis* physiology.

In contrast to LpqS and Rv2963, MmcO has high similarity to several well characterized multicopper oxidases (MCO)/ferroxidases in other domains of life[317,318]. In eukaryotes from yeast to humans, MCOs oxidize Fe^{2+} to Fe^{3+}, making it less toxic to cells. MCOs such as *Saccharomyces cerevisiae* Fet3p facilitate the receptor-mediated transport of Fe into cells[319]. Interestingly, MmcO is lipidated[320] and, like Fet3p in yeast, is membrane-anchored in *M. tuberculosis*, although most if not all known bacterial MCOs are soluble periplasmic proteins. Fet3p, along with the Fe permease Ftr1p, is involved in Fe transport and also provides Cu resistance in yeast[321]; therefore, my colleagues and I speculated that MmcO may perform similar functions in *M. tuberculosis*. It was previously determined that a related periplasmic MCO in the Gram-negative bacterium *Pseudomonas aeruginosa* was important for Fe acquisition[322]. However, experiments in my laboratory to test this hypothesis have so far proved negative for *M.*

tuberculosis[321]. Furthermore, deletion of *mmcO* in *M. tuberculosis* results in a Cu-sensitive phenotype that is observed using an agar plate-based assay, but not a liquid-based assay. This result differs from the more robust Cu-sensitive phenotype observed with a *mymT* mutant that has phenotypes both in liquid and on solid medium[323]. Like a *mymT* mutant, an *mmcO* mutant is not attenuated for virulence in a mouse infection model[324].

Because *mymT* and *mmcO* mutants have Cu-sensitive phenotypes *in vitro* but no apparent virulence defect *in vivo*, Shi *et al.* tested a double *mymT mmcO* mutant for these phenotypes. Although a double mutant is more sensitive to Cu *in vitro* than either single mutant, it is still as virulent as wild type *M. tuberculosis* in mice[325]. Taken together, it appears that the functions of MymT and MmcO are to combat excess extracellular Cu but that they are not critical for virulence in at least one animal infection model.

MymT was not annotated prior to the study by another scientist, most likely due to its small size (53 amino acids) and lack of homology to any known protein. Like *mymT*, *socAB* was also not annotated in the *M. tuberculosis* H37Rv genome, and only *socB* was annotated as an open reading frame in the *M. tuberculosis* CDC1551 genome. *socAB* encodes what are predicted to be two highly basic small proteins, each of about 50–60 residues. Of all of the RicR-regulated genes, *socAB* are only found in the "tuberculosis complex" of mycobacteria that are generally associated with human and other mammalian infections[326]. A transposon insertion mutation in *socA*, which is likely to inactivate *socB* as well, does not affect Cu resistance or virulence of *M. tuberculosis* H37Rv. Thus, like *lpqS* and Rv2963, the functions of these genes remain to be determined[327].

It is noteworthy that the *socAB* locus is so "alien" that we cannot identify related sequences in any organism or bacteriophage to date. That RicR controls its expression is intriguing. Did the

tuberculosis complex acquire *socAB* to allow it to be a better pathogen in humans and other mammals? Perhaps these genes, as well as others in the RicR regulon, are required during specific phases of infection in higher mammals.

Because inactivation of one or two RicR-regulated genes has no effect on virulence in mice, Shi *et al.* tested the hypothesis that the entire regulon needs to be inactivated to observe a phenotype. Based on the CsoR structure, Shi *et al.* could predict which residues in RicR might be important for coordinating Cu; mutagenesis of one or more of these amino acids could render RicR "Cu-blind" (and thus DNA-bound even in the presence of Cu), resulting in the constitutive repression of the entire regulon. Because RicR represses the *ricR* promoter, Shi *et al.* made a construct where the RicR-binding site was mutated to allow constitutive expression ("$ricRp^c$"), and where the *ricR* coding sequence was mutated to prevent Cu binding ("$RicR_{C38A}$"); thus, $RicR_{C38A}$ would be constitutively expressed to repress the other RicR-regulated promoters. This construct, "$ricRp^c$-$ricR_{C38A}$"results in *M. tuberculosis* that is highly sensitive to Cu *in vitro* and, more importantly, attenuated in mice[328]. Interestingly, a control strain that constitutively expresses wild type *ricR* ("$ricRp^c$-$ricR^+$) is more resistant to Cu than wild type *M. tuberculosis*; this may be due to elevated levels of RicR, which could act as a sink for Cu^+.

It is important to consider that not all Cu-regulated genes may be necessary for Cu homeostasis. For example, Cu may act as a signal to indicate that *M. tuberculosis* has entered an environment that requires the expression of specific genes needed to deal with Cu-independent stresses within a macrophage. Because LpqS, Rv2963, and SocAB do not appear to be required for Cu resistance, perhaps they counteract other yet-to-be-identified antimicrobial factors in the host.

Where exactly does *M. tuberculosis* (or any pathogen) encounter Cu? Several studies in the last few years have strongly indicated that microbes sense Cu and other metals during infections.

Petris and colleagues[329] showed early on that the eukaryotic Cu transporter ATP7A contributes to restricting bacterial growth in cultured macrophages. The Petris group showed that cultured macrophages in which ATP7A gene expression is silenced do not control the growth of a non-pathogenic *Escherichia coli* strain as well as control macrophages can. Because *M. tuberculosis* is mainly found in phagocytic cells, it would not be surprising if ATP7A were important for controlling mycobacterial growth as well, a hypothesis my colleagues and I are currently testing.

Another potential source of Cu is ceruloplasmin (Cp). Cp is a multicopper oxidase that oxidizes Fe^{2+} to Fe^{3+}, and is found in blood plasma and thus in most if not all parts of the body[330]. Although the Cu in Cp has not been previously considered as a source of antimicrobial activity, it could nonetheless have the potential to affect infection. For example, phagocytes may take up Cp, after which phagolysosomal proteases could break down Cp and release its Cu. If bacteria happen to be in the same compartment, the released Cu could potentially kill the microbe in parallel to the ATP7A-dependent mechanism proposed by Petris and colleagues[330]. Because microbes typically induce an immune response that leads to the acidification of the phagolysosomal compartment, one could also imagine that the acidic pH would maintain Cu in its most toxic form (Cu^{+}). Thus, it will be interesting to see if animals that are deficient in Cp are more susceptible to infection by *M. tuberculosis* or other intracellular pathogens such as *Salmonella*.

Although much research has revealed that *M. tuberculosis* uses several independent pathways to deal with Cu toxicity, several key questions remain. For one, *mctB* is not CsoR- or RicR-regulated and therefore represents a third independent Cu resistance pathway. The mechanism of regulation of *mctB* is unknown. Furthermore, the mechanism of action of many Cu-responsive

proteins has yet to be determined. CtpV appears to be required for Cu export, but this has not been shown definitively. All Ctp proteins are P-type ATPases, and there are 11 Ctp proteins in *M. tuberculosis*; although not all of them are regulated in a Cu-dependent manner, we cannot rule out that one or more of them function in Cu efflux. Interestingly, CtpA and CtpB are predicted to be Cu-binding ATPases[331,332]. If they do indeed bind Cu, it is possible these are required for metallating extra-cytoplasmic Cu-binding proteins. Also, it is curious as to why an organism such as *M. tuberculosis* requires two distinct Cu-responsive regulons, which begs to ask if there is a biphasic, fine-tuned, temporal response to Cu in the host. Additionally, a Cu chaperone, with features of well characterized Cu chaperones[333], has not been identified in *M. tuberculosis*. It is possible that there is no protein chaperone, or that there is a non-classical type of chaperone, or that mycobacteria use one or more small molecules such as mycothiol to mobilize Cu. However, this last option seems unlikely as it would not be clear how this could be carefully regulated. Along these lines, although it is generally presumed that most Cu-binding proteins work outside of the cytoplasm, Cu nonetheless enters the bacterial cytoplasm and interacts with proteins such as the transcriptional regulators CsoR and RicR and the metallothionein MymT; how then is the metal displaced from the regulator and disposed of to restore transcriptional repression? It seems unlikely that mycothiol or similar molecules would be sufficient to deal with the necessary rapid changes in Cu-regulated gene expression.

With the advent of improved technologies for quantifying metals in biological systems[334] as well as a growing interest in the role of nutritional immunity in host-pathogen interactions[335], there is little doubt that the understanding of how microbes and their hosts regulate metal homeostasis will lead to the improved treatment of many devastating diseases.

2.7.4 Iron

Low haemoglobin level is a common condition that is associated with *tuberculosis* due to nutritional deficiency, malabsorption syndromes, bone marrow suppression and iron utilization. Study from mice suggests that excess iron may enhance the growth of *Mycobacterium tuberculosis* and may also worsen infection outcome[336]. Low level of iron may also increase the risk of treatment failure. It is found in the body in a range of complex binding proteins, such as transferrin, lactoferrin, and ferritin, and is essential for the growth of practically all bodily systems. Pathogens are concentrated by high similarity up taking mechanisms, which is critical for both parasite and host cell strength. Iron is a critical element for all organisms since it serves as a major component in metabolic pathways and enzyme performance. To prevent free radical damage to proteins, ribonucleic acids, and cell membranes, cells regulate iron reactivity, availability, and instability. Iron is essential, and iron deficiency has been shown to reduce cell proliferation and immunological function. Iron is required for the growth of *tuberculosis* in macrophages, and high iron consumption has been linked to an increase in active *tuberculosis* and mortality[337]. The pathogenesis, development, and metabolism of *tuberculosis* are all dependent on iron obtained from the host's resources. As a result, anemia due to iron deficiency is common among tuberculosis patients. Shellfish, legumes, red meats, turkey, liver and other organs meats are very rich in iron[338].

2.7.5 Vitamin C

Vitamin C is an antioxidant that strengthen body natural defence, it increases blood antioxidant levels up to 30%, which helps the body defence system fight inflammation, vitamin C helps

improve the absorption of iron as a result of this absorption and it helps to reduce the risk of anemia among people prone to iron deficiency. Vitamin is abundant in many fruits and vegetables[339].

Vitamin C is necessary for overall health, as well as immune cell activity. It is not produced by the body on its own, and its requirements are influenced by a variety of factors such as health and age. Vitamin C is required for the production and repair of collagen in epithelial tissues, which serve as the body's natural anti-infection defenses. This antioxidant vitamin encourages healthy cell growth, healthy circulation, and detoxifies the cells of the entire body, among other things. Vitamin C is also the most essential antioxidant in the blood, and large amounts of vitamin C in neutrophils are required to prevent oxidative stress[340].

Despite the fact that mean plasma vitamin C concentrations normally fluctuate around 50 mol/L, neutrophils acquire millimolar vitamin C concentrations against a concentration gradient, indicating that this vitamin plays a significant function during immunological activation. As a result, neutrophils' low vitamin C levels during infectious episodes may jeopardize their function. Meta-analyses have shown that vitamin C intakes of at least 200 mg/day can reduce the incidence of respiratory infections; but, gram dosages of vitamin C are required because to increasing demands for the vitamin during a continuing infection[341].

2.7.6 Vitamin E (Vit E)

Vitamin E protects immune cells from oxidative damage, it enhances immune response by stimulating T-cell differentiation and proliferation. Deficiency of vitamin E increases the risk of being infected with *tuberculosis*. It is found in plant-based oils, nuts, seeds, fruits, vegetables, Red bell pepper, Almonds, sunflower seeds and wheat germ oil[342].

Vitamin E inhibits the formation of prostaglandin E2 (an immunosuppressive substance), as well as modulating cytokine production and lowering histamine levels. It is distinguished from other fat-soluble vitamins by a long isoprenoid side chain attached at the 2 position of a 6-chromanol ring, which confers unique biochemical properties: it is a powerful antioxidant, it supports membrane stability, and it inhibits platelet aggregation. Vitamin E has been demonstrated to have a variety of health benefits, including anti-infection defense. Vitamin E boosts the body's defenses, improves humoral and cell immune responses, and boosts phagocytic functions. Vitamin E is a powerful membrane antioxidant that neutralizes free radicals and protects the structural components of cell membranes from oxidation and it's also involved in T-cell differentiation and proliferation, as well as infection prevention[343].

2.7.7 Vitamin D (Vit D)

Vitamin D may have a key role in the innate and adaptive immunological responses to *Mycobacterium tuberculosis* infection, as well as the disease's progression. The TLRs on macrophages allow *Mycobacterium bacilli* to enter the body. The expression of CYP27B1 oxidase by macrophages is induced by activation of the TLR signaling system and exposure to inflammatory stimuli[99]. This oxidase transforms 25(OH)-Vit. D to the active form 1,25(OH)-Vit. D, which promotes macrophage antimicrobial activity in an autocrine manner via the VDR/RXR signaling pathway, causing endogenous synthesis of cathelicidin hCAP-18 and its antimicrobial peptide LL-37. The LL37 has the ability to bind with bacterial wall molecules and perforate the cytoplasmic membrane, causing the bacterial cell to die[344].

Vit. D appears to cause autophagy in infected macrophages as well. Autophagy is a process that causes phagolysosomes to mature and intracellular pathogens to be degraded. While MTB strives to prevent autophagy[345], MTB lipoproteins promote it by activating the TLR2/1, CD14, and VDR

signaling pathways. Autophagy is prevented when the expression of LL-37 is silenced, which leads us to believe that this antibacterial mechanism is solely dependent on the cathelicidin/LL-37 combination. Another study found that oral administration of 5000 IU Vit. D and 500 mg b.d. phenylbutyrate consistently enhances the production of LL-37 in macrophages and lymphocytes, as well as an increase in macrophage MTB intracellular killing[346].

Vit. D is also essential for the efficiency of MTB infection acquired immunity. Th1 cells secrete IFN, which stimulates macrophages to create antimicrobial chemicals, as well as autophagy, phagosome maturation, and other anti-MTB effects. Th2 cells secrete IL-4 and IL-5, which trigger an antibody-mediated response. These anti-mycobacterial effects do not appear to arise in the absence of Vit. D[347].

2.7.8 Hepicidin

Hepicidin is a hormone that is made in the liver, it is an iron-regulating peptide. It controls the movement of iron to blood plasma from intestinal cells absorbing iron. Therefore, hepicidin is a protein in human that is encoded by the hepcidin antimicrobial peptide (HAMP). The major function of hepcidin is to regulate the entry of iron into humans. Hepcidin is abnormally high in an inflammatory process with low serum iron due to iron trapping within liver cells and macrophages[348].

Hepcidin is also known to alter immunological reaction against *Mycobacterium tuberculosis* infection. It also has the capacity to change or modulate immunological reaction[349].

Iron is an essential element required by almost all organisms, due to its role in a range of key cellular processes such as DNA synthesis and respiration. During infection, iron availability to both host and pathogen may impact on infectious outcome. Since both the host and the pathogen require iron, the pathogen must be able to scavenge iron from the host, and the host alters its iron

distribution in response to infection. This redistribution may serve as a protective mechanism against some pathogens, such as the extracellular bacterium *Vibrio vulnificus*[350], which is termed siderophilic because excess iron enhances its pathogenicity. Host iron status impacts on the severity of many infectious diseases including TB[351], HIV[352], and malaria[353].

In humans, the majority of iron in circulation is derived from the recycling of senescent red blood cells by macrophages and a relatively small amount is obtained daily through the absorption of dietary iron[354]. Since excretion of iron is not modulated, iron storage and release must be tightly regulated in order to prevent iron deficiency, iron toxicity, and to prevent excess iron availability for pathogens. Hepcidin, a 25 amino acid peptide hormone produced in the liver, is a major regulator of iron homeostasis[355]. Hepcidin regulates systemic iron levels as well as the redistribution of tissue iron by binding to the iron exporter ferroportin, causing it to be degraded[356]. Ferroportin is highly expressed on macrophages and enterocytes[357]. Hepcidin decreases serum iron concentrations by redistributing iron into macrophages, and impairing dietary iron absorption. As a result, excess hepcidin can cause anaemia since iron availability for erythropoiesis is decreased. On the other hand, low hepcidin leads to excessively high systemic iron levels as a result of increased dietary iron uptake and iron release from erythrophagocytic macrophages. Hereditary haemochromatosis is an iron overload disorder resulting from genetic mutations leading to defective hepcidin production[358]. Since hepcidin regulates the distribution of iron in the body, it has variable impacts on the outcome of infection, depending on the niche of the pathogen[359]. For example, high hepcidin decreases susceptibility to some blood-borne bacteria[360] and can also protect against liver stage *Plasmodium* infection[361]. Macrophage iron is increased when hepcidin is high, and this could result in increased replication of macrophage-

tropic pathogens. Therefore, changes in hepcidin and iron homeostasis are likely to have entirely different effects on pathogenicity depending on the nature of the pathogen[362,263].

The regulation of hepcidin is complex; a number of factors including iron, erythroid drive, inflammation and hypoxia are involved[364]. Increased systemic iron induces hepcidin via the bone morphogenetic protein (BMP)/SMAD signalling pathway, of which Bmp6 is an essential regulator[365]. Hepcidin is also induced by a wide range of infectious stimuli in mice, for example *Vibrio vulnificus*, Influenza A virus, *Candida albicans*, *Plasmodium* species, and *Salmonella typhimurium*[366,367,368]. During infection, inflammation can induce hepcidin through the interleukin 6 (IL6)/ signal transducer and activator of transcription 3 (STAT3) pathway. Persistent upregulation of hepcidin can lead to anaemia of chronic disease (ACD)[369]. Conversely, anaemia and erythroid drive inhibit hepcidin production at least in part through erythroferrone[370].

Tuberculosis (TB) is an airborne infection, transmitted through the inhalation of *M.tb* infected droplets into the lungs, where it is phagocytosed by resident alveolar macrophages[371,372]. It is well documented that *M.tb* requires iron in order to survive, and iron and haemoglobin have been associated with BCG growth *in vitro*[373], but little is known about the role of hepcidin in *M.tb* infection *in vivo*. Recent studies in humans highlight an associative relationship between high hepcidin and increased susceptibility to TB, although a number of these studies may be confounded by HIV co-infection[374]. In addition, high hepcidin and ferritin have also been associated with increased risk of progression to TB disease in close household contacts of *M.tb* infected individuals[375]. In mice, iron overload in beta-2 microglobulin knockout animals has been associated with increased susceptibility to *M.tb* infection[376]. However, more recently

neither hepcidin knockout mice, nor wild-type mice with increased iron levels were shown to have increased susceptibility to *M.tb*[377].

2.7.9 Beta subunit of RNA Polymerase (rpoB)

rpoB is a universal gene in bacteria. RNA polymerase (RNAP) is an important enzyme in the transcriptional process and is the final target for regulatory pathways controlling gene expression in all living organism. RNAP is solely responsible for synthesizing mRNA, rRNA and tRNA in bacteria. A portion of the MTB gene encoding the rpoB was amplified by PCR using degenerate oligonucleotides and used hybridization probe to isolate plasmide clones carrying the entire rpoB gene of MTB[378].

2.7.10 KatG gene

KatG gene is a protein that plays an important role in antiTB therapy, it is a virulence determinant that activates the prodrug isoniazid (INH), the primary first line antibiotic drug used against TB infection. KatG gene enzmyes function primarily as catalase to remove hydrogen peroxide by dismutation[379].

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Chapter Three

Methodology

The methodological issues discussed in this segment of the thesis include the methods used in collecting data, sources of data, model specifications as well as estimation techniques.

3.1 Sample Population

This study included one hundred (100) *Mycobacterium tuberculosis* positive patients were grouped into four categories.

$$n = \frac{Z^2.P.(1-p)}{d^2}$$

n is the number of sample

Z is the standard deviation at 95% = 1.96

P is the estimated proportion = 8.0%

D is the margin of error = 0.05

$$n = \frac{1.96^2 \times 0.08(0.93)}{(0.05)^2}$$

$$n = \frac{.28}{0.0025}$$

$$n = 114$$

3.2 Research Design

This study adopted experimental research design. This was because the variables were described without any form of manipulation.

3.3 Ethical approval

Ethical approval to carry out this research was given by the Oyo State Research Ethics Review Committee, Ministry of Health, Oyo State Secretariat, Ibadan, Nigeria.

Inclusion criteria

All patients were *Mycobacterium tuberculosis* positive, patients with age-range between 20 to 55 years old were recruited for the study.

Controls

Apparently healthy individuals never tested positive for tuberculosis.

Exclusion criteria

Patients with history of hypertension, diabetes mellitus, HIV-positive patients and pregnant women.

Sample Collection:

Sputum and blood samples were collected from patients attending four governments tuberculosis Clinics designed for the treatment of tuberculosis. The clinics included Government Chest Hospital, Jericho, Oniyanrin Health Centre, Sabo Health Centre and Alafia Hospital. Apparently healthy non-TB individuals served as controls after thorough screening.

One hundred *Mycobacterium tuberculosis* positive patients that were grouped into four caterogies. This study included drug sensitive tuberculosis patients recruited after screening with GenXpert machine. Sputum samples of MDR-TB and DS-TB were cultured and the isolate were used for genetic characterization. Groups i-iii were treated with micronutrient supplementation and anti-TB drugs as an intensive phase for two months.

i. 25 PTB patients with anti-TB drugs plus 25mg zinc supplement.
ii. 25 PTB patients with anti-TB drugs plus 50mcg selenium supplementation.
iii. 25 PTB patients with anti-TB drugs plus 200mg ferrous sulfate (calculate ferrous equiv.).

iv. 25 PTB patients with anti-Tb drugs only.

v. 25 apparently normal Tb free individuals

Participants were screened for the presence of tuberculosis by using ziehl – Neelsen staining techniques to stain the sputum while genxpert was used to categorized patients into *Mycobacterium tuberculosis* negative, drug sensitive *Mycobacterium tuberculosis* positive and multi drug resistant (MDR-TB). Blood samples were collected from all participants who met the inclusion criteria before commencement of treatment and after two months of treatment with anti-TB drugs and micronutrient supplementations.

Seven ml (7ml) of venous blood was collected from each patient as baseline (before treatment). Also, after two months of intensive treatment with anti-TB drug alone and anti-TB drugs plus micronutrients supplementation (groups 1-3), another 7ml of venous blood was collected from each patients. At each time of blood collection, 2.0ml of the seven (7ml) blood sample was poured into EDTA bottle for the analysis of haematological parameters; 3.0ml into lithium heparin bottles for the analysis of trace elements and immunological parameters. 2.0ml of blood was put into Sodium citrate bottle for the analysis of markers of coagulopathy. The sample in the lithium heparin bottle was separated by centrifugation at 3000 revolution per minute and the plasma stored at -20°C until ready for analysis.

3.4 Sputum Analysis

3.4.1 Sputum Analysis using Ziehl-Neelsen Staining Techniques

Principle

When sputum smear is stained with carbol fuchsin, it solubilizes the Lipoidal material present in the mycobacterial cell-wall. When heat is applied, the carbol fuchsin penetrates further through the lipoidal wall and then enters into the cytoplasm, the cell then appears red. This then decolourized with acid alcohol (3% Hcl in 95% alcohol). After this, the smear is countered stained with Methylene Blue. All other cells except MTB will absorbed the counter stain (blue colour), while the tubercle bacilli retained the red colour of the carbol fuchsin[1].

Procedure

A sputum smear (1 x 2cm) was made on clean, grease free slide. Smear was allowed to air dry and gently heat fixed. Smear on the slide was covered with carbohl fuchsin stain. Heat was applied to the slide flooded with stain until vapour just rises. The stain was allowed to remain on the slide for 5 minutes. The stain was washed off under running tap water. It was decolourized with 3% acid alcohol for 30 seconds to 1 minute. It was rinsed with tap water. Methylene blue was added to the smear for 1 – 2 minutes. The slide was then rinsed with tap water and allowed to air-dry. The smear was examined under the microscope using X100 (oil immersion) objective.

Positive result: Bright red straight or slightly curved rods, occurring singly or in small groups, beaded or in clusters[2].

3.4.2 Sputum Analysis using Genexpert

The sputum tubercle bacilli positive samples from Ziehl-Neelsen techniques were further analysed using Genexpert equipment at molecular level to categorise into MTB positive (drug sensitive or MDR) and MTB negative.

Principle:

The genexpert MTB/RIF detects DNA sequences specific for *Mycobacterium tuberculosis* and rifampicin resistance by polymerase chain reaction (PCR). It is based on the Cepheid Genxpert system, a rapid, simple to use nucleic acid amplification tests (NAAT[1]). This diagnostic test (also known as the Xpert M.tb/RIF) test is based on a semi-quantitative, real-time PCR for the detection of *M. tuberculosis* complex DNA in sputum samples and the detection of rifampicin (RIF) resistance associated mutations of the rpoB gene in samples from patients at risk for RIF resistance[3].

3.4.3 Decontamination with n-acetyl l-cysteine-Sodium Hydroxide (NALC/NaOH + Na Citrate) Method:

Tools, reagents and specimens were arranged in a BSC class 2 and the working area was covered with paper towels and sprayed with 5% phenol. 5ml of sputum was transferred into a 50 ml sterile screw-capped conical centrifuge tube. An equal volume of NALC-NaOH/Na Citrate solution was added to specimen. The cap was replaced, tightened and vortex for 20 seconds. The tube was inverted 5 times to ensure that NALC-NaOH solution comes in contacts with the entire surface of the tube. The tubes were allowed to stand at room temperature for 15 minutes for decontamination. Sterile 0.067M phosphate buffer (pH 6.8) was added to 40ml mark of the digested-decontaminated specimen to reduce the continued action of NaOH and lower the viscosity of the mixture. The tube was recapped tighly and inverted several times to mix the contents. It was then centrifuged at 4^{o}C and 3,000xg for 15 minutes by using aerosol from centrifuge safety cups.

After centrifugation, the supernatant was poured off into a splash proof discard container with 5% phenol inside. The sediment was re-suspended in 2ml of phosphate buffered saline (PBS) and vortex to mix[4].

3.4.4 Inoculation: Lowenstein-Jensen and Pyruvic acid medium (PRVT slopes were brought to room temperature and appropriately labelled. The excess condensate carefully removed by inversion. With the aid of sterile plastic disposable pipette, 4 drops (0.2mls) of NaOH/NALC treated sputum were inoculated into LJ and PRVT slopes, allowing the drops to run from the mouth of the tube to the bottom.

The inoculated slopes were loosely capped and incubated in a slant position. After one week, the caps were tightened and the slopes straightened to continue incubation for 8 weeks. Every week, the cultures were checked for the presence of growth

3.5 Identification

The noticeable colonies which may be raised, cream (buffy) termed eugenic growth for *Mycobacterium tuberculosis*, but flat, and yellowish in case of Non Tuberculosis Mycobacteria were smeared on a grease free slide and stained using hot ZN to identify AFB. Slopes with colonies positive for AFB were further tested for.

3.5.1 Rate of Growth: Which had been observed right from the beginning of cultural incubation (fast grower). The slow growers colonies came up at about week four (4).

3.5.2 Temperature at Which Growth Occurs: The two distinct types colonies were sub-cultured in fresh LJ medium and incubated at different temperatures of 25.32 & 36 degree Celcius. See table in the next chapter for result.

3.5.3 Pigment Production: The positive cultures were deliberately left in the light for 2 hours, after which they were re-incubated at 37 degree Celcius overnight. The colonies were examined for the development of a yellow pigment.

3.5.4 Niacin Accumulation Test: This test was performed by adding 1ml o sterile distilled water to the slant of a mature BA, Ogawa and LJ culture media. The culture was placed in a slanting position so that the fluid covers the mycobacterial growth. 0.5 ml of the fluid was removed and added to a clean, screw-capped test tube. 0.5 ml each of 4% alcoholic aniline and 10% aqueous cynogen bromide solutions. It was mixed gently. Yellow colour indicates presence of niacin. Positive and negative controls were run along with the test[5].

3.5.5 Growth on P-Nitrobenzoic Acid (PNB) Medium

One loopful of growth was picked with 3mm diameter-loop and mixed with 5ml of sterile distilled water (4mg/ml-suspension). Then, 0.1ml of the suspension was inoculated onto two slants of PNB containing and PNB free media. The slants were incubated at 37^{o}C for 4 weeks. The cultures were observed for growth at 4th day and 4th week of incubation. Negative and positive controls were run along with the test.

3.5.6 SD Bioline Test: From solid culture, using a sterile wire loop, pick a loopful of colony of the AFB positive culture andemulsified into 100 micro liter extraction buffer. The fubes were tighly closed and vortex for the sample suspension to mix properly. SD Bioline Testing Kit was allowed to attain ambient room temperature prior to testing. The foil pouch were removed and placed on a flat surface inside the BSC. They were labelled with the sample identification number. Tubes containing the sample suspension placing area and the sample tubes were recapped.

It was timed for 15 minutes with stop watch. Positive result showed two red bands while negative result showed a single band[6].

3.5.7 Drug Sensitivity Testing (DST): Antibiotic susceptibility test was performed by proportion method as described by Akiko (2001), NIMR (2007) and FMOH (2009). The *Mycobacterium tuberculosis* isolates were tested against Isoniazid (Fisher), rifampicin (Sigma), streptomycin (Sigma) and ethambutol (Sigma). Pure drug crystals of isoniazid, rifampicin, Kanamycin, Amikacin, Ofloxacin, Capreomycin and ethambutol were produced by Sigma scientific laboratories and Fisher Laboratory, USA. Two loopfull of the pure isolate were picked and emulsified in 2ml of sterile distilled water containing six beeds in a sterile tubes, this was tightly capped and vortex to have turbidity of 1 McFarland standard. 4 drops of 1 in 100 suspension each of this isolate suspension were inoculated into LJ DST slopes containing the two of the first line and five second line anti-tuberculosis drugs, three control slopes of LJ DST without anti-tuberculosis drugs were inoculated with lowest suspension (10^{-4}) of isolate into C^3 slope, 1 in 1000 (10^{-3}) into C^2 slope and 1 in 100 (10^{-2}) into C^1 slope and the drug slopes as stated earlier. The inoculated slopes were loosely capped and incubated at 37 degree Celcius in a slant position. After one week, the caps were tightened and the slopes straightened to continue incubation for 6 weeks. The slopes were read for growth at 28days and again at 42 days for sensitive strains. The growth were recorded as follows: Confluent growth: 3+ More than 100 colonies: 2+1- 100 colonies: Acual number was recorded[7].

3.5.8 Preparation of Drug Solutions

3.5.9 Drug Potencies

The true potency of a drug is the number of micrograms of active drug per milligram total weight of the product. Thus desired activity (mg/ml) = (weight of drug) x (potency)/(volume of solvent).

3.5.10 Isoniazid

Isoniazid potency is 1g to 1g substance. 20.0mg of isoniazid was dissolved in 40.0mls of sterile distilled water. This gives 2.0mg/ml solution. 2.0mls of this stock solution was aseptically diluted with 50.0mls of sterile distilled water to give 20.0mg/ml stock solution. It was sterilized by membrane filteration. 10mls of stock solution were added to 100mls of LJ medium, mixed gently, dispensed in 6mls volumes and inspussated at 90°C for 50 minutes[8].

3.5.11 Rifampicin

Rifampicin potency is generally > 980.0mg/ml. 80.0mg/potency of rifampicin was dissolved in 5.0mls of absolute methanol. The solution was further diluted with 5.0ml of 95% ethanol to give 8000.0mg/ml stock solution (rifampicin is self sterilizing and does not require further sterilization). 5.0mls of stock solution were added to 1000mls of LJ medium, mixed gently, dispensed in 6mls volumes and inspissated at 90°C for 50 minutes[8].

3.5.12 Streptomycin

Because the potency may vary from 667mg to 800mg/mg. 40.0mg/potency of streptomycin sulphate was dissolved in 50.0mls of sterile distilled water to give 800.0mg/ml stock solution. 10mls of stock solution was added to 1000mls LJ medium. It was mixed gently, dispensed in 6mls volumes and inspissated at 50°C for 50 minutes[8].

3.5.13 Ethambutol

Ethambutol potency is 1g to 1g substance. 20.0mg of ethambutol powder was dissolved in 100.0mls of sterile distilled water to give 200.0mg/ml stock solution. 5mls of stock solution was added to 500mls of LJ medium. It was mixed gently, dispensed in 6mls volumes and inspissated at 90°C for 50 minutes[8].

3.5.14 The Proportion Method

A representative sample of 5.0mg to 10.0mg from the subculture within 1 to 2 weeks after appearance of growth using an inoculation loop was placed into a sterile McCartney bottle (14mls screw capped bottle) containing 1.0ml distilled water and 10 glass beads. The mixture was homogenized on a vortex mixer for up to 1 minute and the opacity of the suspension was adjusted by the addition of sterile distilled water to a standard suspension containing 1mg/ml of tubercle bacilli.

Two serial dilutions were made from the suspension, 10^2 and 10^4 using the calibrated inoculating loop and sterile McCartney vials containing 1.0ml of distilled water. 0.1ml of 10^{-2} and 10^{-4} suspensions were inoculated onto 2 slants of drug free (control) medium. Then 10^{-2} dilution of the suspension was inoculated onto a series of drug containing media. The suspensions were spread over on the whole surface of the medium and kept at slanting position with loosen caps. The slants were incubated at 37^{o}C. When the growth appears on the control media, cap was tightened and incubation continued for 4 weeks.

When enough growth, more than 100 colonies for 10^{-2} suspension and more than 50 colonies for 10^{-4} suspension, was observed on the drug free medium at 4 weeks of incubation, the growth on all media was read. Strains showing drug susceptibility at 4 weeks, further reading at 6 weeks was done before reporting susceptibility. Quality control strains – H37RV was included in each batch of testing.

3.5.15 Reading Interpreting and Reporting:

The seeded media were examined for contamination after 1 week. The first reading of drug susceptibility test result was done at 4 weeks (28 days) of incubation at 37°C. The growth on the drug containing medium was compared with the growth on the drug free medium at 10^{-4} dilution. When the growth on the drug containing medium was none or less than that of a drug free medium at 10^{-4} dilution, it was classified as susceptible/sensitive.

The following formula was used to calculate the % of resistant.

Number of colonies on drug containing medium = % resistance

Number of colonies on the drug free medium at 10^{-4} dilution

Resistance: The criteria for resistance is 1% of growth for all the drugs.

Susceptible: No growth or less than 1% of colonies growth compared to the controls.

Multi-drug resistance *M. tuberculosis* was defined as resistance to at least isoniazid and rifampicin.

Poly-drug resistance *M. tuberculosis* was defined as resistance to more than one drug but not isoniazid and rifampicin at the same time.

Mono-drug resistant *M. tuberculosis* was defined as resistance to only one drug[9].

3.5.16 Molecular Identification of Isolates

- **Bacteria DNA Extraction**

DNA was extracted using the protocol stated by (1). Briefly, Single colonies grown on medium were transferred to 1.5 ml of liquid medium and cultures were grown on a shaker for 48 h at 28 °C. After this period, cultures were centrifuged at 4600g for 5 min. The resulting pellets were resuspended in 520 µl of TE buffer (10 mMTris-HCl, 1mM EDTA, pH 8.0). Fifteen microliters

of 20% SDS and 3 µl of Proteinase K (20 mg/ml) were then added. The mixture was incubated for 1 hour at 37 ºC, then 100 µl of 5 M NaCl and 80 µl of a 10% CTAB solution in 0.7 M NaCl were added and votexed. The suspension was incubated for 10 min at 65 ºC and kept on ice for 15 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 min and centrifugation at 7200g for 20 min. The aqueous phase was then transferred to a new tube and isopropanol (1: 0.6) was added and DNA precipitated at –20 ºC for 16 h. DNA was collected by centrifugation at 13000g for 10 min, washed with 500 µl of 70% ethanol, air-dried at room temperature for approximately three hours and finally dissolved in 50 µl of TE buffer[10].

3.5.17 PCR Analysis

- **Bacteria PCR**

PCR sequencing preparation cocktail consisted of 10 µl of 5x GoTaq colourless reaction, 3 µl of 25mM MgCl2, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 pmol each 27F 5'- AGA GTT TGA TCM TGG CTC AG-3' and - 1525R, 5′-AAGGAGGTGATCCAGCC-3′ primers and 0.3units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water 8µl DNA template. PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) with a Pcr profile consisting of an initial denaturation at 94°C for 5 min; followed by a 30 cycles consisting of 94°C for 30 s, 50°C for 60s and 72°C for 1 minute 30 seconds ; and a final termination at 72°C for 10 mins. And chill at 4oC.GEL (2,3)[10]

Integrity:

The integrity of the amplified gene fragment was checked on a 1% Agarose gel ran to confirm amplification. The buffer (1XTAE buffer) was prepared and subsequently used to prepare 1.5%

agarose gel. The suspension was boiled in a microwave for 5 minutes. The molten agarose was allowed to cool to 60°C and stained with 3µl of 0.5 g/ml ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 minutes to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel. Two microliter (2 l) of 10X blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 4µl of each PCR product and loaded into the wells after the 100bp DNA ladder was loaded into well 1. The gel was electrophoresed at 120V for 45 minutes visualized by ultraviolet trans-illumination and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a 100bp molecular weight ladder that was ran alongside experimental samples in the gel[10].

Purification of Amplified Product

After gel integrity, the amplified fragments were ethanol purified in order to remove the PCR reagents. Briefly, 7.6 µl of Na acetate 3M and 240 µl of 95% ethanol were added to each about 40µl PCR amplified product in a new sterile 1.5 µl tube eppendorf, mix thoroughly by vortexing and keep at -20°C for at least 30 min. Centrifugation for 10 min at 13000 g and 4°C followed by removal of supernatant (invert tube on trash once) after which the pellet were washed by adding 150 µl of 70% ethanol and mix then centrifuge for 15 min at 7500 g and 4°C. Again remove all supernatant (invert tube on trash) and invert tube on paper tissue and let it dry in the fume hood at room temperature for 10-15 min. then resuspend with 20 µl of sterile distilled water and kept in -20oC prior to sequencing. The purified fragment was checked on a 1.5% Agarose gel ran on a

voltage of 110V for about 1hr as previous, to confirm the presence of the purified product and quantified using a nanodrop of model 2000 from thermo scientific[11].

SEQUENCING

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 were used for all genetic analysis[11].

Molecular Analysis of MDR-coding Genes

Molecular investigations of MDR-coding genes in the TB-resistant isolates were by simple PCR on the extracted DNA using ESBL-coding regions specific primers. Primer sequences were as earlier documented. Reaction cocktail used for all PCR per primer set included (Reagent Volume µl) - 5X PCR SYBR green buffer (2.5), MgCl2 (0.75), 10pM DNTP (0.25), 10pM of each forward and backwards primer (0.25), 8000U of taq DNA polymerase (0.06) and made up to 10.5 with sterile distilled water to which 2 µl template was added. Buffer control was also added to eliminate any probability of false amplification Table below shows the primer sequence and PCR profile used in amplifying each fragment. PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) using the appropriate profile as designed for each primer pair[12].

Gene		Primer sequence 5'-3'	
RpoB	rpoBF	CCACCCAGGACGTGGAGGCGATCACAC	An initial denaturing 5min at 94ºC, then 35 cycles of 94ºC for 30s, 57ºC for 30s 72ºC for 60s and terminate at 72ºC for 10min
	rpoBR	CGTTTCGATGAACCCGAACGGGTTGAC	
KatG	katG –F	CATGAACGACGTCGAAACAG	An initial denaturing 5min at 94ºC , then 35 cycles of 94ºC for 30s 48ºC for 30s and 72ºC for 60s and terminate at 72ºC for 10min
	katG-R	CGAGGAAACTGTTGTCCCAT	

Agarose Gel Electrophoresis

Agarose gel (1.5%) prepared as previous was used to confirm positive amplification. Ten microlitres of each PCR product were loaded into the wells with the 100bp DNA ladder loaded into well 1. The gel was electrophoresed at 120V for 45 minutes visualized by ultraviolet trans-illumination and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a 100bp molecular weight ladder that was ran alongside experimental samples in the gel[13].

3.6 Determination of Blood Parameters

3.6.1 Determination of Iron Concentration using spectrophotometric method

The iron concentration was determined using commercially prepared reagent kit produced by Abbott Laboratories, Abbott Park, IL 60064, USA.

Principle:

The iron assay utilizes an acidic media to release ferric iron from transferrin. The ferric iron is converted to the ferrous form by the action of hydroxylamine hydrochloride. The released ferrous iron reacts with ferene to produce a colored iron-ferene complex. The absorbance of the iron-ferene complex is measured at 604 nm and the colour intensity is proportional to the concentration of iron present in the sample. Thiourea and detergent are added to prevent copper interference and turbidity, respectively[14].

3.6.2 Determination of Ferritin Concentration using Ferritin-ELISA Method

The ferritin concentration was determined using commercially prepared reagent kit as produced by Abbott Laboratories, Abbott Park, IL 60064, USA.

Principle:

The Abbott ferritin ELISA test is based on simultaneous binding of human ferritin to two monoclonal antibodies, one immobilized on microwell plates and the other conjugated with horseradish peroxidase (HRP). After incubation the bound/free separation is performed by a simple solid-phase washing. Then the enzyme HRP in the bound-fraction reacts with the substrate (H_2O_2) and the TMB-substrate and develops a blue color that changes into yellow when the stop solution (H_2SO_4) is added. The colour intensity is proportional to the ferritin concentration in the sample. The ferritin concentration in the sample is calculated based on a standard curve[15].

3.6.3 Determination of C-reactive Proteins (CRP) Concentration using spectrophotometric method

The CRP concentration was determined using commercially prepared reagent kit as produced by Abbott Laboratories, Abbott Park, IL 60064, USA.

Principle:

C-Reactive Protein was determined by using a commercially prepared kit. The principle is based on the fact that when an antigen-antibody reaction occurs between CRP in a sample and polyclonal anti-C-reactive protein antibody, which has been adsorbed to latex particles, agglutination results. This agglutination is detected as an absorbance change, with the magnitude of the change being proportional to the quantity of CRP in the sample. The actual concentration is then determined by interpolation from a calibration curve prepared from calibrators of known concentration. The increase in absorbance at 572 nm is proportional to the CRP concentration[16].

3.6.4 Determination of Immunoglobulin G (IgG) Concentration using immunoturbidimetric method

The IgG concentration was determined using commercially prepared reagent kit as produced by Abbott Laboratories, Abbott Park, IL 60064, USA.

Principle:

The IgG assay is an immunoturbidimetric procedure that measures increasing sample turbidity caused by the formation of insoluble immune complexes when antibody to IgG is added to the sample. Sample containing IgG is incubated with a buffer (R1) and a sample blank determination is performed prior to the addition of IgG antibody (R2). In the presence of an appropriate antibody in excess, the IgG concentration is measured as a function of turbidity[17].

3.6.5 Determination of Complement 3 (C3) Concentration using immunoturbidimetric method

The C3 concentration was determined using commercially prepared reagent kit as produced by Abbott Laboratories, Abbott Park, IL 60064, USA.

Principle:

The compliment 3 (C3) assay is an immunoturbidimetric procedure that measures increasing sample turbidity caused by the formation of insoluble immune complexes when antibody to C3 is added to the sample. Sample containing C3 is incubated with a buffer (R1) and a sample (R2) blank determination is performed prior to the addition of C3 antibody. In the presence of an appropriate antibody in excess, the C3 concentration is measured as a function of turbidity[18].

3.6.6 Determination of Haemoglobin

Haemoglobin was determined using oxyhaemoglobin method

Principle

Blood is diluted in weak alkali (0.04%) ammonium hydroxide, specific gravity 0.88) which lyses the red blood cell and oxyhaemoglobin is released into the solution. This conversion is complete and immediate and the resulting colour is stable[19].

Method

0.1ml of blood was diluted in 20ml of 0.4ml/L ammonia and add 20mg of sodium dithionite. The absorbers is measured in a spectrophotometer at 538mm and 578nm within 10mins. The quotient is calculated in $\frac{A^{538}}{A^{578}}$ and is read in percentage[20].

3.6.7 Determination of Serum Zinc

Serum Zinc was determined colorimetrically using Human monoliquid kit, purchased from fortress Diagnostics, United Kingdom, with product code Bxco462, following manufacturer's instruction.

Principle

Zinc estimation utilizes a chromogen that forms a coloured complex specifically with zinc. The intensity of the coloured measured at 425nm is directly proportional to the zinc concentration in the sample[21].

3.6.8 Determination of Plasma Selenium using atomic absorption spectrometry

Plasma selenium levels were measured by graphite furnace atomic absorption spectrometry (Atomic absorption spectrophotometer ELICO, India model No. SL 173), following manufacturer's instructions.

Principle

Selenium estimation is based on the oxidation of iodide in acidic medium by Selenium (IV) contain in the sample to form elemental iodine which in turn reacts with the excess iodide to form the triiodide anions[22].

3.7.0 Determination of D-Dimer

D-Dimer concentration was determined using commercially prepared reagent kit as produced by Guangzhou Wondfo Biotech Co. Ltd, No. 8 Lizhishan Riad, science city, Luogang District, 510663, Guangzhou, P.R. China.

Principle

The fine care TM D-Dimer test is based on fluorescence immune assay technology, when sample is added into the sample well of the test cartridge, the fluorescence – labeled detector D-Dimer antibodies on the sample pad bind to D-Dimer antigen in blood specimen and they form immune complexes. As the complexes migrate on the nitrocellulose matrix of test strip by capillary action, the complexes of detector antibodies that have been immobilized on test strip. Thus the more D-

Dimer antigens in the blood specimen, the more complexes accumulated on test strip. Signal intensity of fluorescence of detector antibodies reflect the amount of captured D-Dimer[23]

3.7.1 Determination of Prothrombin Time (PT)

Prothrombin test was determined using the coaguchek pro II meter produced by Roche, Roche Diagnostics GmbH, Sandhofer strasse 116, D-68305 Mannheim.

Principle

Electrochemical measurement of prothombin time following activation of blood coagulation with human recombinant tissue factor. The test strip area containing a prothombin reagent, when blood is applied, the reagent is dissolve, and an electrochemical reaction takes place which is transformed into a clotting time[24].

3.7.2 Determination of the Activated Partial Thromboplastin Time (aPTT)

The determination of activated partial thromboplastin time was determined using the coaguchek pro II meter produced by Roche, Roche Diagnostics GmbH, Sandhofer strasse 116, D-68305 Mannheim.

Principle

Electrochemical measurement of activated partial thromboplastin time (aPTT) following activation of blood coagulation with celite. The test strip has an area containing an aPTT reagent. When blood is applied, the reagent is dissolved and an electro chemical reaction takes place which is transformed into a clotting time value[25].

3.7.3 Determination of Plasma Albumin by BCG Technique

Albumin concentration was determined using commercial prepared reagent kit DIALAB Production M55, 2351 Wr. Neudorf Austria Rf-..D97203

Principle

Albumin at pH 4.2 is sufficiently cationic to bind the anionic dye bromcresol green (BCG) to form a blue green coloured complex. The intensity of the blue-green colour is directly proportional to albumin concentration in the specimen. It is determined by measuring the increase in absorbance at 580-630nm[26].

3.8 Quality Control

For the assessment of the accuracy of all the parameters, quality control samples were included in all the analysis.

3.9 Statistical Analysis

The sampling method is a convenient sampling. All statistical analysis were performed using the statistical package for social sciences (SPSS for windows, version 20.0). Student T-test and Anova.

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Chapter Four

Results and Discussion

4.1 Results of Findings

This chapter enumerates the results derived from the methods employed in the assessment of effects of micronutrients supplementation in TB patients and genetic identification of multidrug resistance in MTB. The results were analysed along with the objectives earlier stated in this study and were compared with other similar researches to ensure best practices; thus presentation of the results are shown below: Baseline weights of tuberculosis treatment groups (anti-Tb treatment only, anti-Tb +Fe supplement, anti-Tb +Se supplement and anti-Tb +Zn supplement) and controls were compared using analysis of variance (ANOVA). There were significant (F-test =34.63; $P< 0.001$) differences between the tuberculosis treatment groups and controls, as shown in Table 4.1. After two months of treatment, the body weights increased significantly ($p<0.05$) in tuberculosis groups treated with Fe and Se, but no significant change ($p> 0.05$) were observed in the tuberculosis treatment group treated without mineral supplementation, and patients treated with TB drugs + Zn supplementation when compared with baseline values as shown in Table 4.2.

Table 4.1: Comparison of Baseline Levels of Age and Weight of Tuberculosis Groups and Controls Using ANOVA

	Controls	Anti-Tb (only) group	Anti-Tb +Fe supplement	Anti-Tb + Se group	Anti-Tb +Zn group	\F-test	p-value
Age (years)	45±6.3	48±4.3	45±6.3	43±6.8	50±8.1	0.142	0.991
Weight (Kg)	71.92±7.83	48.48±6.93	46.72±6.91	48.87±6.14	49.04±7.13	34.63	<0.001*

*P value statistically significant at value $p \leq 0.05$
Source: Author's fieldwork, 2023

Table 4.2: Comparison of Weight of Tuberculosis Groups Before and After Treatment

	Baseline Weight (Kg)	Post-treatment (2 months) Weight (Kg)	T- test	P-value
Anti TB (only) group	48.48±6.93	49.00±6.311	0.277	0.777
Anti-Tb +Fe supplement	46.72±6.91	52.84±5.36	3.498	0.001*
Anti-Tb + Se group	48.87±6.14	53.28±4.61	2.872	0.017*
Anti-Tb +Zn group	49.04±7.13	51.92±6.74	1.468	0.117

*P value statistically significant at value $p \leq 0.05$
Source: Author's fieldwork, 2023

Within the groups, ANOVA showed significant (P< 0.05) differences in the values of Hb, Fe, Se, Zn, ferritin, IgG, C3 and CRP, D-dimer and albumin; but no significant (P> 0.05) difference could be observed in the levels of prothrombin time and APTT, as shown in Table 4.3. Patients treated for two months with anti TB drugs only showed there were significant (P < 0.05) increases in the level of Hb(10.4±1.3g/dl), albumin(3.1±0.4g/dl), Fe(12.3±3.2microM/L), Se (26.1±5.9microg/L), Zn(11.3±1.9microM/L) when compared with baseline values. Levels of ferritin (295.0±37.0ng/ml), IgG (17.0±4.0g/L), (P < 0.0001), C3(159.9±26.2Mg/dl), CRP (9.8±6.2mg/L) and D-dimer(0.3724±0.2mg/L) were decreased significantly (P<0.05) when compared with baseline values. Post therapy levels of PT (P = 0.168) and aPTT (P = 0.894) did not show any significant change when compared with the baseline values, as shown in Table 4.4.

Table 4.3: Comparison of Trace Metals, Acute Phase Proteins and Markers of Coagulopathy in PTB before Treatment, PTB patients after Anti -TB drugs only, and after micronutrient using Analysis of Variance (ANOVA)

Parameters	Control	PTB Patients before Treatment,	After Anti-TB drug only	After Anti-TB drugs + Fe	After Anti-TB drugs + Se	After Anti-TB drugs + Zn	F-values	P-values
Hb (g/dl)	14.0±1.2	8.9±1.8	10.4±1.3	13.2±1.4	12.7±1.4	12.3±1.6	22.759	0.001*
Ferritin (ng/ml)	136.8±45.7	335.0±40.0	295.0±37.0	336.2±53	216.7±75.3	262.4±50.3	51.362	0.001*
Albumin (g/dl)	4.2±0.6	2.6±0.5	3.1±0.4	3.7±0.4	3.8±0.4	3.8±0.3	21.089	0.001*
Fe (microM/L)	18.1±3.7	8.6±2.0	12.3±3.2	15.2±3.4	13.2±2.4	9.7±2.8	25.453	0.001*
Se (microg/L)	40.2±8.5	18.4±3.3	26.1±5.9	32.4±6.0	64.5±17.1	27.3±3.8	68.825	0.001*
Zn (microM/L	13.2±2.3	6.9±2.0	11.3±1.9	12.1±1.2	12.4±1.5	13.2±2.4	4.271	0.003*
IgG (g/L)	10.0±2.3	21.0±3.1	17.0±4.0	12.5±2.4	13.0±2.9	13.2±2.8	18.556	0.001*
C3 (Mg/dl)	131.8±19.2	211.6±22.6	159.9±26.2	128.0±40.6	126.5±28.7	129.8±29.1	5.541	0.001*
CRP (mg/L)	5.1±1.6	17.3±15.2	9.8±6.2	8.1±2.3	6.5±1.8	7.04±2.0	7.460	0.001*
D-dimer (mg/L)	0.35±0.14	2.4±2.3	0.3724±0.21	0.40±0.18	0.29±0.15	0.472±0.14	3.915	0.005*
Prothrombin time (s)	11.1±1.5	11.0±2.0	11.7±3.07	11.2±1.2	11.4±1.3	10.8±1.1	1.929	0.110
APTT (s)	34.5±4.2	33.8±5.5	33.6±4.6	34.1±2.8	33.7±3.4	34.0±4.2	0.223	0.924

*P value statistically significant at value $p \leq 0.05$
Source: Author's Fieldwork, 2023

Table 4.4: Comparison of Trace Metals, Acute Phase Proteins and Markers of Coagulopathy between PTB Patients before and after 2 Months Intensive Treatment Anti-TB Drugs Only

Parameters	Baseline before anti-TB treatment (n = 25)	PTB after Anti-TB treatment only (n = 25)	t-test	p-value
Hb (g/dl)	8.9±1.8	10.4±1.3	3.153	0.001*
Ferritin (ng/ml)	335.0±40.0	295.0±37.0	3.670	0.013*
Albumin (g/dl)	2.6±0.5	3.1±0.4	3.735	0.001*
Fe (microM/L)	8.6±2.0	12.3±3.2	4.921	0.001*
Se (microg/L)	18.4±3.3	26.1±5.9	5.679	0.0001*
Zn (microM/L)	6.9±2.0	11.3±1.9	8.060	0.0001*
IgG (g/L)	21.0±3.1	17.0±4.0	4.014	0.0001*
C3 (Mg/dl)	211.6±22.6	159.9±26.2	7.462	0.0001*
CRP (mg/L)	17.3±15.2	9.8±6.2	2.260	0.0001*
D-dimer (mg/L)	2.4±2.3	0.3724±0.2	4.459	0.0001*
Prothrombin time (s)	11.0±2.0	11.7±3.07	1.400	0.168
APTT (s)	33.8±5.5	33.6±4.6	0.134	0.894

*P is statistically significant at value $p \leq 0.05$

Source: Author's fieldwork, 2023

Patients treated with anti TB drugs with Fe supplementation for two months showed (P <0.05) higher levels of Hb (13.2±1.4g/dl), Fe (15.2±3.4microM/L), Se (32.4±6.0microg/L), Zn (12.1±1.2microM/L), albumin (3.7±0.4g/dl) when compared with their baseline values. Levels of ferritin (336.2±53ng/ml), IgG (12.5±2.4g/L), C3 (128.0±40.6Mg/dl), CRP (8.1±2.3mg/L) and D-dimer (0.40±0.18mg/L) decreased significantly (P <0.05) when compared with their baseline values. While PT (P = 0.665) and aPTT (P =0.884) did not show any significant changes when compared with baseline values, as shown in Table 4.5. There were significantly (P <0.05) higher levels of albumin (3.8±0.4g/dl), Hb (12.7±1.4g/dl), Fe (13.2±2.4 µM/L), Se(64.5±17.1µL), Zn(12.4±1.5µM/L) in the patients treated with anti TB drugs + Se supplementation for two months compared with their baseline values. Levels of ferritin (216.7±75.3ng/ml), IgG (13.0±2.9g/L), C3 (126.5±28.7Mg/dl), CRP (6.5±1.8mg/L) and D-dimer (0.29±0.15mg/L) decreased significantly (P <0.05) when compared with their baseline values. Level of PT and aPTT (P > 0.05) did not show any significant value when compared with their baseline values, as shown in Table 4.6.

Table 4.5: Comparison of Trace Metals, Acute Phase Proteins and Markers of Coagulopathy between PTB Patients before and after 2 Months of Intensive Treatment with Anti-TB Drug + Fe Supplementation

Parameters	Baseline before treatment + Fe supplementation (n = 25)	After 2 months Anti-TB treatment + Fe supplementation (n = 25)	t-test	P-value
Hb (g/dl)	10.3±1.7	13.2±1.4	6.725	0.0001*
Ferritin (ng/ml)	374.8±70.4	336.2±53	2.189	0.011*
Albumin (g/dl)	2.7±0.4	3.7±0.4	9.133	0.0001*
Fe (µM/L)	7.3±2.3	15.2±3.4	9.429	0.0001*
Se (µg/L)	19.5±1.9	32.4±6.0	10.277	0.0001*
Zn (µM/L)	7.9±1.3	12.1±1.2	11.759	0.0001*
IgG (g/L)	21.4±3.9	12.5±2.4	9.761	0.0001*
C3 (Mg/dl)	201.6±18.7	128.0±40.6	8.242	0.0001*
CRP (mg/L)	26.7±7.7	8.1±2.3	11.505	0.0001*
D-dimer (mg/L)	1.3±0.5	0.40±0.18	7.913	0.0001*
Prothrombin time (s)	11.3±1.5	11.2±1.2	0.436	0.665
APTT (s)	34.2±3.6	34.1±2.8	0.172	0.864

*P is statistically significant at value $p \leq 0.05$

Source: Author's Fieldwork, 2023

Table 4.6: Comparison of Trace Metals, Acute Phase Proteins and Markers of Coagulopathy between PTB Patients Before and after 2 months Intensive Treatment with Anti -TB Drugs + Se Supplementation

Parameters	Baseline before treatment + Se supplementation (n = 25)	After Anti-TB treatment + Se supplementation (n = 25)	t-test	p-value
Hb (g/dl)	9.5±2.3	12.7±1.4	6.056	0.0001*
Ferritin (ng/ml)	344.4±75.7	216.7±75.3	5.978	0.0001*
Albumin (g/dl)	2.9±0.42	3.8±0.4	7.488	0.0001*
Fe (µM/L)	8.4±21	13.2±2.4	7.457	0.001*
Se (µg/L)	17.6±2.4	64.5±17.1	13.595	0.0001*
Zn (µM/L)	7.3±1.7	12.4±1.5	11.111	0.0001*
IgG (g/L)	23.5±4.9	13.0±2.9	9.404	0.0001*
C3 (Mg/dl)	197.0±41.6	126.5±28.7	6.969	0.0001*
CRP (mg/L)	27.2±8.2	6.5±1.8	12.321	0.0001*
D-dimer (mg/L)	1.2±0.5	0.29±0.15	9.223	0.0001*
Prothrombin time (s)	11.7±1.4	11.4±1.3	0.733	0.467
APTT (s)	33.7±4.0	33.7±3.4	0.107	0.916

*P is statistically significant at value $p \leq 0.05$

Source: Author's fieldwork, 2023

There were significantly (P < 0.05) higher levels of Hb (12.3±1.6g/dl), albumin (3.8±0.3g/dl), Se (27.3±3.8µg/L), and Zn (13.2±2.4µM/L) in patient treated for two months with anti TB drugs + Zn supplementation when compared with their baseline values. Levels of ferritin (262.4±50.3ng/ml), IgG (13.2±2.8g/L), C3 (129.8±29.1Mg/dl), CRP (7.04±2.0mg/L) and D-dimer (0.472±0.14mg/L) decreased significantly (P <0.05) when compared with their baseline values. Fe (microM/L), PT and aPTT did not show any significant changes when compared with their baseline values (P > 0.05), as shown in Table 4.7. The baseline values of Hb (8.9±1.8g/dl), albumin (2.6±0.5g/dl), Fe(8.6±2.0microM/L), Se(18.4±3.3microg/L) and Zn (6.9±2.0 µM/L) were significantly (P < 0.05) lower compared with controls. The baseline values of IgG (21.0±3.1g/L), C3(211.6±22.6Mg/dl), D-dimer(2.4±2.3mg/L) and CRP(17.3±15.2mg/L) increased significantly (P< 0.05) when compared with control. There were no significant (P >0.05) difference with baseline values of PT and aPTT when compared with control. The post-treatment values of Hb (10.4±1.3g/dl), albumin (3.1±0.4g/dl), Fe (12.3±3.2microM/L), Se (26.1±5.9microg/L), and Zn (11.3±1.9microM/L) were significantly (P< 0.05) lower when compared with control. The post-treatment values of D-dimer (mg/L), PT, aPTT did not show any significant (P >0.05) difference when compared with the control, as shown in Table 4.8.

Table 4.7: Comparison of Trace Metals, Acute Phase Proteins and Markers of Coagulopathy between PTB patient before and after 2 months of intensive treatment with Anti-TB drugs + Zn supplementation

Parameters	Baseline before treatment + Zn supplementation (n = 25)	After 2 months Anti-TB treatment + Zn supplementation (n = 25)	t-test	p-value
Hb (g/dl)	9.5±1.1	12.3±1.6	7.140	0.0001*
Ferritin (ng/ml)	348.2±46.1	262.4±50.3	6.290	0.0001*
Albumin (g/dl)	2.8±0.5	3.8±0.3	9.058	0.0001*
Fe (µM/L)	11.0±10.3	9.7±2.8	0.614	0.299
Se (µg/L)	17.6±1.7	27.3±3.8	11.560	0.0001*
Zn (µM/L)	7.7±1.9	13.2±2.4	8.816	0.0001*
IgG (g/L)	21.9±4.5	13.2±2.8	8.210	0.0001*
C3 (Mg/dl)	201.4±15.9	129.8±29.1	10.794	0.0001*
CRP (mg/L)	26.6±9.8	7.04±2.0	9.796	0.0001*
D-dimer (mg/L)	1.4±0.8	0.472±0.14	5.716	0.0001*
Prothrombin time (s)	10.6±1.4	10.8±1.1	0.566	0.574
APTT (s)	34.0±4.6	34.0±4.2	0.016	0.987

*P is statistically significant at value $p \leq 0.05$

Source: Author's Fieldwork, 2023

Table 4.8: Comparison of Trace Metals, Acute Phase Proteins and Markers of Coagulopathy among PTB patients before, after 2 months of intensive treatment with Anti-TB Drugs only and control

Parameters	Baseline before treatment (n = 25)	After Anti-TB treatment (n = 25)	Controls (n = 25)	P_a- value	P_b-value
Hb (g/dl)	8.9±1.8	10.4±1.3	14.0±1.2	0.0001*	0.0001*
Ferritin (ng/ml)	335.0±40.0	295.0±37.0	136.8±45.7	0.0001*	0.0001*
Albumin (g/dl)	2.6±0.5	3.1±0.4	4.2±0.6	0.0001*	0.0001*
Fe (µM/L)	8.6±2.0	12.3±3.2	18.1±3.7	0.0001*	0.0001*
Se (µg/L)	18.4±3.3	26.1±5.9	40.2±8.5	0.0001*	0.0001*
Zn (µM/L	6.9±2.0	11.3±1.9	13.2±2.3	0.0001*	0.001*
IgG (g/L)	21.0±3.1	17.0±4.0	10.0±2.3	0.0001*	0.002*
C3 (Mg/dl)	211.6±22.6	159.9±26.2	131.8±19.2	0.0001*	0.003*
CRP (mg/L)	17.3±15.2	9.8±6.2	5.1±1.6	0.0001*	0.027*
D-dimer (mg/L)	2.4±2.3	0.3724±0.2	0.35±0.14	0.0001*	0.623
Prothrombin time (s)	11.0±2.0	11.7±3.07	11.1±1.5	0.949	0.169
APTT (s)	33.8±5.5	33.6±4.6	34.5±4.2	0.538	0.465

P_a = Significance of difference between baseline values and controls, P_b = Significance of difference between post-treatment and controls

*P is statistically significant at value $p \leq 0.05$

Source: Author's Fieldwork, 2023

The baseline values of Hb (10.3±1.7g/dl), Fe (7.3±2.3µM/L), albumin (2.7±0.4g/dl), Zn (7.9±1.3 µM/L) and Se (19.5±1.9 µg/L) were significantly ($P < 0.005$) lower in patients baseline values when compared with the control. The baseline values of Ferritin (374.8±70.4ng/ml), IgG (21.4±3.9g/L), C3 (201.6±18.7Mg/dl), CRP (26.7±7.7mg/L) and D-dimer (1.3±0.5mg/L) increased significantly ($P < 0.05$) when compared with control while PT and aPTT shows no significant difference between the baseline values and control, as shown in Table 4.9. The post treatment values of Albumin (3.7±0.4 g/dl), Fe (15.2±3.4 µM/L), Se (32.4±6.0 µg/L), and Zn (12.1±1.2 µM/L) were significantly ($P <0.05$) lower in patients with post treatment compared with control. The values of Hb (g/dl), C3(Mg/dl), CRP(mg/L), D-dimer(mg/L), PT and aPTT were not statistically different from the control ($P >0.05$), as shown in Table 4.9.

Table 4.9: Comparison of Trace Metals, Acute Phase Proteins and Markers of Coagulopathy among PTB patients before, after 2 months of intensive treatment with Anti-TB Drugs + Fe supplementation and control

Parameters	Baseline before treatment (n = 25)	After 2 months Anti-TB treatment + Fe supplementation (n = 25)	Controls (n = 25)	P_a-value	P_b-value
Hb (g/dl)	10.3±1.7	13.2±1.4	14.0±1.2	0.0001*	0.096
Ferritin (ng/ml)	374.8±70.4	336.2±53	136.8±45.7	0.0001*	0.0001*
Albumin (g/dl)	2.7±0.4	3.7±0.4	4.2±0.6	0.0001*	0.006*
Fe (µM/L)	7.3±2.3	15.2±3.4	18.1±3.7	0.0001*	0.004*
Se (µg/L)	19.5±1.9	32.4±6.0	40.2±8.5	0.0001*	0.0001*
Zn (µM/L)	7.9±1.3	12.1±1.2	13.2±2.3	0.0001*	0.029*
IgG (g/L)	21.4±3.9	12.5±2.4	10.0±2.3	0.0001*	0.017*
C3 (Mg/dl)	201.6±18.7	128.0±40.6	131.8±19.2	0.0001*	0.677
CRP (mg/L)	26.7±7.7	8.1±2.3	5.1±1.6	0.0001*	0.151
D-dimer (mg/L)	1.3±0.5	0.40±0.18	0.35±0.14	0.0001*	0.253
Prothrombin time (s)	11.3±1.5	11.2±1.2	11.1±1.5	0.560	0.825
APTT (s)	34.2±3.6	34.1±2.8	34.5±4.2	0.785	0.652

P_a = Significance of difference between baseline values and controls, P_b = Significance of difference between anti-Tb drugs+Fe supplementation and controls

*P is statistically significant at value $p \leq 0.05$

Source: Author's Fieldwork, 2023

The baseline values of Hb (9.5±2.3g/dl), albumin (2.9±0.42g/dl) and Se (17.6±2.4microg/L) were significantly ($P < 0.05$) lower when compared with the controls. The values of ferritin (344.4±75.7ng/ml), IgG (23.5±4.9g/L), C3 (197.0±41.6Mg/dl), CRP (27.2±8.2mg/L) and D-dimer (1.2±0.5mg/L) increased significantly ($P < 0.05$) when compared with the control. There were no significant difference ($P >0.05$) in the baseline values of PT and aPTT when compared with the control, as shown in Table 4.10. The post treatment values of Hb (12.7±1.4g/dl), albumin (3.8±0.4g/dl) and Se (64.5±17.1 µg/L) were significantly ($P < 0.05$) lower when compared with control. Ferritin (216.7±75.3ng/ml) and IgG (13.0±2.9g/L) were significantly higher ($P < 0.05$) when compared with the control, as shown in Table 4.10. Zn (µM/L, C3 (Mg/dl), CRP (mg/L), D-dimer(mg/L), PT and aPTT were not statistically significant ($P > 0.05$) when compared with control, as shown in Table 4.10.

Table 4.10: Comparison of Trace Metals, Acute Phase Proteins and Markers of Coagulopathy among PTB patients before, after 2 months of intensive treatment with Anti-TB Drugs + Se supplementation and control

Parameters	Baseline before treatment (n = 25)	After Anti-TB treatment + Se supplementation (n = 25)	Controls (n = 25)	P_a-value	P_b-value
Hb (g/dl)	9.5±2.3	12.7±1.4	14.0±1.2	0.0001*	0.005*
Ferritin (ng/ml)	344.4±75.7	216.7±75.3	136.8±45.7	0.0001*	0.0001*
Albumin (g/dl)	2.9±0.42	3.8±0.4	4.2±0.6	0.0001*	0.008*
Fe (µM/L)	8.4±21	13.2±2.4	18.1±3.7	0.0001*	0.0001*
Se (µg/L)	17.6±2.4	64.5±17.1	40.2±8.5	0.0001*	0.0001*
Zn (µM/L)	7.3±1.7	12.4±1.5	13.2±2.3	0.0001*	0.127
IgG (g/L)	23.5±4.9	13.0±2.9	10.0±2.3	0.0001*	0.004*
C3 (Mg/dl)	197.0±41.6	126.5±28.7	131.8±19.2	0.0001*	0.449
CRP (mg/L)	27.2±8.2	6.5±1.8	5.1±1.6	0.0001*	0.516
D-dimer (mg/L)	1.2±0.5	0.29±0.15	0.35±0.14	0.0001*	0.197
Prothrombin time (s)	11.7±1.4	11.4±1.3	11.1±1.5	0.159	0.453
APTT (s)	33.7±4.0	33.7±3.4	34.5±4.2	0.411	0.430

P_a = Significance of difference between baseline values and controls, P_b = Significance of difference between anti-Tb drugs+Se supplementation and controls

*P is statistically significant at value $p \leq 0.05$

Source: Author's Fieldwork, 2023

There were significantly (P <0.05) decreased in the levels of Hb (9.5±1.1g/dl), albumin (2.8±0.5g/dl), Fe(11.0±10.3microM/L), Se(17.6±1.7microg/L) when compared with the control. Ferritin (348.2±46.1ng/ml), IgG (21.9±4.5g/L), C3 (201.4±15.9Mg/dl), CRP (26.6±9.8mg/L), D-dimer (1.4±0.8mg/L) increased significantly (P < 0.05) when compared with control. Both PT and aPTT were not statistically significant (P > 0.05), as shown in table 4.11. Hb (12.3±1.6 g/dl), Alb (3.8±0.3g/dl), Fe(9.7±2.8 μM/L) and Se(27.3±3.8μg/L) were found to be significantly (P < 0.05) lower when compared with the control. Post treatment values of IgG (g/L), Ferritin (ng/ml) were observed the increased and statistically significant (P < 0.05) when compared with control, as shown in table 4.11. Zn (μM/L), C3 (Mg/dl), CRP (mg/L), D-dimer (mg/L), PT and aPTT were not statistically significant (P > 0.05) when compared with the control, as shown in Table 4.11.

Table 4.11: Comparison of Trace Metals, Acute Phase Proteins and Markers of Coagulopathy among PTB patients before, after 2 months of intensive treatment with Anti-TB Drugs + Zn supplementation and control

Parameters	Baseline before treatment + Zn supplementation (n = 25)	After Anti-TB treatment + Zn supplementation (n = 25)	Controls (n = 25)	P_a- value	P_b-value
Hb (g/dl)	9.5±1.1	12.3±1.6	14.0±1.2	0.0001*	0.0001*
Ferritin (ng/ml)	348.2±46.1	262.4±50.3	136.8±45.7	0.0001*	0.0001*
Albumin (g/dl)	2.8±0.5	3.8±0.3	4.2±0.6	0.0001*	0.009*
Fe (µM/L)	11.0±10.3	9.7±2.8	18.1±3.7	0.002*	0.0001*
Se (µg/L)	17.6±1.7	27.3±3.8	40.2±8.5	0.0001*	0.0001*
Zn (µM/L)	7.7±1.9	13.2±2.4	13.2±2.3	0.0001*	0.376
IgG (g/L)	21.9±4.5	13.2±2.8	10.0±2.3	0.0001*	0.008*
C3 (Mg/dl)	201.4±15.9	129.8±29.1	131.8±19.2	0.0001*	0.332
CRP (mg/L)	26.6±9.8	7.04±2.0	5.1±1.6	0.0001*	0.358
D-dimer (mg/L)	1.4±0.8	0.472±0.14	0.35±0.14	0.0001*	0.603
Prothrombin time (s)	10.6±1.4	10.8±1.1	11.1±1.5	0.268	0.497
APTT (s)	34.0±4.6	34.0±4.2	34.5±4.2	0.658	0.631

P_a = Significance of difference between baseline values and controls, P_b = Significance of difference between anti-Tb drugs+Zn supplementation and controls

*P is statistically significant at value $p \leq 0.05$

Source: Author's Fieldwork, 2023

The levels of Hb (13.2±1.4g/dl), albumin (3.7±0.4g/dl), Fe(15.2±3.4microM/L) and Se (32.4±6.0microg/L) increased significantly ($P < 0.05$) when compared with patient with anti TB drugs only. There were significantly reduction in the levels of IgG(12.5±2.4g/L), and C3 (128.0±40.6Mg/dl) when compared with patients on anti TB drugs alone. Zn (µM/L), CRP (mg/L), D-dimer (mg/L), PT and aPTT were not statistically significant ($P > 0.05$), as shown in Table 4.12. The levels of Hb (12.7±1.4g/dl), albumin (3.8±0.4g/dl) and Se (64.5±17.1µg/L) increased significantly in patients with Se supplementation when compared with patients on anti TB drugs only. CRP (6.5±1.8mg/L), C3(126.5±28.7Mg/dl), IgG(13.0±2.9g/L) and Ferritin(216.7±75.3ng/ml) were found to be significantly ($P < 0.05$) reduced when compared with patients on anti TB drugs only, as shown in table 4.12. There were significant ($P < 0.05$) increased in the levels of Hb (12.3±1.6g/dl), Alb(3.8±0.3g/dl) and Zn(13.2±2.4 µM/L) in patients treated with anti TB drugs + Zn supplementation when compared with patients treated with anti TB drugs only, as shown in Table 4.12. Ferritin (262.4±50.3ng/ml), Fe (9.7±2.8microM/L), IgG(13.2±2.8g/L) and C3(129.8±29.1 Mg/dl) decreased significantly when compared with patients treated with anti TB drugs only ($P < 0.05$). The levels of Se (µg/L), CRP (mg/L), D-dimer(mg/L), PT and aPTT were not statistically significant ($P > 0.05$) when compared with patients on anti TB drugs only, as shown in Table 4.12.

Table 4.12: Comparison of Trace Metals, Acute Phase Proteins and Markers of Coagulopathy among PTB patients after Anti -TB drugs only, after anti- TB drugs + Fe, after anti TB drugs + Se, after anti TB drugs + Zn supplementation

Parameters	After Anti-TB drug only	After Anti-TB drugs + Fe	After Anti-TB drugs + Se	After Anti-TB drugs + Zn	P_a-values	P_b - values	P_c-value
Hb (g/dl)	10.4±1.3	13.2±1.4	12.7±1.4	12.3±1.6	<0.001*	0.0001*	0.0001*
Ferritin (ng/ml)	295.0±37.0	336.2±53	216.7±75.3	262.4±50.3	0.011*	0.0001*	0.043*
Albumin (g/dl)	3.1±0.4	3.7±0.4	3.8±0.4	3.8±0.3	0.001*	0.001*	0.001*
Fe (µM/L)	12.3±3.2	15.2±3.4	13.2±2.4	9.7±2.8	0.0001*	0.778	0.001*
Se (µg/L)	26.1±5.9	32.4±6.0	64.5±17.1	27.3±3.8	0.003*	0.0001*	0.562
Zn (µM/L)	11.3±1.9	12.1±1.2	12.4±1.5	13.2±2.4	0.178	0.061	0.015*
IgG (g/L)	17.0±4.0	12.5±2.4	13.0±2.9	13.2±2.8	0.0001*	0.0001*	0.0001*
C3 (Mg/dl)	159.9±26.2	128.0±40.6	126.5±28.7	129.8±29.1	0.001*	0.001*	0.017*
CRP (mg/L)	9.8±6.2	8.1±2.3	6.5±1.8	7.04±2.0	0.429	0.007*	0.191
D-dimer (mg/L)	0.3724±0.2	0.40±0.18	0.29±0.15	0.472±0.14	0.908	0.221	0.678
Prothrombin time (s)	11.7±3.07	11.2±1.2	11.4±1.3	10.8±1.1	0.130	0.130	0.347
APTT (s)	33.6±4.6	34.1±2.8	33.7±3.4	34.0±4.2	0.969	0.859	0.897

P_a = Significance of difference between anti-Tb drugs onlyand Anti-Tb +Fe supplementation, P_b = Significance of difference between anti-Tb drugs onlyand Anti-Tb +Se supplementation , P_b = Significance of difference between anti-Tb drugs onlyand Anti-Tb +Zn supplementation

*P is statistically significant at value $p \leq 0.05$

Source: Author's Fieldwork, 2023

4.2 Genetic Characterisation

As shown in Figure 4.4, the agarose gel demonstrates the positive amplification of the 16S regions at the band size approximately 1500bp, as shown in figure 4.1. The gel image indicates a positive only in MDR2 which indicate the presence of KatG gene at the band size approximately 230bp, as shown in figure 4.2. The gene image indicates a positive amplification in all the MDR samples indicating the presence of rpoB gene at the band size approximately 330bp, as shown in figure 4.3. The phylogenetic analysis showed that MDR1, MDR2 and DS1 had clusters of both *Mycobacterium tuberculosis* and *Mycobacterium bovis*. Meanwhile, there was no cluster of *Mycobacterium bovis* in DS2.

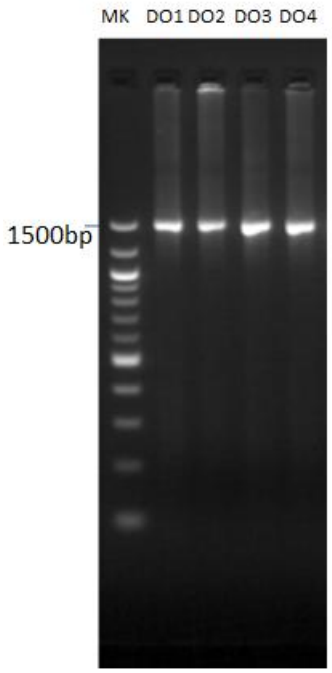

Figure 4.1: Molecular Amplification of Selected Mycobacteria Samples Using Agarose Gel

Note:

*DO1 = multidrug resistant TB sample one

*DO2 = multidrug resistant TB sample two

*DO3 = drug sensitive TB sample one

DO4 = drug sensitive TB sample two

Source: Author's Fieldwork 2023

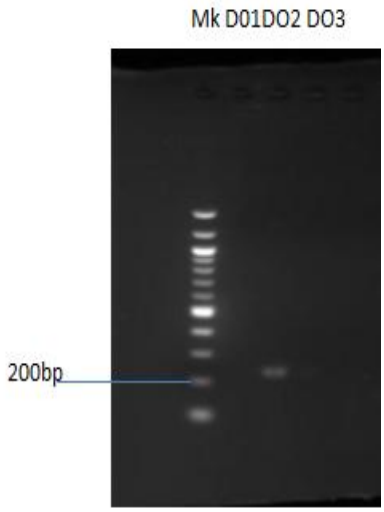

Figure 4.2: Molecular Identification of Virulence Gene in Mycobacterium Tuberculosis Isolates using Agarose Gel Electrophoresis of the PCR Products of KatG gene Amplified

Note:

*DO1 = multidrug resistant TB sample one

*DO2 = multidrug resistant TB sample two

*DO3 = drug sensitive TB sample one

DO4 = drug sensitive TB sample two

Source: Author's Fieldwork 2023

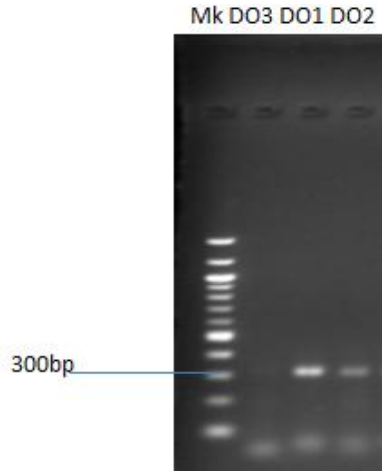

Figure 4.3: Molecular Identification of Virulence Gene in Mycobacterium Tuberculosis Isolates using Agarose Gel Electrophoresis of the PCR Products of rpoB gene Amplified

Note:

*DO1 = multidrug resistant TB sample one

*DO2 = multidrug resistant TB sample two

*DO3 = drug sensitive TB sample one

DO4 = drug sensitive TB sample two

Source: Author's Fieldwork 2023

Table 4.13: NCBI Blast showing the Sequence Identity of the isolates edited sequences

Sample ID	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident
DO1(MDR1)	Mycobacterium tuberculosis	2595	2595	99%	0	99.79%
DO2 (MDR2)	Mycobacterium tuberculosis	2499	2499	99%	0	99.93%
Do3 (DS 1)	Mycobacterium tuberculosis	2604	2604	99%	0	99.86%
Do4 (DS 2)	Mycobacterium tuberculosis	2584	2584	99%	0	99.72%

Source: Author's Fieldwork, 2023

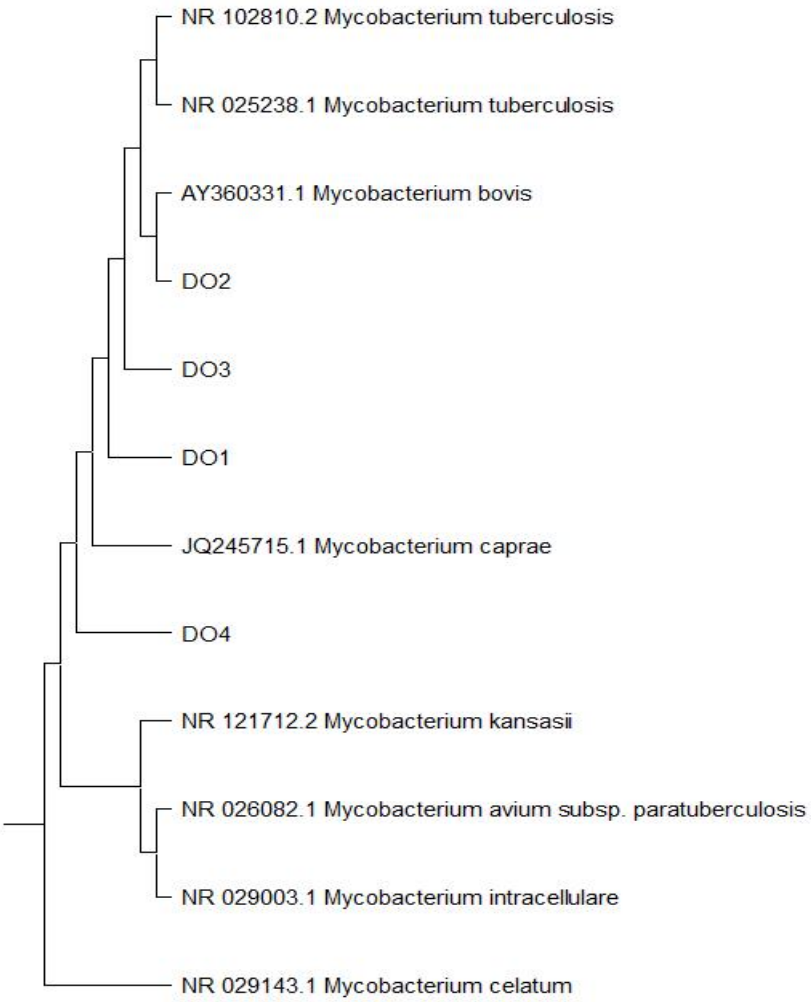

Figure 4.4: Phylogenetic Analysis Showing the Genetic Relatedness between the Isolated Mycobacteria and Reference Sequence Generated From the Ncbi Data Base

Note:

*DO1 = multidrug resistant TB sample one

*DO2 = multidrug resistant TB sample two

*DO3 = drug sensitive TB sample one

DO4 = drug sensitive TB sample two

Source: Author's Fieldwork 2023

Table 4.14: Pairwise Genetic Distance Showing the Extent of Similarity Between the Sequenced Tb Isolates

	DO1 (MDR1)	DO2 (MDR 2)	DO3 (DS 1)	DO4 (DS 2)
DO1	0.00591			
DO2	0.00496			
DO3	0.00743	0.00591		
DO4	0.00785		0.00641	

Note:

*DO1 = multidrug resistant TB sample one

*DO2 = multidrug resistant TB sample two

*DO3 = drug sensitive TB sample one

DO4 = drug sensitive TB sample two

Source: Author's Fieldwork 2023

4.3 Discussion of Findings

Pathophysiology of tuberculosis include metabolic disorders, immunological disorders with macro- and micro-nutrients insufficiencies[1]. Impaired immune system (T-cell suppression), and chronic inflammation have been implicated in the loss of body weight and reduction in the production of leptin[2]. The significant underweight observed in the tuberculosis patients recruited for this study agrees with the previous workers, where they reported that the body mass index was significantly lower in PTB patients, which also correlated negatively with severity of TB infection[3]. This weight loss could be due to malnutrition, anorexia and inflammation.

Selenium is an important trace metal that is crucial in the body immune status. Selenium supplementation in PTB infection may help to strengthen the immune response, to counteract oxidative cellular damage related with inflammation and infection. Selenium may modulate the sensitivity of host to pathogens and immune system against microorganism[4]. The present study revealed low level of plasma Se in PTB patients. This could be due to malnutrition caused by anorexia in tuberculosis infection. Surprisingly in patients treated with anti-TB and Fe supplementation, the Se level increased significantly after 2 months of treatment, compared with patient treated with anti TB drugs only. Some researchers observed lower baseline level of plasma selenium in the tuberculosis patients used for their study[5, 6]. Malnutrition, malabsorption and usurpation of Fe, Se, and Zn in tuberculosis could account for the significantly lower levels reported in tuberculosis patients recruited for this study. In this study, after two months of intensive treatment, the body weight of patients treated with Fe, and Se supplementation increased significantly. Meanwhile, the body weight of PTB patients treated with only anti-TB drugs and those treated with anti-TB drugs + Zn did not show any significant changes when compared with baseline levels. Study shows that patients on anti TB drugs + selenium

supplementation demonstrated weight gains[7]. A study reported that a group of PTB patients treated with selenium supplementation as adjuvant had significant increase in their weight[8]. They associated this increase in body weight to the fact that selenium has immunomodulatory potentials that could enhance the effect of the anti-TB drugs. The weight gain observed in the Fe supplemented treatment might be due to the fact that Fe enhances the production of red blood cells, and oxygen transfer needed for metabolic activities in the body[9]. These micronutrients have been reported to play significant roles in immune responses against infectious agents[10]. Iron acquisition mechanisms by microorganism and its effects on the host immune and inflammatory response also affects the competence of adequate immune responses[11]. Zinc is an important element in nutrition, a constituent of many enzymes like carbonic anhydrase, alcohol dehydrogenases, RNA polymerase, alkaline phosphatase and many others. Low level or deficiency of zinc affects the host defense mechanism by decreasing phagocytosis and also lowering the number of circulating T-cells[12]. Iron is an important nutritional elements and plays essential roles in electron transport, oxygen transfer, cellular respiration, redox and metabolic activities such as differentiation of gene expression, iron acquisition mechanism is higher in PTB infection hence there is reduced in host plasma iron[13]. The significantly lower level of iron in PTB patients recruited for this study could be due to malabsorption; loss of appetite and sepsis. This nutritional disorder and iron deficiency might be the cause of anaemia in the tuberculosis patients[14]. Iron and zinc are both required in haem metabolism. Low level of haemoglobin have also been associated with zinc deficiency in tuberculosis patient[15]. This study also corroborates the findings of some researchers who observed low level of iron concentration in tuberculosis patients[16,17]. However, after two months of treatment with iron, zinc and selenium supplementation, the levels of plasma iron and haemoglobin improved significantly. This study

corroborates the findings of other studies that moderate and timely iron supplementation as adjuvant therapy have an inflammation lowering effect[18,19]. Selenium in erythrocytes has a protective effect against oxidative damage on erythrocyte, thereby maintains the levels of iron and haemoglobin in tuberculosis patients[20,21,22].

Although there is limited data on the level of plasma zinc in patients with pulmonary tuberculosis infection, researchers have established that cases of pulmonary tuberculosis are associated with low serum zinc level[23]. Zinc plays an essential role in the functioning of body immune system. It is also a constituent of antioxidant mechanism. Low level of zinc is associated with pulmonary tuberculosis infection[24]. Normal level of zinc is important to control the M. tuberculosis infection and to also support in cell mediated immunological response[25]. In this study, the baseline level of plasma zinc was significantly lower in all groups of pulmonary tuberculosis patients. This low level of plasma zinc in tuberculosis patients might be due to redistribution of zinc from plasma to other tissues. A study in United Kingdom showed that *Mycobacterium tuberculosis* patients were observed to have a significantly lower level of plasma zinc along with other micronutrients[26]. Another study also reported significantly lower levels of zinc in PTB patients[27]. In a study conducted in Ethiopia, a decrease in the level of plasma zinc in pulmonary tuberculosis positive patients was reported[28]. In this present study, the level of zinc in patients treated with anti TB drugs plus zinc supplementation increased significantly when compared with baseline level and patients on anti TB drugs only.

C-reactive protein is a marker of inflammation and severity of infection. It is produced by the hepatocytes in response to a variety of inflammatory cytokines[29]. It activates macrophages to invade the sites of infection. CRP can therefore be used to determine the inflammatory status of an infection[30]. Several studies have reported increases in the levels of CRP in various

tuberculosis infections. Baseline level of CRP was significantly higher in PTB patients used for this study. This corroborates the reports of other studies who reported increased levels of CRP and ferritin in PTB patients[31.32]. A study titled predictive value of CRP for tuberculosis discovered that an elevate plasma CRP is associate with anaemia in tuberculosis patients[33]. Increased baseline levels of CRP might be due to increased cellular activation and pro-inflammatory cytokines released[34]. After two months of intensive treatment with anti-tuberculosis drugs only, and anti-tuberculosis drugs supplemented with either iron, zinc or selenium, the level of CRP decreased significantly compared with the baseline levels in patients treated with or without micronutrient supplementation. This might be due to decrease in the bacterial load and macrophage activation in the treated patients. This study agrees with the report of other researcher who observed a decreased level of CRP after two months of treatment with anti- TB drugs[32].

Immunoglobulin G (IgG) is an antibody that accounts for about 75% of serum antibodies in humans and are very common in blood circulation and extracellular fluid; allowing it to control infections in the body tissues. IgG responses are affected by the infective state of Mycobacterium tuberculosis, since it is a very high sensitive and specific parameter for the diagnosis of tuberculosis[35]. It was observed that a humoral response occurs in the host with active MTB infection which may cause an increase in the level of IgG. Baseline IgG and C3 levels were significantly elevated in PTB patients before commencement of treatment. A group of scientists also reported an elevated serum level of IgG in PTB patients before treatment[36,37]. The elevated plasma IgG may also be due to the fact that IgG plays significant roles in inflammation and innate immune response typified by microbial infection.

C3 is a modulator of immune response generated by both classical and alternative pathways during infection[38]. The elevated C3 levels observed in PTB patients before treatment could be attributed to bacteria induced production of C3 for the host survival, this study collaborate with other research work who reported significantly higher baseline levels of C3 in pulmonary tuberculosis patients in his study[39]. However, in the present study, the levels of IgG and C3 reduced significantly in PTB patients on anti-tuberculosis drugs + micronutrient supplementation, after two months of intensive treatment, when compared with patients on anti-tuberculosis drugs only. This might be due to the enhanced bactericidal activity of the anti-tuberculosis drugs by metal dependent immune cells in the PTB patients or the results of possible decrease in the inflammatory processes when anti-TB was supplemented with iron, zinc or selenium as adjuvant therapy. A study also observed a decrease in the level of IgG after two months of treatment of PTB patients[40]. The decrease in the level of IgG might be due to the effects of trace elements on phagocytic activities of some immune cells, thus causing a reduction in inflammation and enhances immunity[41].

Ferritin is described as a blood protein that stores iron in the body cells. It is an acute phase reactant that responds to inflammation[42]. Increased baseline plasma ferritin levels were observed in the PTB patients. The elevated levels of ferritin could be a sign of acute or chronic inflammation in the tuberculosis patients. Since iron is an important micronutrient for the survival of microbes including mycobacterium tuberculosis, elevated ferritin levels could therefore be beneficial to the patients for its potential to reduce the availability of iron for microbial proliferation. A study reported that pulmonary tuberculosis patients had an elevated level of ferritin and CRP[43]. Other researchers in their studies also reported a baseline hyper-ferritinemia in their pulmonary tuberculosis patients[44]. Mycobacterium tuberculosis has therefore

been linked with hyper-ferritinemia[45]. After eight weeks of treatment with anti-TB supplemented with iron, zinc and selenium, the ferritin levels decreased significantly in patients with anti-TB drugs only, patients with anti TB-drugs plus selenium supplementation and patients with anti TB drugs plus zinc supplementation. However there was an increase in ferritin level in patients with anti-TB and iron supplementation. Therefore, the host iron status may be an important and yet under-evaluated factor in TB prevention, therapy and vaccine design[46].

Albumin is the most abundant circulatory protein that is critical for transportation of molecules, creation of oncotic pressure, maintenance of body tissues, binding ligands for substances and antioxidant activities[47]. Significantly lower level of albumin observed in the tuberculosis patients recruited for this study could be associated with malnutrition in the patients. This could also be due to inflammation (since albumin is a negative acute phase reactant protein). Previous researchers reported significantly lower levels of albumin and zinc in PTB patients[48,49]. After 8 weeks of treatment, it was observed that the plasma levels of albumin increased significantly in the patients treated with anti-TB drugs and micronutrients supplementation, this study corroborates with another study who reported that selenium supplementation increases the level of serum albumin if taken as adjuvant therapy during treatment with anti-TB[50]. A study observed that PTB patients on zinc supplementation had an increased plasma albumin level when compared to their baseline level[51]. There was a study which concluded that to reduce the severity of PTB infection, nutritional supplement may be needed as adjuvant with anti TB regiment[31].

D-dimer is a product of fibrin degradation and a specific indicator of hypercoagulation in the PTB patients[52]. Tuberculosis has been implicated as an inducer of inflammation, coagulation and disarrangement in fibrinolytic system[53]. It is therefore evident that the PTB patients used for this study had coagulopathy based on the significantly higher levels of D-dimer in them. This study

agrees with previous researchers that reported altered hemostatic activity and hypercoagulopathy in the PTB patients[54]. The hypercoagulation observed in these PTB patients could be the consequence of deep vein thrombosis (DVT) induced by chronic inflammation in the PTB patients[55]. The increased level of D-dimer may also be associated with the circulation of pro-inflammatory cytokines. This agrees with previous researchers who reported a high level of D-dimer and CRP in PTB patients[56]. The novel of this study is the declined levels of D-dimer after intensive phase treatment with or without trace element supplementation.

Prothrombin test (PT) and aPTT are markers of coagulation used to assess the effectiveness of clotting factors. There were no significant changes in baseline values of PT and aPTT in the PTB patients compared with controls. This study is consistent with the finding of another study which reported that there was no significant difference in the levels of PT and aPTT in PTB patients[57]. They further explained that some cytokines like tumor necrosis factor-alpha and interleukin-6 (IL-6) emerging from TB granulomatous lesions were thought to influence the insignificantly prolonged PT and aPTT they observed.

Selenium is an important trace metal that is crucial in the body immune status. Selenium supplementation in PTB infection may help to strengthen the immune response, to counteract oxidative cellular damage related with inflammation and infection selenium may modulate the sensitivity of host to pathogens and immune system against microorganism[58]. The present study revealed low level of plasma Se in PTB patients. This could be due to malnutrition caused by anorexia in tuberculosis infection. Surprisingly in patients treated with anti-TB and Fe supplementation, the Se level increased significantly after 2 months of treatment, compared with patient treated with anti TB drugs only. Other studies also observed lower baseline level of plasma selenium in the *tuberculosis* patients used for their study[56].

Mutations or parallel gene transfer facilitated by phages, plasmids, chromosomal aberrations through antibiotic use-induced selection are possible mechanisms by which bacteria acquire resistance to antibiotics[59]. *Mycobacterium tuberculosis's* development of drug resistance embodied the essence of Darwin's theory of evolution. DNA sequences specific for M*ycobacterium tuberculosis* and rifampicin resistance by polymerase chain reaction were carried out by using genexpert MTB/RIF. Also, genetic characterization of drug sensitive and multi-drug resistant *M. tuberculosis* was carried out in this study. The cluster observed in the MDR tuberculosis are similar to *M. bovis.* This study agrees with previous workers who reported that the virulence factors of *M. bovis* are the same as those of *M. tuberculosis*, as both organisms can cause identical clinical disease in humans and are genetically very similar[60] .

The phylogenetic analysis which shows that MDR and DS had clusters of both *Mycobacterium tuberculosis* and *Mycobacterium bovis* in this findings may hypothesize that gene mutation of *M. tuberculosis* could mimic *M. bovis*. This study therefore corroborates the report of a researcher who observed that *M. bovis* and *M. tuberculosis* genomes show 99.95% identity at the nucleotide level[61]. Other study shows that the glycolytic enzymes *glpK*, *pykA* and *pdhA* of both M. tuberculosis and M. bovis are similar. The *rpoB* gene codes is the target of rifampicin, an essential drug in the treatment of tuberculosis and other mycobacterial infections. Mutations in the *rpoB* gene alter the structure of this protein and cause drug resistance[62]. In this study, MDR tubercle bacilli demonstrated a presence of rpoB gene at the band size approximately 330b. This seems to corroborate the report of another study which says that rifampicin resistance associated mutations occurred mostly in rpoB codon 531 (60%; 273/470 and 320/515)[63]. This study also demonstrated *Kat*G gene in MDR group. Mutations in several genes like *Kat*G, *inh*A, *oxy*R-*aph*C, *kas*A and *ndh* have been implicated in the resistance of MDR *M. tuberculosis* to Isoniazid. This study confirms the report of a study that MDR strains of *M. tuberculosis* contains mutated KatG gene[64].

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Chapter Five

Conclusion

5.1 Summary of Findings

Significantly lower levels of plasma Se, Zn and Fe were observed in all PTB patients recruited for this study. The lower levels of the elements could be associated with oxidative stress, mal-absorption and anorexia in the PTB patients. Baseline levels of ferritin, IgG, C3, CRP and D-dimer were higher significantly in all PTB patients when compared to controls. In contrast PT and aPTT did not show any significant change in all PTB patients compared to controls. After 2 months of treatment, ferritin, IgG, C3, CRP and D-dimer decreased significantly compared with the baseline values in patients with anti-TB drugs + Fe supplementation, anti-TB drugs + Se supplementation, and anti-TB drugs + Zn supplementation. Levels of PT and aPTT did not show any significant difference before and after treatment in all PTB patients when compared with controls. This study identified *rpoB* gene and *Kat*G gene in MDR strains of *M. tuberculosis*.

5.2 Conclusion

All TB patients in the four groups demonstrated higher levels of D-dimer which might be due to chronic intravascular coagulation. Increased levels of ferritin, C3 and CRP could suggest chronic inflammation. The consumption of micronutrients supplementation seemed to show significant effects on the body weight, haemoglobin concentration, ferritin, IgG, C3, CRP and D-dimer in

pulmonary tuberculosis patient. It may be necessary to include Fe, Se and Zn supplementation as adjuvant therapy in the management of tuberculosis to enhance metabolic activities, reduce inflammation, prevent iron deficiency anaemia, reduce intravascular coagulation, and to increase immune response of patients to infection. rpoB and KatG genes were confirmed in MDR-TB samples. Similar clusters observed in MDR-TB, M. *bovis* and some DS-TB may indicate the possible points of mutation that may lead to drug resistance. Although, this study is the first to report this. It may be necessary to always do phylogenetic analysis of samples to identify which sample has a cluster common to MTB and *M. bovis,* as this may help in reducing MDR-TB infection and possibly reduce the number of death.

5.3 Recommendations

Supplementation of anti-TB therapy with iron, selenium and zinc in PTB patients may be of help in the treatment outcome of tuberculosis. Addition of these supplements as adjuvants in the treatment of tuberculosis may help to:

1. Reduce inflammation
2. To prevent iron deficiency anaemia.
3. Reduce intravascular coagulation
4. Increase immune response to infection.

Therefore, the management of TB may require micronutrients supplementation as an adjuvant therapy.

Also, it may be necessary to always carryout phylogenetic analysis of TB samples to identify which samples have similar clusters of MDR-strains and M. *bovis* in order to reduce MDR-TB.

5.4 Contribution to Knowledge

Iron, selenium and zinc supplementations may enhance metabolic processes and hasten recovery in PTB patients on anti-TB therapy.

Phylogenetic analysis of TB samples may enhance reduction in drug resistance TB as it may help in evolutionary history of *Mycobacterium tuberculosis*.

5.5 Suggestion for Further Studies

- Carry out a more genetic analysis to examine the clusters observed in MDR-TB and some DS-TB samples if they could be due to mutation.
- Carry out lung function tests on patients with micronutrients supplementation as adjuvants, and with patients on anti-TB drug only.

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Appendix I

Ethical Approval

TELEGRAMS..... TELEPHONE.....

MINISTRY OF HEALTH

DEPARTMENT OF PLANNING, RESEARCH & STATISTICS DIVISION

PRIVATE MAIL BAG NO. 5027, OYO STATE OF NIGERIA

Your Ref. No. .....
All communications should be addressed to the Honorable Commissioner quoting
Our Ref. No. AD 13/479/ 4465

22nd November, 2022

The Principal Investigator,
Department of Biology Sciences,
Faculty of Natural and Applied Sciences,
Lead City University,
Ibadan, Nigeria.

Attention: Owolabi Ekundayo

ETHICS APPROVAL FOR THE IMPLEMENTATION
OF YOUR RESEARCH PROPOSAL IN OYO STATE

This is to acknowledge that your Research Proposal titled: "Genetic Characterization of Multidrug Resistant Tuberculosis and Biochemical Responses of Tuberculosis Patients during Treatment with or without Micronutrient Supplementation." has been reviewed by the Oyo State Ethics Review Committee.

2. The committee has noted your compliance. In the light of this, I am pleased to convey to you the full approval by the committee for the implementation of the Research Proposal in Oyo State, Nigeria.

3. Please note that the National Code for Health Research Ethics requires you to comply with all institutional guidelines, rules and regulations, in line with this, the Committee will monitor closely and follow up the implementation of the research study. However, the Ministry of Health would like to have a copy of the results and conclusions of findings as this will help in policy making in the health sector.

4. Wishing you all the best.

Dr. Abbas Gbolahan
Director, Planning, Research & Statistics
Secretary, Oyo State, Research Ethics Review Committee

Appendix II

MTB Positive Patient

Appendix III

PCR Analysis of MDR1 (DO1)

ACATCGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGAAAGGTCTCTTC
GGAGATACTCGAGTGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCCTGCACTT
CGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATAGGACCCCGGGATGCATGTC
TTGTGGTGGAAAGCGCTTTAGCGGTGTGGGATGAGCCCGCGGCCTATCAGCTTGTTG
GTGGGGTGACGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGTCCGG
CCACACTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACGGCCTTC
GGGTTGTAAACCTCTTTACCATCGACGAAGGTCCGGGTTCTCTCGGATTGACGGTAG
GTGGAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTG
CGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGCGTTGT
TCGTGAAATCTCACGGCTTAACTGTGAGCGTGCGGGCGATACGGGCAGACTAGAGT
ACTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAG
GAACACCGGTGGCGAAGGCGGGGTGTCTGGGCAGTAACTGACGCTGAGGAGCGAAA
GCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGT
ACTAGGTGTGGGTTTCCTTCCTTGGGATCCGTGCCGTAGCTAACGCATTAAGTACCC
CGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCA
CAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTT
TGACATGCACAGGACGCGTCTAGAGATAGGCGTTCCCTTGTGGCCTGTGTGCAGGTG
GTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGC
GCAACCCTTGTCTCATGTTGCCAGCACGTAATGGTGGGGACTCGTGAGAGACTGCCG
GGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGTCCA
GGGCTTCACACATGCTACAATGGCCGGTACAAAGGGCTGCGATGCCGCGAGGTTAA
GCGAATCCTTAAAAGCCGGTCTCAGTTCGGATCGGGGTCTGCAACTCGACCCCGTGA
AGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGG
CCTTGTACACACCGCCCGTCACGTCATGAAAGTCGGTAACACCAGAAGCCAGTGGC
CTAACCCTCGGAT

Appendix IV

PCR Analysis of MDR2 (DO2)

CCCGCTGGCGGCGTGCATACACATGCAAGTCGAACGGAAAGGTCTCTTCGGAGATA
CTCGAGTGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCCTGCACTTCGGGATA
AGCCTGGGAAACTGGGTCTAATACCGGATAGGACCACGGGATGCATGTCTTGTGGT
GGAAAGCGCTTTAGCGGTGTGGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGT
GACGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGTCCGGCCACACT
GGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACA
ATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACGGCCTTCGGGTTGT
AAACCTCTTTCACCATCGACGAAGGTCCGGGTTCTCTCGGATTGACGGTAGGTGGAG
AAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCG
TTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGCGTTGTTCGTGA
AATCTCACGGCTTAACTGTGAGCGTGCGGGCGATACGGGCAGACTAGAGTACTGCA
GGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACAC
CGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGG
GAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGTACTAGGT
GTGGGTTTCCTTCCTTGGGATCCGTGCCGTAGCTAACGCATTAAGTACCCCGCCTGG
GGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCG
GCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATG
CACAGGACGCGTCTAGAGATAGGCGTTCCCTTGTGGCCTGTGTGCAGGTGGTGCATG
GCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TTGTCTCATGTTGCCAGCACGTAATGGTGGGGACTCGTGAGAGACTGCCGGGGTCAA
CTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCA
CACATGCTACAATGGCCGGTACAAAGGGCTGCGATGCCGCGAGGTTAAGCGAATCC
TTAAAAGCCGGTCTCAGTTCGGATCGGGGTCTGCAACTCGACCCCGTGAAGTCGGGA
GTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTAC
ACACACT

Appendix V

PCR Analysis of DS3 (DO3)

ATCGAACGCTGGCGGCGTGCTTACACATGCAAGTCGAACGGAAAGGTCTCTTCGGA
GATACTCGAGTGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCCTGCACTTCGG
GATAAGCCTGGGAAACTGGGTCTAATACCGGATAGGACCACGGGATGCATGTCTTG
TGGTGGAAAGCGCTTTAGCGGTGTGGGATGAGCCCGCGGCCTATCAGCTTGTTGGTG
GGGTGACGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGTCCGGCCA
CACTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG
CACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACGGCCTTCGGG
TTGTAAACCTCTTTCACCATCGACGAAGGTCCGGGTTCTCTCGGATTGACGGTAGGT
GGAGAAGAAGCACCGGCCAACAACGTGCCAGCAGCCGCGGTAATACGTAGGGTGC
GAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGCGTTGTT
CGTGAAATCTCACGGCTTAACTGTGAGCGTGCGGGCGATACGGGCAGACTAGAGTA
CTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGG
AACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGC
GTGGGGAGCGAACAGGATTAGATCCCCTGGTAGTCCACGCCGTAAACGGTGGGTAC
TAGGTGTGGGTTTCCTTCCTTGGGATCCGTGCCGTAGCTAACGCATTAAGTACCCCG
CCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACA
AGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTG
ACATGCACAGGACGCGTCTAGAGATAGGCGTTCCCTTGTGGCCTGTGTGCAGGTGGT
GCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGC
AACCCTTGTCTCATGTTGCCAGCACGTAATGGTGGGGACTCGTGAGAGACTGCCGGG
GTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGTCCAGG
GCTTCACACATGCTACAATGGCCGGTACAAAGGGCTGCGATGCCGCGAGGTTAAGC
GAATCCTTAAAAGCCGGTCTCAGTTCGGATCGGGGTCTGCAACTCGACCCCGTGAAG
TCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCT
TGTACACACCGCCCGTCACGTCATGAAAGTCGGTAACACCCGAAGCCAGTGGCCTA
ACCCTCGGGAT

Appendix VI

PCR Analysis of DS4 (DO4)

AACGGCGTGCTTAACACATGCAAGTCGAACGGAAAGGTCTCTTCGGAGATACTCGA
GTGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCCTGCACTTCGGGATAAGCCT
GGGAAACTGGGTCTAATACCGGATAGGACCACGGGATGCATGTCTTGTGGTGGAAA
GCGCTTTAGCGGTGTGGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGACGG
CCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGTCCGGCCACACTGGGAC
TGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG
CGCAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACGGCCTTCGGGTTGTAAACC
TCTTTCACCATCGACGAAGGTCCGGGTTCTCTCGGATTGACGGTAGGTGGAGAAGAA
GAACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTC
CGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGCGTTGTTCGTGAAATCT
CACGGCTTAACTGTGAGCGTGCGGGCGATACGGGCAGACTAGAGTACTGCAGGGGA
GACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGGTG
GCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGC
GAACAGGATACGATACCCTGGTAGTCCACGCCGTAAACGGTGGGTACTAGGTGTGG
GTTTCCTTCCTTGGGATCCGTGCCGTAGCTAACGCATTAAGTACCCCGCCTGGGGAG
TACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGG
AGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATGCACA
GGACGCGTCTAGAGATAGGCGTTCCCTTGTGGCCTGTGTGCAGGTGGTGCATGGCTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGT
CTCATGTTGCCAGCACGTAATGGTGGGGACTCGTGAGAGACTGCCGGGGTCAACTC
GGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCACA
CATGCTACAATGGCCGGTACAAAGGGCTGCGATGCCGCGAGGTTAAGCGAATCCTT
AAAAGCCGGTCTCAGTTCGGATCGGGGTCTGCAACTCGACCCCCTGAAGTCGGAGT
CGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACAC
ACCGCCCGTCACGTCATGAAAGTCGGTAACACCCGAAGCCAGTGGCCTAACCCTCG
GGAGGGAAAT

Bio-data

A. Personal Data:

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2.	**Address:**	House 2, Road C7, Iwo road Ojo Expressway Power house Busstop Akobo Extension Ibadan
3.	**E-mail Address:**	dowoblowo2@gmail.com
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B. Educational Background -

School Attended	**Dates**	**Qualifications**
• College of Medicine, University College Hospital	1990-1994	AIMLS/BMLS
• Lead City University, Ibadan	2018-2020	M.Sc. Molecular Biology and Genomics
• Lead City University, Ibadan	2020-2024	PhD in view

C. Work Experience with Dates

• Alafia Hospital 1938 Ltd. Ibadan	1995 till date
• Banby Specialist Hospital Ibadan	2006 till date

D. Awards and Fellowship

Fellow Institute of Professional Managers and Administrators	-	2020
Fellow Corporate Institute of Strategic Research	-	2022

E. Membership of Academic/Professional Bodies

Medical Laboratory Science Council of Nigeria

American Society for Biochemistry and Molecular Biology

African Society for Laboratory Medicine

F. Publications

- Owolabi Ekundayo, *Iron Status in the Modulation of Multidrug Resistance in Mycobacterium tuberculosis (MTB) Infections in Ibadan*. **Lead City University, Ibadan, 3rd International Conference,** 2-4 Nov. 2022, 33-34.

G. Major Conferences/Workshop Attended with Dates

-	COVID 19: Overview, situation update, Control Effort & Prevention Measures (WHO Oyo State)	16-17 March, 2021
-	FASCON	2-4 November, 2022
-	Fertility prognosis and diagnosis (National Andrology Team)	8-9 February, 2023
-	Impact of Emerging Digital Health Technologies (OYHMB)	8-10 May, 2023

H. Referees

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   08038613172

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_____ _____

Signature **Date**

The University Compliance Certification

This is to certify that the thesis by Ekundayo Joseph OWOLABI with the Matric Number LCU/PG/002022 in the Department of Biological Sciences, Faculty of Natural and Applied Sciences, Lead city University Ibadan, is in full compliance with the approved University format and style.

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