# THE PRODUCTION AND GENE POLYMORPHISMS OF TUMOR NECROSIS FACTOR- ALPHA IN PATIENTS WITH TUBERCULOSIS IN THE VHEMBE DISTRICT, LIMPOPO PROVINCE

**BY**

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# ABSTRACT

**Background:** Tuberculosis (TB) is an old, irresistible infection caused by *Mycobacterium tuberculosis* (*Mtb*). The causative agent has antigens that can stimulate the production of cytokines via the mononuclear phagocyte system. Although mutations in immune-related genes may directly impact a host's ability to control the infection when exposed to *M.tb*, the pro-inflammatory cytokine Tumor Necrosis Factor- α is crucial in host defence against TB and granuloma formation. Therefore, this study aimed to assess the production and gene polymorphisms of TNF-α in patients with TB in the Vhembe district, Limpopo province.

**Methods:** This study recruited thirteen TB patients from three healthcare facilities in the Vhembe district, Limpopo province. Collected samples (sputum) were analysed using the Allplex/Anyplex kit, to detect the presence of Mycobacterium tuberculosis. Serum was collected from the blood and used to determine the levels of TNF-α in the participants, using the DIAsource ELISA kit. Genomic DNA was extracted from blood samples and purified using the Zymo kit from Inqaba Biotec. The purified DNA was quantified using the Nanodrop 8 from Thermo Fischer. The quantified DNA was analyzed by MassARRAY system to sequence various TNF-α SNPs. Data analysis for this study was carried out using the Jamovi software for windows version 2.5.3.

**Results:** This study found that about 69% of the participants were male and 62% were ≤48 years of age. The majority of the participants (62%) were unemployed. Additionally, 46% of the sputum samples were positive for *Mtb.* There was a high prevalence (69%) in participants with moderate TNF-α levels. Furthermore, this study found ten SNPs namely: rs10242595, rs1524107, rs1799724, rs1799964, rs1800629, rs1800630, rs1800750, rs3093662, rs309376, and rs361525. The following alleles were reported to have higher frequency than the other: rs10242595 A* (58%), rs1524107 C* (58%), rs1799724 C* (100%), rs1799964 T* (93), rs1800629 G* (88), rs1800630 C* (92), rs1800750 G* (85), rs3093662 A* (73%) and rs361525 A* (100). The allele frequency in rs309376 was evenly distributed G* (50%) and A* (50%).

**Conclusion:** Our findings suggest that there's a correlation between TNF-α levels and risk factors and also, there's a significant association between TNF-α SNPs and TNF-α expression levels.

**KEYWORDS:** *Tuberculosis, Granuloma, Cytokine, Tumor necrosis factor, Polymorphism, Risk factors.*

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## Declaration

I, Mmasehlare Stellah Molepo declare that "***The production and gene polymorphisms of Tumour Necrosis Factor- alpha in patients with tuberculosis in the Vhembe district, Limpopo province***" is my work and has been carried out under the supervision of Prof A.N Traore, Dr M Magwalivha and Prof N Potgieter. I further declare that this dissertation has been composed solely by me and it has not been submitted, in whole or in part, to any institution for a degree or diploma and has been complemented by referenced works duly acknowledged.

11/02/2025

…….. …….

Signature Date

# Acknowledgement

Firstly, I would like to thank God for His continuous guidance and blessings throughout this research journey. His divine light has been my guiding star and I'll forever be grateful.

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# Dedication

This dissertation is dedicated:

- To my incredible parents, whose unwavering love and endless support have shaped me into who I am today. Your guidance and sacrifices have been the foundation of all my achievements.
- To my wonderful siblings, for the bond we share and the joy you've brought into my life. Your encouragement and laughter have been my constant companions.
- And to my beloved son, the light of my life. Your innocence, curiosity, and love inspire me every day to be the best version of myself.

# Abbreviations

| | |
|---|---|
| BCG | Bacillus Calmette-Guerin |
| BMI | Body mass index |
| CD | Cluster of differentiation |
| DNA | Deoxyribonucleic acid |
| DTH | Delayed-type hypersensitivity |
| ELISA | Enzyme-linked immunosorbent assay |
| FDA | Fluorescein diacetate |
| HIV/AIDS | Human Immunodeficiency virus/Acquired immune deficiency syndrome |
| ICT | Immunochromatographic |
| IFN-$\gamma$ | Interferon gamma |
| IGRAs | Interferon-Gamma Release Assays |
| IL- | Interleukin |
| IKK | Inhibitor of kappa Kinase |
| LAM | Lipoarabinomannan |
| LEDs | Light-emitting diodes |
| LTBI | Latent tuberculosis infection |
| MALDI-TOF | Matrix-assisted laser desorption/ionization- Time of light |
| MAPKs | Mitogen-activated protein kinases |
| MDR | Multi-drug resistance |
| MHC | Major Histocompatibility Complex |
| *Mtb* | *Mycobacterium tuberculosis* |
| NF-κB | Nuclear factor kappa |
| NRAMP | Natural resistance-associated macrophage protein |
| NTM | Non-tuberculous mycobacteria |
| PBS | Phosphate buffer solution |
| PCR | Polymerase chain reaction |
| pg/mL | pictograms per millilitre |
| PPD | Pure Protein Derivative |
| PRR | Pattern recognition receptors |
| RIP 1 | Receptor- Interacting Protein Kinase 1 |

| | |
|---|---|
| RNA | Ribonucleic acid |
| RNIs | Reactive nitrogen intermediates |
| ROIs | Reactive oxygen intermediates |
| SNP | Single nucleotide polymorphism |
| TB | Tuberculosis |
| TGF-β | Transforming Growth Factor- beta |
| TLRs | Toll-like receptors |
| TNF-α | Tumor Necrosis Factor-Alpha |
| TNFR | Tumor necrosis factor receptor |
| TRADD protein | Tumor Necrosis Factor Receptor Type 1- Associated DEATH Domain |
| TRAF 2 | Tumor Necrosis Factor Receptor-Associated Factor 2 |
| TST | Tuberculin skin test |
| WHO | World Health Organisation |
| XDR | Extensive-drug resistance |

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# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 BACKGROUND

Tuberculosis (TB) is a contagious disease caused by the bacterium *Mycobacterium tuberculosis (Mtb)*. Although it can affect other bodily parts, it mainly affects the lungs (WHO, 2020). During the transmission, the bacterium spreads within the frame of airborne beads when an infected individual is in contact with another individual. The mycobacterium is transmitted through airborne droplets and enter the respiratory tract, where they travel to the alveoli-typically in the middle or lower lobes of the lungs-initiating colonization and triggering an immune respponse. The disease can be latent, where the bacteria remain inactive in the body, or active, where symptoms manifest, and the disease can be transmitted to others. Frequent symptoms experienced by TB patients are anorexia, hemoptysis, night sweats, persistent coughing, and lethargy (Ellner et al. 2000).

With millions of new cases being reported yearly, TB remains a global health issue. In 2023, the World Health Organization (WHO) reported an estimated 10.8 million individuals contracted TB, and 1.25 million of those cases resulted in death (WHO, 2023). TB is present in all countries and all age groups, but is still prevalent in developing countries with high rates of HIV/AIDS, malnutrition, and poor healthcare infrastructure (WHO, 2023). At the moment, people with weakened immune systems as a result of HIV infection account for the majority of TB cases in South Africa and other Sub-Saharan countries. As a result, countries in Sub-Saharan Africa—where HIV prevalence is relatively high—are currently dealing with a catastrophic epidemic that is significantly increasing disease and mortality rates *(Abdool* et al. *2009).*

The immune system is crucial in the body's defence against *Mtb*. The interaction between *Mtb* and the host's immune system determines whether the infection will be contained, progress to active, or be cleared entirely. When *Mtb* enters the body, it is engulfed by macrophages which attempt to destroy the bacteria (Martino et al. 2019). However, the bacterium tends to survive since it has evolved a mechanism that helps it to survive within the host. The ability of this *Mtb* to repress the phago-lysosome process

and multiply within the macrophages occurs within the macrophages discharging cytokines that will fortify the resistant cells to come to the site of infection. The presence of these resistant cells occurs within the arrangement of a granuloma encompassing the caseating cells to be removed to eradicate the infection (Flynn et al. 1993). At this point, cytokines such as the TNF-α, Interleukins, and interferon-gamma (IFN-ɣ) are discharged in arrangement to fortify or recruit more cells to come to the location of infection. This may result in either the disposal of the *Mtb*, keeping it at torpid, or the infection advancing to the dynamic stage (Ellner et al. 2000).

Tumor Necrosis Factor-alpha is one of the key cytokines in the immune response against *Mtb* infection, and it is important for the integrity of the granuloma (Kaufmann et al. 2005). TNF-α is important for security against mycobacterial diseases (Ellner et al. 2000), and it has been observed that an increase in TNF-α has occurred among individuals receiving treatment for TB infection during the period of deterioration. The pro-inflammatory cytokine TNF-α is detrimental to many immune system illnesses and is necessary for host defense against intracellular pathogens like mycobacteria. This cytokine encourages macrophage phagocytosis and intracellular killing, and it helps with granuloma organization and function, in the human immune response to *Mtb.*

## 1.2 RATIONALE

TNF-α plays a vital role in the immune response to *Mtb*. It helps form granulomas, which are essential for containing the infection (El-Tahan et al. 2016). Understanding the production levels and genetic variations of TNF-α in TB patients can provide insights into the disease's pathogenesis and progression. Several TNF-α gene promoter region polymorphisms, such as -308G/A, -238G/A, and others, have been associated with altered TNF-α production (El-Tahan et al. 2016). These polymorphisms can influence an individual's immune response to TB, potentially leading to differences in disease susceptibility and outcomes. Numerous studies have detailed connections between SNPs of immune-related genes and the chance of *Mtb* illnesses (Jafari et al. 2016). Research conducted in China revealed that SNPs of the cytokine TNF-α have indicated critical affiliation with TB vulnerability (Shouquan et al. 2020). Another study conducted in Arabia has shown that TB risks are related to polymorphisms in candidate genes

related to the immune system and inflammatory reactions (Jafari et al. 2019). The magnitude of penalties of these SNPs, and their likeliness for driving phenotypic alterations amid strains is of incredible concern. Few studies have examined and investigated the implications of TNF-α gene polymorphism in TB patients. Hence, this study aims at assessing the production and gene polymorphisms of TNF-α in TB patients.

## 1.3 OBJECTIVES

### 1.3.1 PRIMARY OBJECTIVE

To assess the production and gene polymorphism of tumor necrosis factor-alpha in patients with tuberculosis in the Vhembe district Limpopo province.

### 1.3.2 SECONDARY OBJECTIVES

The set specific objectives are:

- To investigate the factors associated with an increased risk of TB.
- To determine the serum levels of TNF- α in patients with latent TB versus active TB.
- To determine the association of TNF- α SNPs with the development and progression of TB.

## 1.4 PROBLEM STATEMENT

Despite existing literature on TNF-α and its role in TB, there is limited research focusing specifically on the Vhembe District population. This gap highlights the need for localized studies that explore the prevalence of specific TNF-α gene polymorphisms and their correlation with serum TNF-α levels among TB patients. Such research could provide insights into genetic susceptibility to TB within this population and inform targeted therapeutic strategies.

## 1.5 STRUCTURE OF THE DISSERTATION

This dissertation investigates the production and gene polymorphisms of Tumor Necrosis Factor-alpha in patients with TB in the Vhembe district Limpopo province. The

study aims to explore the relationship between TNF-α production, gene polymorphism, and TB susceptibility.

The dissertation is divided into six chapters. Chapter one introduces TB, TNF-α, the research objectives, and the problem statement. Chapter two is literature review on the role of TNF-α in TB, genetic polymorphisms, and immune response. Chapter three describes the research methodology, including sample collection, and laboratory procedures. Chapter four represents findings of TNF-α production and gene polymorphism analysis in TB patients. Chapter five discusses the findings and implications for TB susceptibility. Chapter six outlines the limitations and futures recommendations.

# CHAPTER 2
# LITERATURE REVIEW

## 2.1 BACKGROUND

Tuberculosis (TB) is a long-term illness that can persist for months or even years if not treated properly. It is characterized by forming granulomas, clusters of immune cells that contain the bacteria and prevent its spread (WHO, 2018). Individuals infected with TB can face death if the infection is not treated. Symptoms such as malaise, night sweats, and constant cough are usually associated with active TB. Moreover, symptoms of TB infection in the lungs include hacking, chest pains, and coughing up blood. The affected zone or area of the body determines the symptoms one can have when infected with TB (Churchyard et al. 2017). In any case, on the off chance that an uninfected individual went through time with a TB-infected individual or an individual with side effects of TB, the uninfected individual ought to be tested. In essence, there is a massive chance that members of the family or even colleagues of the individual infected contract TB. This is typically due to the close relativeness, or the ability to share the same space (WHO, 2022).

TB is a global health issue, with a significant burden in low- and middle-income countries. The WHO estimates that approximately 10 million people contract TB each year, with about 1.5 million deaths (WHO, 2022). TB disproportionately affects vulnerable populations, including those with weakened immune systems, malnutrition, and living in crowded conditions. TB is a disease that can affect the entire family, and their vulnerability may not only be due to the path of transmission but also because of the mental impact that the disease can have on the whole family (Churchyard et al. 2017). Similar to the stigma associated with HIV, TB also bears the shame of poverty and filthy living conditions, which are prevalent in much of South Africa (Churchyard et al. 2017).

## 2.2 ETIOLOGY OF *MYCOBACTERIUM TUBERCULOSIS*

TB is a significant global health concern, primarily caused by the *Mycobacterium tuberculosis* (*Mtb)*. This pathogen is highly adept at surviving within the human host, often leading to a latent infection that can persist for years. Understanding the etiology of *Mtb* is crucial for developing effective prevention and treatment strategies. This report delves into *Mtb's* origins, characteristics, and mechanisms, providing a detailed overview of its etiology.

### 2.2.1. *MYCOBACTERIUM TUBERCULOSIS*

The intracellular, facultative *Mtb* bacterium has a rod-like form and is 2-4 meters long (Schnoller, 2012). More than 100 species of mycobacteria are found in nature, and most of them are not harmful (Chandler et al. 2011). Based on a 94.3% sequence similarity in the 16S ribosomal RNA gene, more than 120 members of the genus Mycobacterium were discovered and categorized (Saini et al. 2020). Furthermore, for microscopic identification, they feature a unique complex cell wall envelope that may be stained in various ways with the Ziehl-Neelsen acid-fast stain (Ahmed, 2017). The organism is an aerobe that reproduces slowly, does not form spores, and is non-motile. A cell wall skeleton of peptidoglycan, mycolic acids specific to mycobacteria, and arabinogalactan forms a hydrophobic permeability barrier (Jensen et al. 2005).

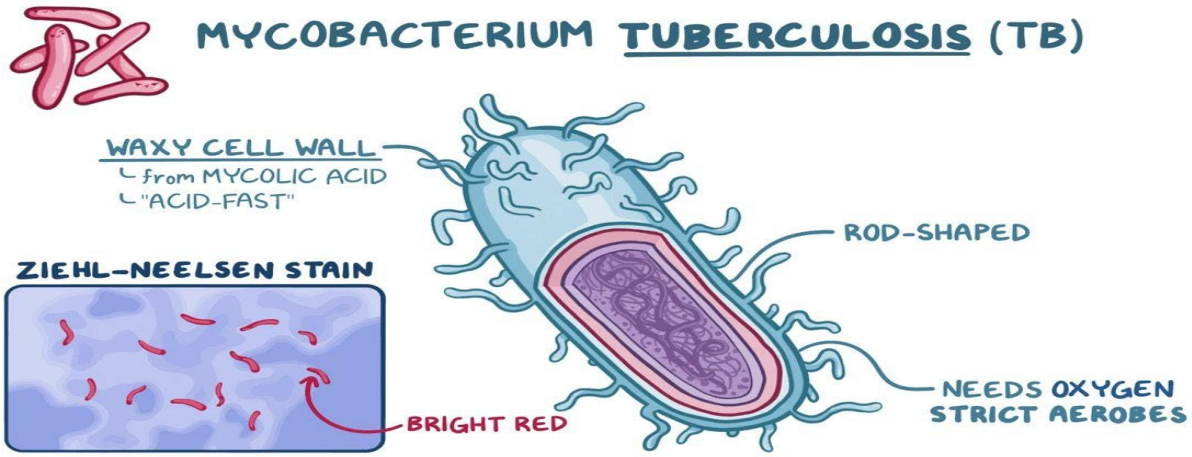

Figure 2.1: Mycobacterium tuberculosis morphology (Adopted from https://www.osmosis.org/learn/Mycobacterium_tuberculosis_(Tuberculosis))

### 2.2.2 TRANSMISSION

Transmission of *Mtb* occurs through the air when a person with active TB disease in their lungs or throat coughs, speaks or sings. These actions release tiny droplets containing the bacteria into the air, which people can inhale nearby (Figure 2.2). When droplets with *Mtb* are suspended in the air for several hours, especially in enclosed spaces with poor ventilation, people nearby can inhale these infectious droplets. Once inhaled, the bacteria can settle in the lungs and multiply (Shiloh, 2016). The immune system can often contain the bacteria, leading to a latent TB infection. People with LTBI do not feel sick, do not have symptoms, and cannot spread TB to others. If the immune system cannot contain the bacteria, or if the bacteria become active later, the infected individual develops active TB disease. This is when symptoms appear, and the infected individual becomes contagious (WHO, 2012).

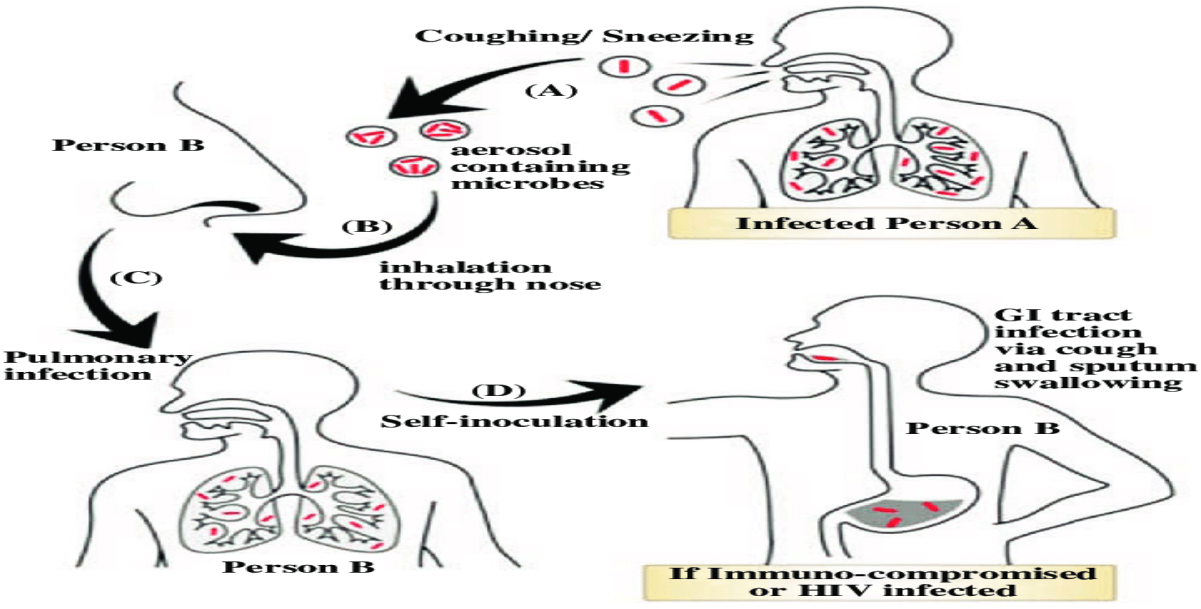


Figure 2.2: Transmission of Mycobacterium tuberculosis (Adopted from https://mungfali.com/explore/Tuberculosis-Chain-of-Infection)

### 2.2.3 PATHOGENESIS OF TUBERCULOSIS

The immune response to *Mtb* is complex and involves both innate and adaptive immunity (Mayer-Barber and Barber, 2015):

**Innate Immunity:** Alveolar macrophages and dendritic cells use pattern recognition receptors (PRRs), like Toll-like receptors (TLRs), to identify *Mtb* when infected. Upon detection, these immune cells become activated by pro-inflammatory cytokines, such as TNF-α. TNF-α is essential for the formation and upkeep of granulomas, improves phagocytosis, and triggers the death of infected cells, all of which help to control the infection.

**Adaptive Immunity:** The production of interferon-gamma (IFN-γ) by activated T cells, namely Th1-type CD4+ T-helper cells, is a hallmark of the adaptive immune response. The stimulation of macrophages and the improvement of their microbicidal activities depend on IFN-γ. Furthermore, infected macrophages can be killed by cytotoxic CD8+ T cells, which release the bacteria and permit additional immunological attack or containment within new granulomas.

**Cytokine Dynamics:** An essential part of the immune response in TB is the involvement of cytokines, particularly TNF-α. Numerous cells, such as macrophages, dendritic cells, and T cells, produce TNF-α, which has multiple functions in managing TB infection. Gene polymorphisms in the TNF-α gene could impact its production and, in turn, the host's vulnerability to TB and the disease's advancement. According to recent research, several polymorphisms in the TNF-α gene's promoter region are linked to changed cytokine production, which affects how healthy granulomas form and how well the immune system responds.

*Mtb* enters the body through the lungs and resides in the alveoli (Frieden et al. (2003)). The first stage of infection is a primary infection, with three possible outcomes wherein *Mtb* can either be eradicated, remain in place, or persist (Jensen et al. 2005). While *Mtb* invades the immune response in the active state, resulting in an active phase, the immune system may suppress the infection in the retention situation, leading to latent TB (Nicod, 2007). The immune system has positioned macrophages in the alveoli as an anti-infection precaution. As the host's second line of defence, these innate immune

system macrophages allow the body to eliminate invasive mycobacteria and stop infection (Scott et al. 2004). Essentially, the outcome is the quality of the host's defences and the balance between them and the invasive mycobacteria.

After being ingested by macrophages, mycobacteria multiply slowly, dividing their cells every 25 to 32 hours (Nicod, 2007). Regardless of whether the infection gets under control or worsens, macrophages produce proteolytic enzymes and cytokines to break down the bacteria (Korf et al. 2006). Macrophages and T lymphocytes try to contain the infection by forming granulomas, even though; the bacilli may adapt to survive (Geng et al. 2003). The wall's integrity is disrupted in patients with deficient immune systems, which makes it possible for the bacilli to escape and spread to nearby alveoli or organs (Scott et al. 2004). Infected individuals develop a cough that progresses to the production of purulent sputum or sputum containing traces of blood. This can occur due to the rupture of a dilated vessel within a cavity, the destruction of an intact vessel in the cavity wall, or the formation of an aspergilloma in a pre-existing cavity (Scott et al. 2004).

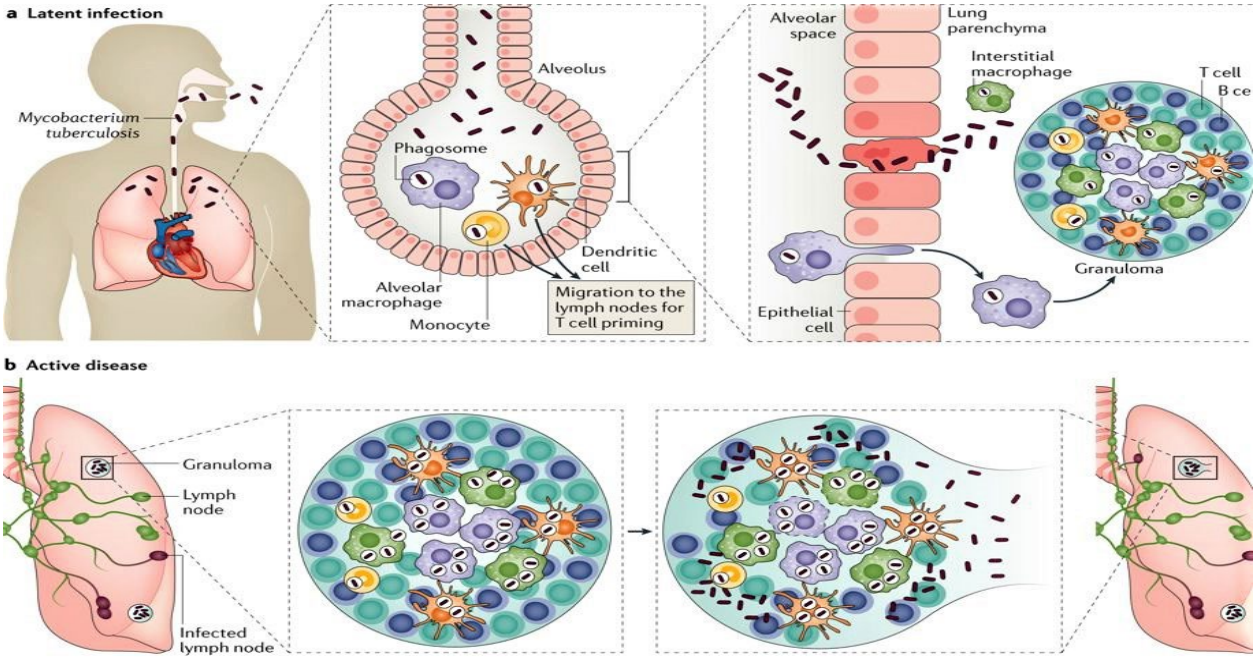


Figure 2.3: Pathogenesis of Mycobacterium tuberculosis (Adopted from: https://mavink.com/explore/Pathogenesis-of-Mycobacterium-Tuberculosis)

### 2.2.4 TYPES OF TB

#### 2.2.4.1 LATENT TUBERCULOSIS INFECTION (LTBI)

In the absence of clinical and radiographic findings, LTBI is characterized by a reactive tuberculin skin test (TST), from which the response of delayed-type hypersensitivity (DTH) to intradermal injection of purified proteins derived from *Mtb* or a T cell response to M. tuberculosis-specific antigens (Ag) is indicated (Horsburgh and Rubin, 2011). Latently infected individuals typically do not have tubercle bacilli recoverable from sputum or other places, suggesting a low bacillary burden (Sia and Wieland, 2011). Treatment for LTBI entails 9 months of isoniazid treatment, four months of rifampin treatment (a transcriptional inhibitor) (Sterling et al. 2011), or two months of rifampin plus pyrazinamide treatment (a sterilizing drug that is said to target dormant bacilli living in an acidic environment) (Jasmer et al. 2002). However, the last combination regimen is no longer advised due to the increased risk of hepatoxicity among HIV-seronegative people (Jasmer et al. 2002)

According to the earliest medical trial experiment, a once-weekly regimen of isoniazid with rifapentine and isoniazid for just three months had a far higher completion rate and is just as effective as a regular self-administered nine-month daily regimen of isoniazid (Sterling et al. 2011). McDermott defined *Mtb* persistence as the “capacity of drug-susceptible organisms to survive drug attack when subsisting in an animal body” (McDermott, 1958). Thus, in the traditional sense, host immune defences cause LTBI, whereas *Mtb* persistence is linked to antibiotic pressure; nevertheless, there seems to be a phenotypic relationship between these two events, which could indicate comparable physiological states of the organism (Jasmer et al. 2002).

In the case of LTBI, persistent bacilli seem to be more vulnerable to sterilizing medications such as rifampin and pyrazinamide than to isoniazid bactericidal medication. The length of TB treatment was cut from 18 months to 9 months with the addition of rifampin to the anti-TB regimen, and it was further shortened to the current six months with the addition of pyrazinamide (Karakousis, 2009). Certain medications, such as isoniazid, are classified as bactericidal because they destroy quickly proliferating bacilli, whereas other medications, such as pyrazinamide and rifampin, are

more successful at eliminating persistent, non-replicating organisms (Dooley et al. 2020). Antibiotic tolerance (Jasmer et al. 2002) refers to the reduced responsiveness to death by cell wall-antibiotics, such as isoniazid, a common characteristic of LTBI and persistent bacilli. This phenomenon is not specific to mycobacteria; it has also been observed in several other organisms, such as *Streptococcus pneumoniae, Streptococcus pyogenes, Escherichia coli,* and *Treponema pallidum* (Balaban et al. 2004).

The genetic susceptibility component of TB appears to comprise several genes and distinct genes that function differently in several groups (Maartens and Wilkinson 2007). TB is a highly complex disease. Numerous polymorphic genes, such as interferon-gamma (IFN-$\gamma$) (Pacheco et al. 2008) and natural resistance-associated macrophage protein (NRAMP1/SLC11A1) (Yuk et al. 2024), have been linked to TB (Möller and Hoal 2010). These findings highlight the significance of these genes for TB susceptibility. The complexity of the immune system and the multifactorial character of complex diseases mean that gene interactions significantly influence an individual's risk of acquiring a complex disease, compared to single polymorphisms (Ritchie et al. 2001; Tsai et al. 2003; Williams et al. 2000).

A novel method for determining susceptibility to complicated diseases is gene-gene interaction analysis. Gene-gene interactions, commonly referred to as epistasis, are described as interactions between genes located exceptionally far apart within the genome and do not share a common locus. One gene can either suppress (antagonistic epistasis) or promote (synergistic epistasis) the expression of another gene in gene-gene interactions (Nagel, 2005). Currently, there is limited information on gene-gene interactions in infectious diseases like TB has been published (Velez et al. 2010). Gene-gene relationships have been discovered in non-infectious illnesses like rheumatoid arthritis (Martinez et al. 2006), diabetes (Bergholdt et al. 2005; Koeleman et al. 2004), asthma (Kim et al. 2006; Lee et al. 2004), and Parkinson's disease (Deng et al. 2004; Pankratz et al. 2003).

### 2.2.4.2 Clinical reactivation of tuberculosis infection (active TB)

A primary infection or the reactivation of the dormant, controlled infection may be the cause of clinical TB in humans. HIV/AIDS-related secondary immunosuppression is the most frequent reason for *Mtb* reactivation. Recently, overt clinical illness and the reactivation of dormant *Mtb* bacilli have hampered therapy with neutralizing TNF antibody, infliximab, or soluble TNFR, etanercept. Within 12 weeks of starting TNF-neutralizing medication, reactivation occurs (Keane, 2005), and it frequently manifests in extrapulmonary disease (disseminated infection in the lymph node, peritoneum, and pleura). Compared to the frequency of other opportunistic infections linked to infliximab therapy, the frequency of TB was much greater. As a result, active TB may appear shortly after infliximab or etanercept treatment is initiated (Keane, 2005).

The primary consequence of HIV/AIDS-which is a worldwide health concern, especially across Southern Africa and Asia, is the reactivation of infection in places where TB is endemic (WHO, 2020). Notably, this is enough to recall that latent/persistent infections can reactivate when CD4 cells and cytokines are depleted. In addition to the tragedy experienced by those who are afflicted by both diseases, most households cannot afford the expenses associated with TB and HIV/AIDS. Controlling TB and HIV/AIDS requires an immediate and significant increase in health sector spending to boost access to preventative and curative health services as well as foreign financial support (Jacobs et al. 2007).

### 2.2.5 FACTORS ASSOCIATED WITH AN INCREASED RISK OF TB

**Medical factors**- Several health conditions might elevate the possibility of contracting TB, including immunosuppression and chronic conditions. A debilitated immune system deprives the body of battling infection, permitting inactive TB to be dynamic within the host. It moreover makes it somehow easy to contract TB in the initial stage (Narasimhan et al. 2013). According to previous reports (Narasimhan et al. 2013; WHO, 2023), the following conditions can turn off a person's immune system:

- **Age:** Very young and significantly older adults typically have weak immune systems.

- **Chemotherapy:** These drugs suppress the immune system in addition to fighting cancer.
- **Corticosteroids:** A person's immune system is naturally weakened if they use oral steroids for an extended time, equal to taking 15 mg of prednisolone for a month or more.
- **HIV/AIDS:** Individuals living with HIV are believed to be at least 16 times more likely to get TB. The risk of TB increases when HIV gets worse or progresses to AIDS.
- **Organ transplants:** People must take immunosuppressive drugs continuously for the rest of their lives to prevent the body from rejecting a transplanted organ.
- **Tumor necrosis factor inhibitors:** These biologic drugs are frequently prescribed to treat rheumatoid arthritis, psoriatic arthritis, and Crohn's disease.
- **Diet and nutrition:** Malnutrition contributes to TB infection. Severe malnutrition weakens the immune system and contributes to weight loss. People with lower body mass index have a twofold higher risk of developing TB than people with higher body mass index (BMI). Iron and vitamin D levels can matter when it comes to specific supplements. High blood iron levels may promote the growth of mycobacteria, increasing a person's susceptibility to TB. Because vitamin D inhibits the growth of mycobacteria, deficiency can increase the risk.
- **Location:** Those born in areas with more common TB are more likely to be infected with the bacteria. Since it may be difficult to control the movement of an individual within their area, therefore education on prevention strategies can be important.
- **Living conditions**: TB can spread quickly when people are crowded together and primarily in poorly ventilated homes and workplaces.
- **Substance abuse:** Overuse of drugs or substances is more common in people who have TB, and the risk increases twofold with cigarette smoking. The risk of TB spreading can also be increased by the illicit use of medications, both injections and non-injections and by consuming 40 grams or more of alcohol each day (Narasimhan et al. 2013).

### 2.2.6 DIAGNOSTIC METHODS FOR *MTB*

A diagnosis of TB disease is made based on medical history, physical examination, chest x-ray, and other laboratory tests. Laboratory clinical diagnosis includes microscopy, sputum culture, and nucleic sequencing (Figure 2.4).

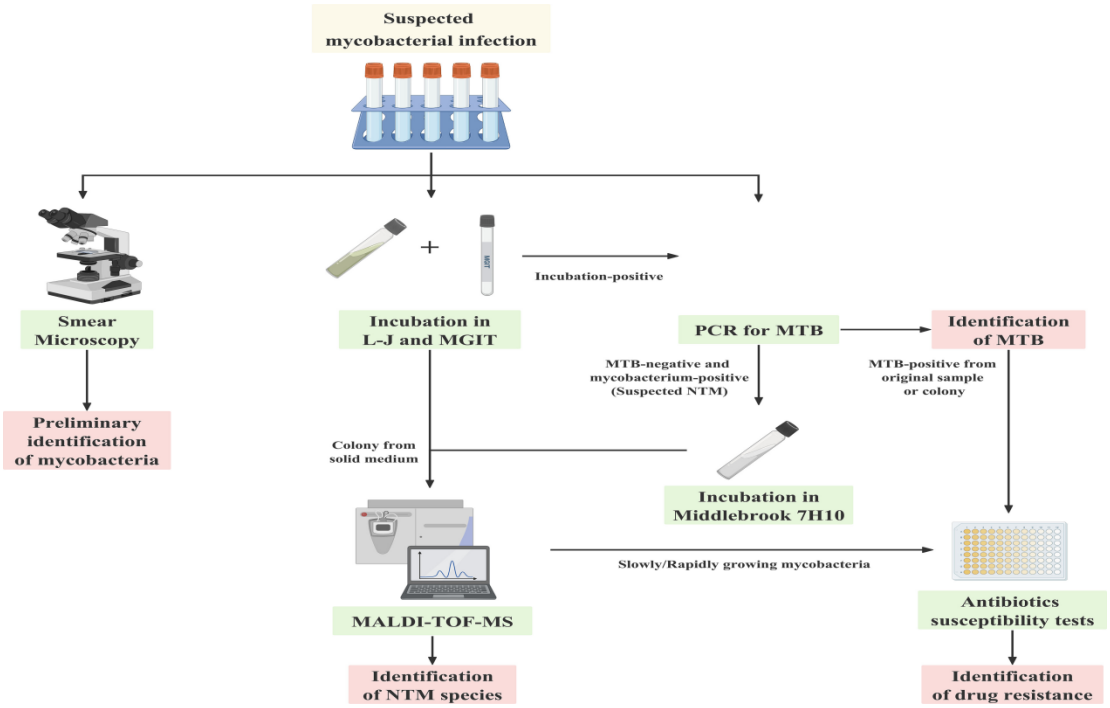


Figure 2.4: Flow-chart on the identification of *M.tb* (Adopted from: https://journals.asm.org/doi/10.1128/spectrum.03179-23).

#### 2.2.6.1 Microscopy

In underdeveloped nations, sputum smear microscopy is still one of the fundamental techniques for recognizing *Mtb*. The most common method is using a carbofuran solution for acid-fast staining. Since *Mtb*'s lipid-rich cell wall is resistant to acid-containing reagent decolourisation, microscopic analysis of smears made from sputum, alveolar lavage fluid, or other specimens can reveal the presence of acid-fast organisms. Smear microscopy's primary drawback is its poor sensitivity, which varies greatly (20–80%) among studies and is especially bad in paucibacillary TB, such as extrapulmonary TB, juvenile TB, or TB co-infected with HIV (Steingart et al. 2006). The degree of specificity may differ significantly based on the local incidence of nontuberculous mycobacterium (NTM) infections.

Smear microscopy can have a specificity of up to 98% in areas where NTM is uncommon (Pai et al. 2016). According to Steingart et al. (2006), fluorescence microscopy can increase smear microscopy's sensitivity while reducing labour costs and enhancing productivity (WHO, 2022). One potential limitation of fluorescence microscopy is the potential for false-positive results due to the non-specific incorporation of fluorochrome dye (Boyd and Marr, 1975). Fluorescent staining has been reported to be unstable (Minion et al. 2011). Fluorescence microscopy engages a robust light source- a high-pressure mercury vapour lamp or halogen, which is costly and delicate, in contrast to conventional microscopy, which uses conventional artificial light (Foulds and O'Brien, 1998). LED microscopy can be applied in resource-constrained contexts because light-emitting diodes (LEDs) are more resilient, maintainable, and have an elongated battery life expectancy than bright light sources. As a result, the WHO advises that LED microscopy can take the role of traditional fluorescence microscopy (WHO, 2022). According to Kanade et al. (2016), fluorescein diacetate (FDA) is a novel stain solution in which only living cells actively change the nonfluorescent FDA into the green fluorescent molecule after enzymatic activity.

The negative or positive response of patients to TB medication, can be predicted through quantitative culture results and detect feasible *Mtb* using the FDA staining technique (Datta et al. 2015). "TBDx" is a cutting-edge smear microscopy system that automatically inserts slides into a microscope, focuses, and takes digital pictures. Computer algorithms are subsequently used to classify smears as positive or negative (Lewis, 2012). These novel microscopic techniques have the potential to identify *Mtb*, but further testing is necessary to confirm how well they work in real-world clinical settings. In high-TB burden locations, sputum smear microscopy is a relatively quick, low-cost, and specific method for diagnosing *Mtb*. Therefore, it remains a valuable approach for diagnosing *Mtb*, particularly in nations with low resources. The primary constraint of microscopy is its low sensitivity in diagnosing TB, particularly in paucibacillary specimens. Moreover, standard microscopy cannot distinguish between living and dead bacilli, which makes early treatment failure or drug resistance detection challenging. Smear microscopy performance is typically well short of what is currently required for TB diagnosis in clinical practice.

### 2.2.6.2 Culture

a). Solid and Liquid Culture

Since *Mtb* isolation is crucial for both illness diagnosis and the identification of medication resistances, culture remains the gold standard for TB diagnosis, as advised by the WHO. Conventional *Mtb* culture can be carried out on liquid (Middlebrook 7H9 Broth) or solid (Lowenstein–Jensen) media. In contrast to liquid culture, which is faster, more sensitive, and more convenient (growth is recognized automatically), solid culture is notably less expensive and less likely to be contaminated by other bacteria or fungi (Rageade et al. 2014; Chien et al. 2000). It is necessary to decontaminate samples (like sputum) contaminated with typical flora before working in the laboratory. N-acetyl-L-cysteine (NALC), which inhibits the growth of Mtb but kills fast-growing bacteria and fungi, can be used in the laboratory in combination with a standard decontamination reagent like NaOH (Hassan et al. 2020).

b). Rapid Identification of Positive Cultures

Early anti-TB therapy is based on rapid identification assays that can differentiate between *Mtb* complex and NTM following positive cultures. Conventional biochemical tests are laborious and require two to three weeks to complete (Procop, 2016; Orikiriza et al. 2017). One of the *Mtb*-specific antigens released during bacterial development is *Mtb* protein 64 (MPT-64). The foundation of immunochromatographic (ICT) assays is the detection of MPT-64 antigen using a double-sandwich enzyme-linked immunosorbent assay. According to a recent evaluation, ICT assays have a high specificity (range, 99.2 to 100%) and high sensitivity (range, 98.1 to 98.6%) for the quick detection of *Mtb* complex (Brent et al. 2011). Furthermore, ICT assays do not require extra special equipment and are quick and easy to perform. Consequently, for the quick identification of *Mtb* complex from positive cultures, the WHO advises utilizing ICT tests (Oommen and Banaji, 2017).

### 2.2.6.3 Immunological diagnosis

a). Antibody Detection

The humoral immune response's identification of *Mtb* antigens by antibodies is the foundation of serologic testing. The WHO advises against using commercial serologic

assays to diagnose TB due to their low diagnostic sensitivity and specificity, which can lead to misdiagnosis and waste of resources (Steingart et al. 2011).

b). Antigen Detection

Based on sandwich enzyme-linked immunosorbent assay principles, circulating *Mtb* antigens can be identified from clinical specimens such as sputum, serum, and urine. According to Strohmeier and Fenton (1999), lipoarabinomannan (LAM), a particular element of the *Mtb* cell membrane, may be a biomarker for detecting TB. Li et al. (2021) stated that the urine lateral flow LAM test FujiLAM has a sensitivity and specificity of 70% and 93% in adult TB, respectively; however, in children with TB those same values are reported to be 51%, and 87%, respectively. Furthermore, it has been demonstrated to function more effectively and have a higher diagnostic sensitivity in individuals with HIV infection or in adults and children with a CD4+ T cell count <200 cells/μL (Li et al. 2021).

c). Tuberculin Skin Testing (TST)

TST is a traditional technique that uses pure protein derivative (PPD) of tuberculin to identify type IV hypersensitivity. Patients with *Mtb* infection can generate sensitized T cells that can identify *Mtb* antigens. A range of soluble lymphokines are released to enhance vascular permeability, local redness, swelling, and induration when the sensitized T cells are triggered by *Mtb* antigens once more (Scheynius et al. 1982; Kowalewicz Kulbat et al. 2018). As a result of TST, the average diameter of induration is measured 72 hours after PPD injection. According to Abubakar et al. (2018), an average diameter of induration of less than 5 mm or no response is regarded as unfavourable, and more than 5 mm is regarded as positive.

The following factors can influence TST results:

(I) **Bacillus Calmette-Guerin (BCG) vaccination:** BCG vaccination may impact TST specificity since PPD and BCG share antigenic components. According to Farhat et al. (2006), the BCG vaccination has little to no impact on TST throughout infancy, particularly ≥10 years after immunization. The decision of whether to multiply after receiving BCG vaccinations has an impact on TST

results as well. Compared to the present BCG one-time vaccine, the effect of BCG booster immunization on TST is more noticeable (Gao and Lin, 2015).

(II) **NTM infection:** Except in populations with a high frequency of NTM infection and a very low incidence of TB infection, NTM is not a clinically significant cause of false-positive TST results (Farhat et al. 2006).

(III)**The immune status of the host:** The accuracy of TST is dependent on the host's immune system because it relies on an immunological response unique to *Mtb*. Consequently, in patients with immunocompromised circumstances, the sensitivity of TST for TB diagnosis is decreased (Jung et al. 2012). TST sensitivity dropped to 31% in hemodialysis patients, according to a systematic review of research on *Mtb* infection in immunocompromised populations (Ferguson et al. 2015). According to a different study, immunosuppressed organ transplant recipients are likely to experience an allergy to the tuberculin antigen, which can result in TST results that are falsely negative (Pennington et al. 2018).

d). Interferon-gamma (IFN-γ) Release Assays (IGRAs)

Menzies et al. (2007) highlighted that the IGRAs are predicated on the release of IFN-γ by lymphocytes exposed to *Mtb*-specific antigens (TBAg), such as culture filtrate protein 10 (CFP-10) and early secreted antigenic target 6 (ESAT-6). IGRA results have been noted to show a greater specificity than TST for the diagnosis of *Mtb* infection since they are unaffected by prior BCG vaccination and most infections are produced by NTM. The two most often used IGRAs that are sold commercially are Quanti FERON-TB (QFT; Qiagen) and T-SPOT.TB (T-SPOT; Oxford Immunotec) (Menzies et al. 2007).

### 2.2.7 PREVENTION AND CONTROL OF TB

a) Early Detection and Treatment:

Early detection is made easier by routinely screening high-risk groups, such as medical personnel, HIV-positive individuals, and those who have close contact with TB patients. Antibiotics such as isoniazid, rifampicin, ethambutol, and pyrazinamide should be used in combination to treat active TB cases for at least six months. Directly Observed Treatment, Short course (DOTS) lowers the possibility of medication resistance by ensuring that patients follow their prescribed course of treatment (WHO, 2019).

b) Vaccination:

Infants in nations where TB is more prevalent are given the Bacillus Calmette-Guérin (BCG) vaccine which offers a defence mechanism against serious TB infections, including TB meningitis in children (WHO, 2019).

c) Infection Control in Healthcare Settings:

To lower the risk of transmission, patients with active TB, particularly those with drug-resistant TB, should be isolated in well-ventilated rooms. Personal protection equipment (PPE) like N95 respirators should be used by healthcare professionals when treating TB patients. The use of high-efficiency particulate air (HEPA) filters and ultraviolet germicidal irradiation (UVGI) are two examples of airborne infection control strategies that can be used to lessen the transmission of tuberculosis in medical institutions (WHO, 2019).

d) Public Health Interventions:

Latent TB infection can be detected and treated early by identifying and screening people who have had intimate contact with TB patients. In order to lessen stigma and motivate people to seek medical attention as soon as possible, it can be helpful to increase knowledge about TB symptoms, transmission, and the significance of finishing treatment (WHO, 2019).

e) Addressing Social Determinants:

One way to lower the risk of TB transmission is to improve ventilation and reduce overcrowding in households and public areas. The chance of acquiring active TB can be decreased and the immune system strengthened by making sure that people are getting enough nutrition and taking care of other health issues like diabetes and HIV (WHO, 2019).

f) Research and Development:

Global TB control requires ongoing research into novel vaccinations, shorter treatment durations, and more efficient diagnostic methods. Treatment and management of extensively drug-resistant TB (XDR-TB) and multidrug-resistant TB (MDR-TB) require the development and application of techniques (WHO, 2019).

## 2.3 TUMOR NECROSIS FACTOR-ALPHA

### 2.3.1 GENETIC STRUCTURE OF THE TNF-α GENE

The TNF-α gene is located on the human genome at chromosome 6p21.3. This region is part of the MHC class III, which is known for containing genes that are involved in immune response, inflammation, and other related pathways. The TNF-α gene is composed of several exons and introns, typical of a eukaryotic gene. The gene is composed of approximately 3.5 kb of DNA in its full structure, spanning several exons and introns. The first exon of the TNF-α gene encodes the leader peptide, which is a short sequence that helps direct the nascent protein to the correct cellular compartment. Exons 2-4 code for the mature TNF-α protein, which is a type II transmembrane protein before being cleaved to release the soluble form. Exon 5 encodes for the 3' untranslated region (UTR) of the gene, which is important for post-transcriptional regulation and mRNA stability.

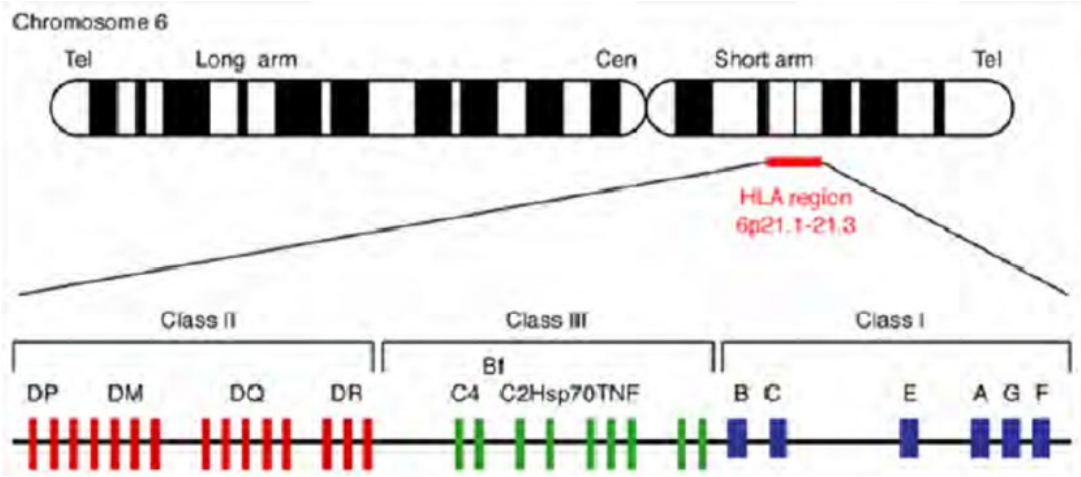


Figure 2.5: Localization of TNF- α gene on chromosome 6 (Adopted from: https://www.researchgate.net/figure/Localization-of-TNF-a-gene-on-chromosome-6-6p213-TNF-gene-codifies-for-a-protein_fig1_216856060)

The mature TNF-α protein is composed of 157 amino acids, and it is produced as a membrane-bound precursor that can be cleaved by TNF-α-converting enzyme (TACE) to release the soluble form of the cytokine. The promoter region of the TNF-α gene plays a critical role in its transcriptional regulation and determines the level of TNF-α expression. The promoter region is particularly important in regulating the inducibility of TNF-α in response to stimuli such as pathogen exposure or inflammatory signals (e.g.,

from LPS or cytokines). Located upstream of the gene, the promoter region contains several key elements:

- NF-kB binding sites: The TNF-α promoter contains several binding sites for NF-kB, a major transcription factor involved in immune and inflammatory responses.
- AP-1 binding sites: These are involved in the regulation of the gene in response to stress and inflammation.
- Sp1 and C/EBP binding sites: These are involved in the regulation of transcription in various immune cells.

### 2.3.2 TUMOR NECROSIS FACTOR-ALPHA AND ITS BIOLOGICAL ROLE

Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine that is mostly produced by T cells and macrophages and is involved in the immunological response of the body. It is a multipurpose cytokine that regulates cell division, proliferation, and apoptosis in addition to its activities in inflammation (Idriss et al. 2000). TNF-α is first produced as a membrane-bound precursor known as pro-TNF, which is then broken down into its mature, soluble form by the TNF-α converting enzyme, or TACE (Idriss et al. 2000). This cytokine's wide-ranging functional influence is facilitated by its primary binding to two receptors, TNFR1 and TNFR2, which are expressed on a variety of cell types. TNF-α has a critical role in the immune response, especially when infectious illnesses like TB are present. This cytokine plays a major role in the granulomatous inflammation associated with TB, helping to regulate the amount of *Mtb* that is contained within the granulomas (Flynn, 2004). TNF-α is essential because its suppression or lack causes granuloma disintegration and unchecked bacterial growth (Keane et al. 2001). Furthermore, TNF-α is essential for macrophage activation because it increases their bactericidal properties and stimulates the synthesis of other pro-inflammatory cytokines. Additionally, it causes immune cells to be drawn to the infection site, which intensifies inflammation and the body's defences against invaders.

### 2.3.3 MECHANISM OF ACTION OF TNF-α

The way that TNF-α works is by activating its receptors, TNFR1 and TNFR2, which set off distinct signalling pathways that occasionally, overlap. The widely expressed TNFR1

mainly triggers the transcription of several inflammatory genes by activating the mitogen-activated protein kinases (MAPKs) and nuclear factor kappa B (NF-κB) pathways (McFarlane et al. 2002). The inflammatory response is mediated by additional inflammatory cytokines, chemokines, and adhesion molecules produced as a result of this signaling cascade. Although it is primarily present in immune cells, TNFR2 has a role in immune cell survival and proliferation in addition to its contribution to the inflammatory response (Naude et al. 2011).

Mechanistically, adaptor proteins including TRADD, TRAF2, and RIP1 are drawn in to form a complex when TNF-α binds to TNFR1, activating the IκB kinase (IKK) complex. This causes the IκB proteins to be phosphorylated and degraded, which in turn activates NF-κB (Naude et al. 2011). In addition to primarily activating the NF-κB pathway, the TNFR2 receptor has been linked to immune cell survival and proliferation, which strengthens the immunological response (Grell et al. 1995). Furthermore, in specific situations, TNF-α signalling via its receptors can trigger necroptosis or apoptosis, which helps the immune system, suppress and get rid of diseased cells.

### 2.3.4 THE FUNCTION AND CONTROL OF TNF-α IN TUBERCULOSIS INFECTION

TNF-α may function as a fragile immunological mediator in the pathophysiology and defence against TB. When TB was first identified as cachexin, TNF-α was discovered to be associated with the disease. Up until this point, TB research has continued to reveal new components of this cytokine that are extremely relevant to both the disease process and novel mediation techniques. The necessity of TNF-α for containing dynamic TB is demonstrated by the cytokine's multiple cellular sources, the intricacy of the processes regulating its quantity, and the mediator's substantial polyfunctionality. The fundamental mechanism leading to infection outcome is symbolized by TNF-α's ability to regulate granuloma formation and TB integrity. Treatment for autoimmune diseases and other chronic inflammatory non-infectious conditions has shown promise in targeting the TNF-α signaling system (Queval et al. 2017).

The promoter region of this gene typically regulates TNF’s increase during illness recovery, suggesting that it may be a crucial cytokine for host defence against a wide range of harmful microbes (Yuk et al. 2024). An individual's potential for cytokine

production is administered by a significant hereditary component, and there are recognizable contrasts between people. The TNF-α promoter polymorphism has an imperative effect on its transcriptional activity, concurring with research (Queval et al. 2017). TNF-α has been the focus of extensive research because of its dual function in protecting the host and promoting disease pathology in TB. This cytokine is the main cytokines in the immune system's reaction to *Mtb*. Below are the different roles played by TNF-α in TB infection:

**a). TNF-α in Granuloma Formation:** Granulomas are arranged collections of immune cells that develop to enclose microorganisms around infection sites. TNF-α is essential for the development and upkeep of granulomas. It facilitates the recruitment of immune cells to the infection site, such as T cells and macrophages, aiding in the creation of a barrier that stops *Mtb* from spreading. Research indicates that mice deficient in TNF-α or its receptors are unable to generate appropriate granulomas and ultimately succumb to disseminated TB, underscoring the crucial function of this cytokine in limiting the infection (Flynn, 2004; Bean et al. 1999).

**b). Protective vs. Pathogenic Roles of TNF-α in TB:** TNF-α has a dual role in TB: it is protective when it comes to granuloma formation and mycobacterium containment. The pathophysiology of a disease can be aggravated by excessive or unregulated production of TNF-α, which can cause significant inflammation and tissue damage. For example, elevated TNF-α has been linked to more lung tissue damage in TB patients, indicating that TNF-α needs to be strictly regulated even if it's essential for defence (Keane et al. 2001). Genetic variations in the TNF-α gene promoter region, such as TNF-α -308 G/A, might cause varying amounts of cytokine production and affect the severity and susceptibility to TB (Yuk et al. 2024). Certain genotypes can cause an excess or decrease in TNF-α production, which can affect the amount of tissue damage and bacterial containment.

**c). TNF-α Targeting Therapies in TB Treatment:** TNF-α has been a target for therapeutic therapies aimed at balancing its pathogenic and protective effects because of its crucial involvement in TB. Anti-TNF-α treatments have been created and are used

to treat autoimmune illnesses like rheumatoid arthritis. Examples of these medications include soluble TNF receptors (e.g., etanercept) and monoclonal antibodies (e.g., infliximab, adalimumab). Their application to TB patients is not without risk; either, TNF-α inhibitors have been shown in several clinical situations to hinder granuloma formation and raise the possibility of reactivating latent TB infections (Wallis et al. 2004). Therefore, when using anti-TNF-α medications in individuals at risk of acquiring TB, thorough screening for latent TB infection and monitoring during therapy are essential.

#### 2.3.4.1 Host Immune Response to Mycobacterium Tuberculosis Infection

There is evidence that although Th1 cytokines are produced at high levels near the site of infection in TB patients, their systemic levels may be lowered (Vankayalapati et al. 2003). In contrast, Th2 cytokine levels may be elevated. The Th1 cytokines, namely IFN-α, IL-2, and IL-12, facilitate cell-mediated immunity- a crucial mechanism for managing mycobacterial infections. IFN-α secretion is the primary result of Th0 cell development towards a Th1 phenotype, which is driven by IL-12 released by DC and infected macrophages. Although IFN-α production is sufficient to completely eradicate the infection, it plays a crucial role in managing mycobacterial infections. Th2 cytokines IL-4 and IL-5, as well as the regulatory cytokine IL-10, are produced at higher rates as the infection progresses. These cytokines may reduce or impede the Th1-mediated response that protects against mycobacterial infection, while also promoting the humoral immune response. Furthermore, the autocrine factors TNF-α and TGF- are secreted by mononuclear cells, and these factors have differing impacts on the bactericidal activity of these cells. Strongly stimulating monocytes and macrophages-TNF-α works in concert with IFN—α to produce reactivate oxygen and nitrogen intermediates (ROIs and RNIs), which in turn trigger antimicrobial activity. RNIs are thought to be crucial for eliminating intracellular *Mtb* (Chan et al. 1992), yet it's still unclear if they have a bearing effect on human health (Thoma-Uszynski et al. 2001) conversely, TGF-demonstrate immunosuppressive properties (Hirsch et al. 1994). As a result, the immune response to a mycobacterial infection comprises a wide range of cytokines, each of which has a unique set of cellular effects. The outcome of infection is ultimately determined by the intricate interaction of these cytokine activities.

The ability of the host to create particular T-cell cytokines is necessary for protective immunity. These cytokines stimulate the growth of *Mtb* antigen-reactive T cells and activate macrophages through IFN-ɣ and TNF-α, which causes granulomas to develop (Toossi, 2000). Granuloma formation, however, does not happen right away in reaction to the *Mtb* infection; until enough cells are recruited to stop the growth of *Mtb,* it may take up to three weeks (Toossi, 2000). The bacilli continue to infiltrate and multiply quickly inside macrophages during that period. Thus, the innate system keeps pushing for the recruitment and activation of macrophages- which generate several autocrine factors, the most significant of which is TNF-α (Valone et al. 1988). TNF-α serves multiple vital roles which in turn makes it essential for a persistent protective immune response against *Mtb*.

Typical granulomatous lesions are formed when activated macrophages continue to produce TNF-α, resulting in an increased number of granulomas. These lesions are necessary to eliminate the invasive *Mtb*. Within the granulomas of latent *Mtb*, the bacilli may grow and use macrophages as a means of survival. This results in tubercle formation, which is characterised by core areas of caseous necrosis (Kautmann et al. 2002). As a result, *Mtb* persists in its ability to activate mononuclear cells and increase their production of TNF-α, which perpetuates an inflammatory response. This severe inflammation triggers the immune system's effort to replace the necrotic tissue, which in turn results in the creation of distinctive granulation and fibrous connective tissue associated with *Mtb* infection (Kautmann et al. 2002).

#### 2.3.4.2 Stimulation of TNF-α Production by Mycobacterial Components

Considering that *Mtb* infection was fatal in mice with TNF receptor defects (Ehlers *et al* 1999), and TNF-α/lymphotoxin double mutants (Jacobs et al. 2007), TNF-α plays a critical role in protective immunity against *Mtb* infection. Therefore, it is crucial to understand the many mechanisms by which *Mtb* induces an increase in TNF-α induction. According to early research, a pure protein product of the bacilli is a potent inducer of TNF-α in monocytes (Valone et al. 1988). It has been discovered that the 30 kDa antigen (Ag85B), a significant secretory component of actively replicating *Mtb,* increases the generation of TNF-α by monocytes via an interaction with fibronectin

(Aung et al. 1996). Moreover, it has been demonstrated that the *Mtb* glycolipid lipoarabinomannan [LAM] can strongly induce TNF-α (Barnes et al. 1992).

TLRs, or toll-like receptors, have been extensively linked to controlling the immune system's reaction to mycobacterial infections. It has been demonstrated that TLR2 and TLR4 are essential for mice to be resistant to *Mtb* infection. This is because mice lacking these receptors died from the infection more quickly, possibly because their macrophages were unable to generate enough TNF-α. (Abel et al. 2002; Drennan et al .2004). MyD88, a significant adaptor protein, appears to be crucial for the TLR signaling that *Mtb* stimulates because mice lacking this protein were unable to release inflammatory cytokines like TNF-α (Scanga et al. 2004). Underhill and colleagues (1999) reported that mouse macrophages are stimulated to release TNF-α in a TLR-dependent manner upon exposure to *Mtb* wherein this response was eliminated in cells expressing a dominant negative version of MyD88 (Underhill et al. 1999). Therefore, it seems that TLR2 is the main mediator of TNF-α generation and macrophage activation in response to mycobacteria.

By increasing *Mtb* gene expression in freshly infected monocytes, TNF-α itself can indirectly increase its production by secreting more antigen 85 (Ag85) and increasing TNF-α synthesis (Aung et al. 1996). This initiates a positive-feedback loop that fuels TNF-α production when a *Mtb* infection occurs. Additionally, TNF-α production inside the disease-defining Mtb lesions can be stimulated by IFN-α (Toossi, 1996), which can be produced autocrinely by the T cells (Kemp et al., 1997) or by the alveolar macrophages themselves (Fenton et al. 1997). Contrarily, the anti-inflammatory cytokines TGF-and IL-10 (Toossi, 1996) can inhibit and reverse TNF-α production, which is also triggered by mycobacterial components like Ag85B (Torres et al. 1998), but at a lower starting level than TNF-α (Toossi et al. 1995).

Apart from TLRs, DC-SIGN is a significant *Mtb* receptor on DC and, to a lesser extent, macrophages. By interacting with mannose-capped LAM on their surface, DCSIGN, a pattern recognition receptor, functions as a point of entry for mycobacteria and certain other microorganisms, in contrast to TLRs. TB patients' alveolar macrophages express DC-SIGN, even though it is primarily a DC receptor, according to a significant recent

study (Taillcux et al. 2005). Mycobacteria employ DC-SIGN to inhibit DC-mediated immune responses (Geijtenbeek et al. 2003). Interfering with TLR downstream signalling processes, such as the production of TNF-α by infected cells, mediates this inhibition. It has only been recently possible to interpret the mechanism behind DC-SIGN's suppressive activity. It involves a serine/threonine kinase known as Raf-1, which regulates downstream events such as the acetylation of NF-κB and the prolonged-expression and secretion of the immunosuppressive cytokine IL-10 (Gringhuis et al. 2002).

### 2.3.5 LEVELS OF TNF-α AND ITS VARIABILITY IN TB PATIENTS

TNF-α is a crucial mediator in the immune response, promoting inflammation and granuloma formation, which are essential for containing *Mtb*. However, excessive TNF-α can also lead to tissue damage and exacerbate disease symptoms. TNF-α levels have been assessed in the blood of individuals suffering from several illnesses, including bacterial infections, sepsis, cardiovascular disorders, inflammatory bowel disorders, and chronic rheumatic diseases. Clinically, measuring TNF-α can aid in diagnosing TB, differentiating it from other conditions with similar clinical presentations. Furthermore, monitoring TNF-α levels could provide insights into treatment response, potentially allowing for more personalized treatment regimens. However, the variability in TNF-α levels necessitates a cautious approach, possibly integrating other biomarkers or clinical indicators to improve diagnostic and prognostic accuracy (Nie et al. 2020). While elevated TNF-α levels are a common finding, there is variability in the literature. A Chinese study confirmed increased TNF-α levels in TB patients, while an Ethiopian study reported undetectable levels, attributing this to the suppressive effects of Transforming Growth Factor-beta (TGF- β) (Joshi et al. 2015; Yang et al. 2022). This variability highlights the complex interplay of cytokines in TB and suggests that TNF-α levels may be influenced by genetic, environmental, or co-infection factors. One study noted that the mean serum of TNF-α level in TB patients was significantly higher at 1265.63 pg/mL, compared to 312.06 pg/mL in control subjects (Beig et al. 2023). This marked difference was statistically significant, underscoring the potential role of TNF-α as a biomarker for TB infection.

### 2.3.6 MOLECULAR TECHNIQUES FOR QUANTIFYING TNF-α LEVELS IN BIOLOGICAL SAMPLES

Measuring TNF-α levels involves a variety of molecular and immunological techniques, which can provide valuable insights into the cytokine's role in immune responses, especially in conditions like TB. Below are some of the most used molecular techniques for quantifying TNF-α levels in biological samples (Valaperti et al. 2019) (e.g., blood, serum, or tissue):

#### 2.3.6.1 Enzyme-Linked Immuno-sorbent Assay (ELISA)

ELISA is one of the most widely used techniques for quantifying TNF-α protein levels in biological samples. This technique uses antigen-antibody interactions to detect and quantify TNF-α. A specific antibody that binds to TNF-α is coated onto a microplate. When the sample is added, TNF-α (if present) will bind to the antibody. A secondary antibody conjugated with an enzyme (typically horseradish peroxidase) is then added, followed by a substrate that reacts with the enzyme to produce a measurable colour change. Advantages of ELISA include sensitivity and specificity, high throughput (can process many samples simultaneously), and can quantify both soluble TNF-α levels in plasma or serum and cellular TNF-α secretion. The disadvantage is that it requires well-characterized antibodies for high specificity, limited to measuring only the protein (does not provide information on mRNA levels or gene expression).

#### 2.3.6.2 Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

qRT-PCR is used to measure the mRNA expression of TNF-α, providing insights into how its production is regulated at the transcriptional level. This technique involves the extraction of total RNA from a sample, followed by reverse transcription into complementary DNA (cDNA). The cDNA is then amplified using PCR, and the quantity of the target TNF-α mRNA is determined by measuring the fluorescence of a dye (e.g., SYBR Green) or probe (e.g., TaqMan) in real time. Advantages include that it allows measurement of gene expression (mRNA levels), is highly sensitive and quantitative, and lastly, provides insights into the regulation of TNF-α at the transcriptional level. Disadvantages are it requires careful RNA extraction and cDNA synthesis and does not provide information on TNF-α protein levels.

#### 2.3.6.3 Flow Cytometry (Intracellular Cytokine Staining)

Flow cytometry is commonly used to analyse intracellular TNF-α production in immune cells, such as T cells, monocytes, and macrophages, in response to stimulation. After stimulating immune cells (e.g., with *Mtb* antigens or mitogens), cells are fixed and permeabilized to allow antibodies to enter the cells and bind to intracellular TNF-α. The cells are then analyzed by flow cytometry to quantify the cytokine-producing cells. Its advantages are that it provides information on cytokine production at the single-cell level, can be combined with markers for cell surface markers, allowing the analysis of specific immune subsets (e.g., CD4+ T cells, monocytes) and has high throughput and can analyse large numbers of cells. Its disadvantages are it requires sophisticated equipment and technical expertise, and it is limited to the analysis of cells that are already isolated or cultured.

#### 2.3.6.4 Western Blotting

Western blotting is used to detect and quantify TNF-α protein levels in tissue or cell lysates. In this technique, proteins are first separated by size using gel electrophoresis (SDS-PAGE). After transfer to a membrane, the membrane is incubated with an antibody specific for TNF-α. A secondary antibody conjugated with an enzyme (typically HRP) is then used to visualise the protein, usually through a chemiluminescent reaction. The advantages are that it provides molecular weight information, helping confirm the identity of TNF-α and allows for semi-quantitative assessment of TNF-α levels. The disadvantages include, it is not as sensitive as ELISA or PCR for quantification and lastly, it is labour-intensive and requires protein extraction.

#### 2.3.6.5 Cytokine Bead Array (CBA)

A multiplex assay that can measure multiple cytokines (including TNF-α) simultaneously in a single sample. For this method, magnetic beads, each coated with a capture antibody specific to a particular cytokine, are incubated with the sample. After binding, a secondary detection antibody conjugated with a fluorescent marker is added. The beads are analysed by flow cytometry to detect the presence and quantity of multiple cytokines in a single sample. The advantages are that it has high-throughput analysis of multiple cytokines in parallel (including TNF-α) and the assay is sensitive and quantitative. The

disadvantages are that it requires specialized equipment (flow cytometer) and it is more expensive than traditional ELISA for single cytokines.

#### 2.3.6.6 Luminex xMAP Technology

This technique is similar to the Cytokine Bead Array (CBA) but uses color-coded beads to detect multiple analytes (e.g., TNF-α, IL-6, IFN-gamma) simultaneously. In this multiplexed bead-based immunoassay, the beads are coated with specific antibodies and use a unique color-coded bead mixture to capture cytokines from the sample. A fluorescently labelled detection antibody is used, and the data is collected using a flow-based analyzer. The advantages are that it allows simultaneous quantification of multiple cytokines, including TNF-α and it has high sensitivity and a wide dynamic range. Disadvantages are that it requires specialized equipment (Luminex instrument) and can be more expensive compared to simpler assays like ELISA.

#### 2.3.6.7 Mass Cytometry (CyTOF)

Mass cytometry, or cytometry by time-of-flight (CyTOF), uses metal-tagged antibodies for high-dimensional analysis of cytokine production, including TNF-α, at the single-cell level. CyTOF involves labelling antibodies with heavy metals and analysing cells using time-of-flight mass spectrometry. This allows for the simultaneous detection of multiple markers, including cytokines such as TNF-α, at the single-cell level. The advantages that it allows simultaneous analysis of many parameters (e.g., cytokines, surface markers, and intracellular proteins) and it has high-dimensional analysis of immune responses in individual cells. The disadvantages are it requires specialized, high-cost equipment and it is highly technical and requires expertise.

#### 2.3.6.8 Immunohistochemistry (IHC)

IHC is used for detecting TNF-α expression in tissue sections, which is particularly useful for understanding localized immune responses in TB infection. For this technique, Formalin-fixed paraffin-embedded (FFPE) tissue sections are incubated with a primary antibody specific for TNF-α. A secondary antibody conjugated to an enzyme (usually horseradish peroxidase) is added, and the signal is visualized using a chromogenic substrate. Advantages are it provides spatial localization of TNF-α within tissues and it is useful for examining inflammatory infiltrates in TB lesions.

Disadvantages are it is limited to tissue sections (not suitable for fluid samples) and it is less quantitative compared to methods like ELISA or qRT-PCR.

#### 2.3.6.9 Next-Generation Sequencing (NGS) and RNA Sequencing (RNA-Seq)

RNA-Seq can be used to measure TNF-α mRNA expression on a genome-wide scale, providing deep insights into the transcriptional regulation of TNF-α in TB patients. For this technique, RNA is isolated from the sample, and sequencing libraries are prepared for high-throughput sequencing. The number of reads corresponding to TNF-α mRNA is then counted and compared to control or baseline samples. Advantages are it has high throughput allows for global gene expression analysis and can identify novel regulatory mechanisms or mutations in TNF-α. Disadvantages are it is expensive and data-intensive, and it requires bioinformatic expertise for data analysis.

## 2.4 GENETIC POLYMORPHISMS OF TNF-α

Gene polymorphisms are differences in the DNA sequence that occur on an individual basis within a population. Large segment rearrangements or something as basic as a single nucleotide alteration can be examples of these changes (Maggert et al. 2012). In addition to influencing physiological traits, treatment responsiveness, and illness susceptibility, they are crucial to the variety of genetic traits present in human races. TB and other infectious and inflammatory diseases have received a lot of interest due to polymorphisms in cytokine genes, notably the TNF-α gene, because of their potential impact on the immune system and the progression of the disease.

### 2.4.1 COMMON POLYMORPHISMS IN THE TNF-α GENE

A key feature of the TNF-α gene is the presence of various genetic polymorphisms that influence the expression and function of the TNF-α protein. These polymorphisms are primarily found in the promoter region and can lead to either increased or decreased levels of TNF-α production, which have been linked to diseases like TB, autoimmune disorders, and cancer. Examples of these polymorphisms include:

- **-308 G/A (rs1800629):** One of the most studied polymorphisms, located in the promoter region. The A allele is associated with increased TNF-α production, while the G allele is linked to lower levels of TNF-α. This polymorphism has been

found to influence susceptibility to infectious diseases, including TB. Higher levels of TNF-α production are linked to greater transcriptional activity of the TNF-α gene, which is caused by the presence of the "A" allele at position -308. Pro-inflammatory cytokine TNF-α is essential for the host's defence against the TB-causing *Mtb* (Sinaga and Amir, 2021).

- **-238 G/A (rs361525):** Another important polymorphism in the promoter region. Similar to -308, the A allele is associated with higher TNF-α production and may influence TB susceptibility. However, there is evidence linking the -238 A allele to a reduction in TNF-α production (Wu et al. 2019).
- **-863 C/A and -1031 T/C:** Other polymorphisms in the promoter region that influence TNF-α levels and may be associated with autoimmune diseases and TB. A study conducted on the polymorphisms of cytokine genes and TB in two independent studies revealed that there is a strong correlation between the polymorphisms (-1031 C/T and -863 C/A) of TNF-α gene and the two Chinese populations (Han and Tibetan) in terms of protection against TB. The study further demonstrated that the polymorphism (-857 C/T) of the TNF-α gene is associated with the progression of TB in the Tibetan Chinese population (Wu et al. 2019).

The table below lists various common single nucleotide polymorphisms (SNPs) in the TNF-α gene and their associations with TB. Each row provides information on a specific SNP, including its location, the type of polymorphism, the alleles involved, the functional effects of the polymorphism, its association with TB, and relevant references.

Table 2.1: Summary of TNF-α SNPs and Their Associations

| SNP ID | Location | Polymorphism | Allele | Association with TB | Reference |
|---|---|---|---|---|---|
| rs1800629 | Promoter region | G/A | G (Wild)<br>A (Risk) | An allele associated with increased risk of TB in several populations | Smith et al. (2015), Lee et al. (2017) |

| | | | | | |
|---|---|---|---|---|---|
| rs361525 | Promoter region | G/A | G (Wild)<br>A( Risk) | An allele linked to severe forms of TB | Khan et al. (2018) |
| rs1799964 | Intron 1 | C/T | A (Wild)<br>G (Variant) | Linked to Tibet and Han population as a risk factor for TB susceptibility | Wu et al., 2019 |
| rs1800750 | Promoter region | C/A | C (Wild)<br>A (Variant) | allele associated with lower TB susceptibility | Zhao et al. (2019) |
| rs1799724 | Intron 2 | C/T | T (Wild)<br>C (Variant) | No significant association with TB | Patel et al. (2020) |
| rs11466053 | 3' UTR region | G/T | C (Wild)<br>T (Variant) | T allele linked to TB progression in HIV+ patients | Williams et al. (2014) |
| Rs1800630 | Promoter region | C/A | C (Wild)<br>A (Variant) | Linked to Tibetan and Han populations as a risk factor for TB susceptibility | Wu et al., 2019 |

### 2.4.2 IMPACT OF THE GENE POLYMORPHISMS OF TNF-α ON TB DEVELOPMENT AND PROGRESSION

The relationship between TNF-α gene polymorphisms and TB susceptibility has been examined in numerous publications (Wu et al. 2019; Adane et al. 2021; Sinaga et al. 2021; Sousa et al. 2023). Studies reveal that some variations in the TNF-α gene, namely SNPs, can affect a person's likelihood of contracting TB (Wu et al. 2019). For example, a lot of research has been done on the TNF-α -308G/A polymorphism, which may be linked to a higher risk of TB in several populations (Wu et al. 2019; Adane et al. 2021; Sinaga et al. 2021; Sousa et al. 2023). Research conducted on populations in India, China, and Iran has demonstrated a correlation between the -308A gene and increased susceptibility to TB (Sharma et al. 2010; Joshi et al. 2015; Shahsavar et al. 2016; Wu et al. 2019).

### 2.4.2.1 The effect of TNF-α polymorphisms and its Production levels on TB development

Variations in the TNF-α gene can have a substantial impact on TNF-α production levels, which can therefore alter how well TB patients respond to immunotherapy. The previously reported TNF-α -308A allele has been associated with more TNF-α production than the -308G variant (Adane et al. 2021). Increased TNF-α can help the body fight *Mtb* more effectively in the early stages of the immune response, but too much of it can also harm the tissue and make the illness worse. Conversely, the TNF-α -238G/A polymorphism has been linked to decreased TNF-α production, which may impede the immune system and heighten the vulnerability to TB. The two polymorphisms emphasize the dual function of TNF-α synthesis in infection defence and the possibility of elevated disease pathology (Correa et al. 2005).

### 2.4.2.2 The occurrence of different TNF-α polymorphisms in different populations

Global differences in TB susceptibility and disease progression are partly explained by the substantial population-level variability in TNF-α gene polymorphism frequency. African populations exhibit a higher prevalence of the TNF-α -308A allele in comparison to European or Asian populations (Hu et al. 2015; Mabunda et al. 2015). The increased incidence rates of TB seen in some areas can be partially explained by this regional heterogeneity. Research using a range of ethnic groups, such as African, Asian, and Caucasian cohorts, has demonstrated this variance and its possible influence on the epidemiology of TB (Wu et al. 2019; Mabunda et al. 2015; Sivangala et al. 2014). Different groups have different distributions of the -238G/A and other polymorphisms, which affect immunological responses to TB as well as genetic susceptibility (Thye et al. 2010).

Given its role in the creation and maintenance of granulomas, which aid in the containment of *Mtb*, TNF-α plays a crucial role in the immunological response to TB. Variations in TNF-α production levels can be caused by polymorphisms in the TNF-α gene, which may impact an individual's susceptibility to TB and the disease's clinical development and outcome (Ehlers and Schaible, 2013).

Studies reveal that specific TNF-α gene polymorphisms are linked to either a higher risk of TB development or a more severe form of the illness (Ates et al. 2007; Wu et al. 2019; Sinaga and Amir, 2021). A thorough knowledge of these polymorphisms can help with the creation of individualised treatment plans. Patients with variants corresponding to milder disease may require alternative therapeutic requirements, whilst those with high-risk TNF-α polymorphisms may require more active surveillance and early intervention. Eventually, the discovery of TNF-α polymorphisms can aid in patient classification and improve TB treatment procedures (Ates et al. 2007).

### 2.4.3 METHODS FOR DETECTING TNF-α GENE POLYMORPHISMS

The detection of TNF-α gene polymorphisms typically involves several molecular biology techniques. The choice of method depends on the specific polymorphism being studied and the resources available. Common methods include (El-Tahan et al. 2016):

a). Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) Analysis

This technique uses specific primers to amplify the region of interest in the TNF-α gene. The amplified product is then digested with restriction enzymes that recognize specific sequences within the polymorphic site. The resulting fragments are separated by gel electrophoresis, allowing for the detection of different alleles based on their size.

b). Real-Time PCR

This method is used for the quantification of gene expression and can also be adapted for genotyping. It involves the use of fluorescent probes that hybridize to specific sequences within the TNF-α gene. The presence of polymorphisms can be distinguished based on differences in the fluorescence signal.

c). Single Strand Conformation Polymorphism (SSCP) Analysis

SSCP is a technique that detects mutations based on the conformational changes in single-stranded DNA fragments. It involves amplifying the polymorphic region of the TNF-α gene by PCR, denaturing the PCR products, and then running them on a non-

denaturing polyacrylamide gel. Different conformations caused by nucleotide changes will migrate differently, allowing for the identification of polymorphisms.

d). Sequencing

Direct sequencing of the TNF-α gene can provide comprehensive information about known and novel polymorphisms. High-throughput sequencing techniques, such as next-generation sequencing (NGS), can identify multiple polymorphic sites simultaneously, making it a powerful tool for studying the genetic variation in the TNF-α gene comprehensively.

The examination of TNF-α gene polymorphisms in TB patients not only enhances the understanding of the genetic factors underlying susceptibility to TB but also helps in identifying potential therapeutic targets and personalized treatment strategies. Further research is needed to explore the full spectrum of TNF-α polymorphisms and their functional consequences in diverse populations.

## 2.5 SUMMARY OF LITERATURE REVIEW

The pathophysiology of TB involves a complex interplay between *M. tuberculosis* and the host immune system. Understanding the stages of TB infection and the immune mechanisms involved, particularly the role of cytokines like TNF-α, is essential for developing better therapeutic and diagnostic strategies. Further research into gene polymorphisms and their effects on cytokine production could provide deeper insights into individual susceptibility and disease progression in TB patients. TNF-α is a pivotal cytokine in the immune response, particularly in the context of TB. Its production and signalling are integral to the containment and eradication of *Mtb*, therefore highlighting the necessity of a finely tuned balance in TNF-α activity to ensure effective immunity while preventing excessive inflammation and tissue damage is necessary.

TNF-α plays a pivotal role in host defence against TB by facilitating granuloma formation and containing mycobacteria. However, its regulation is crucial as imbalances can lead to severe pathology. The dual nature of TNF-α in TB underscores the importance of personalized approaches in both genetic assessment and therapeutic interventions to optimize patient outcomes. TNF-α gene polymorphisms play a

significant role in determining susceptibility to TB and the severity of the disease. Correlation studies have identified key SNPs, such as the -308G/A and -238G/A polymorphisms, that influence TNF-α production levels. The variation in the frequency of these polymorphisms among different populations underscores the importance of considering genetic background in TB research and in developing targeted interventions for TB prevention and treatment.

# CHAPTER 3
# MATERIALS AND METHODS

## 3.1 OVERVIEW OF THE RESEARCH METHODOLOGY

The flowchart below (Figure 3.1) summarizes the methodology done.

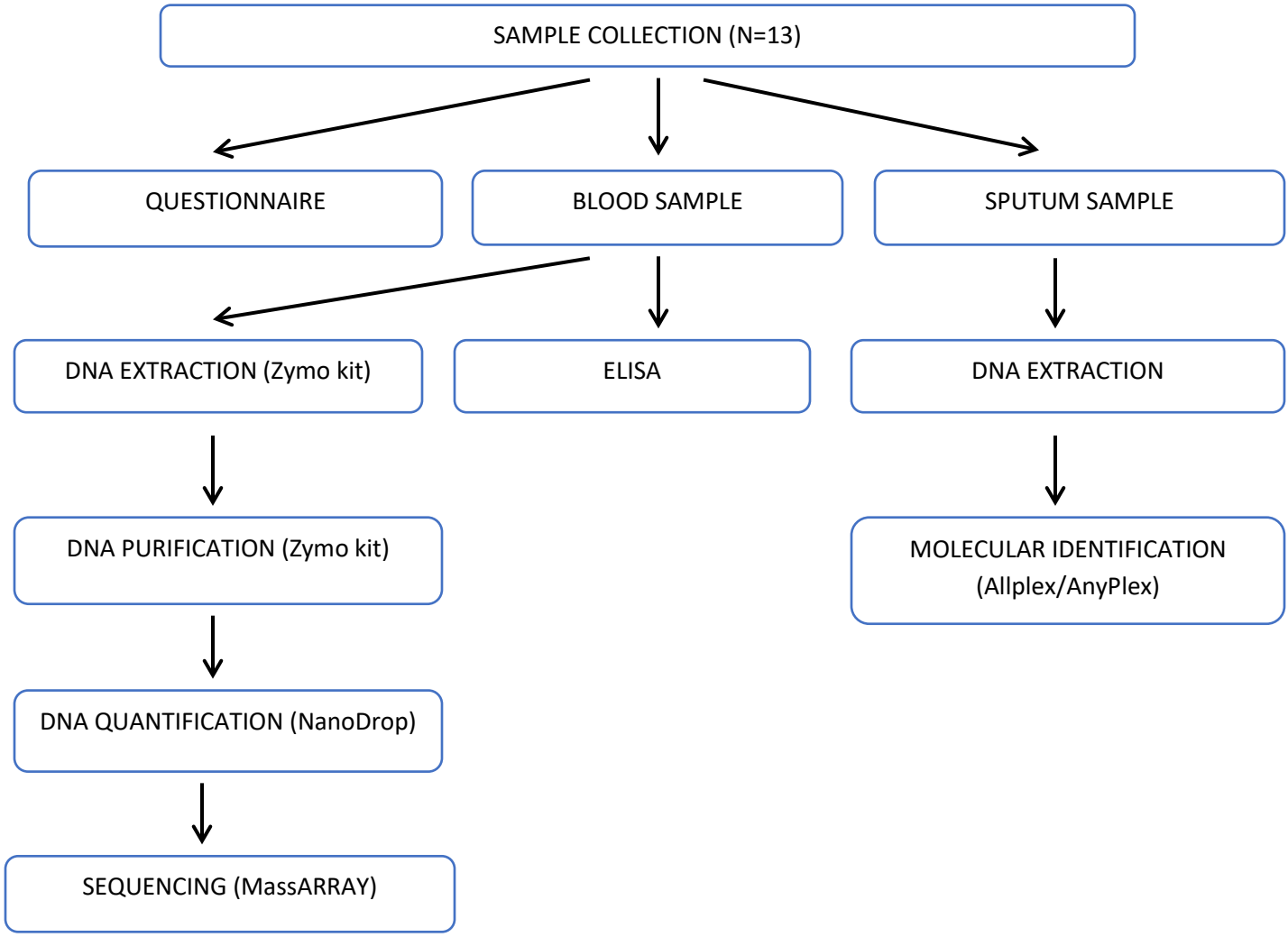


Figure 3.1: A flow chart illustrating the methodology of the research project

## 3.2 STUDY AREA

This study was carried out in the local healthcare facilities in the Vhembe district, which is located in the Limpopo province of South Africa. As of 2023, according to Census S.A., the Vhembe district has a population size of approximately 1.6 million people. The majority of the population is Black African (98.7%), with smaller percentages of Coloured (0.2%), Indian/Asian (0.4%) and White (0.8%) residents. The primary languages spoken in the district are Venda (67.2%), Tsonga (24.8%), Northern Sotho (1.6%) and Afrikaans (1.3%). The district has a population density of about 52.1 people per square kilometre. The district is known for its ethnic diversity, with the Venda people being the majority in the northern part and the Tsonga people forming the majority in the southern part.

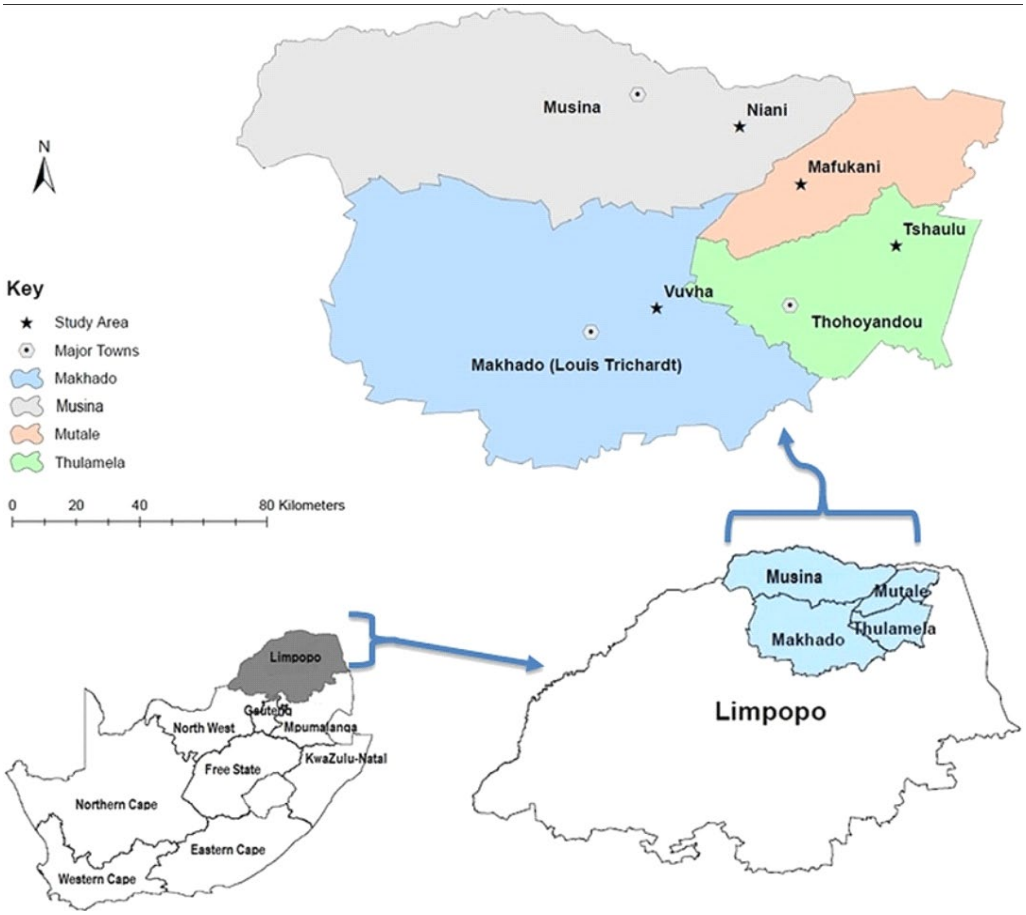


Figure 3.2: Showing the whole map of the Vhembe district (Adopted from https://www.researchgate.net/figure/Map-of-the-Vhembe-District-Limpopo-Province-South-Africa-showing-the-four-villages_fig1_312307903)

### 3.3 ETHICAL CLEARANCE

The ethical clearance for this study was approved by the Research Ethics Committee at the University of Venda (Project no: SMNS/20/MBY/13/2104: Appendix 1). The Department of Health of the Limpopo province (LP_2023-05-013: Appendix 2) and the Vhembe district DOH (S8/4/2/3: Appendix 3) granted permission to carry out this study in their various healthcare facilities. Informed consent was obtained from all the study participants before recruitment into the study, and all experiments were performed per relevant guidelines and regulations.

### 3.4 SAMPLE COLLECTION

For this study, 13 patients were recruited from three healthcare facilities (Shigalo, Malamulele, William Eddie) in the Vhembe district. While this number is relatively small, it was influenced by practical challenges encountered during sample collection. Many potential participants did not attend their scheduled clinic appointments or were lost to follow-up, which significantly limited recruitment and sample collection. Data collection involved obtaining biological samples- specifically blood and sputum- as well as administering structured questionnaires, all of which required participants to be physically present and fully engaged. For sample collection, the professional nurses collected 5ml of blood samples from each patient in an EDTA tube (Sigma Aldrich; St Louis, MI, USA). Meanwhile, sputum samples were collected in a sterile sodium hydroxide (Merck; Rahway, NJ, USA) container to deactivate the mycobacterium. Samples were kept at 4°C in an icebox and were analysed immediately after arriving at the laboratory. Samples were collected from patients adhering to both the clinic appointment and medication provided by the clinics. The identities of all the individuals who participated in this research project were kept confidential by assigning codes according to clinics and sequential numbers (For example, SHG 01 for patient 1 from Shigalo Clinic). This study included 13 participants from which; the socio-demographic data was reported, sputum samples were analyzed using Seegene's Anyplex kit (Seegene; Seoul, South Korea), serum from blood was used for ELISA to determine the serum TNF-α levels, and genomic DNA from each sample was sequenced for possible TNF-α SNPs.

### 3.5 PRE-TREATMENT OF SPUTUM SAMPLE

To ensure that the bacterium is evenly distributed for accurate testing, to ensure that there are enough bacteria available for further processing, and to maintain the pH and prevent contamination, the sputum sample was pre-treated using Merck's PBS solution. Briefly, sputum samples were transferred to 2ml tubes and were vortexed for one minute using the Vortex-Genie 2 Vortex Mixer. The samples were processed after being incubated at room temperature for 15 minutes. 1.5 ml of the material was transferred from the sputum containers to a fresh, sterile tube, and centrifuged for five minutes at 13,000 rpm. The supernatant was removed, and 1 millilitre of Merck's PBS solution was added and mixed well. The tube was centrifuged at 13,000 revolutions per minute (rpm) for five minutes. After removing the supernatant, one millilitre of Merck's PBS solution was added and mixed. A pipette (Thermo Scientific) collected the supernatant from the tubes following a 5-minute centrifugation at 13,000 rpm (Seegene, Seoul, South Korea).

### 3.6 DNA EXTRACTION FROM SPUTUM

The prepared sediment was mixed with 1 millilitre of sterile water, centrifuged using a Biorad centrifuge machine; for 5 minutes at 13,000 rpm, and the supernatant was removed with a pipette (Thermo Scientific). After adding 10 µl of MTB/DRe internal control and 100 µl of DNA extraction solution to the sediment, the mixture was centrifuged (Biorad) for five minutes at 13,000 rpm and vortexed (Vortex-Genie 2 Vortex Mixer) for thirty seconds. After using a cap lock to secure it, the tube was placed on a heat block and left to boil for 20 minutes. The tube was taken out of the heat block and centrifuged (Biorad) for five minutes at 13,000 rpm (Seegene, Seoul, South Korea). Five supernatant microliters were used as a PCR template to identify MTB, MDR, and XDR.

### 3.7 DNA EXTRACTION FROM BLOOD

The DNA was extracted using the Zymo kit (according to the manufacturer's instructions). Briefly, 200µl of the blood sample was added into a microcentrifuge tube, followed by 200µl of BioFluid and Cell Buffer, as well as 20µl of Proteinase K. The mixture was vortexed (Vortex-Genie 2 Vortex Mixer) for 10-15 seconds and then incubated at 55˚C for 10 minutes. After incubation, 200µl of Genomic Binding Buffer was added to the digested sample and the tube was vortexed (Vortex-Genie 2 Vortex

Mixer) for 10-15 seconds. After vortexing, the mixture was transferred into a Zymo-Spin IIC-XLR Column in a Collection Tube. The mixture was centrifuged (Biorad) at 12000 xg for 1 minute. After centrifuging, the flow through and collection tube were discarded. 400µl of DNA Pre-Wash Buffer was added to the spin column in a new collection tube and then centrifuged (Biorad) at 12000 xg for 1 minute. After centrifuging, the collection tube was emptied, and the spin column was returned to the tube. 700µl g-DNA Wash Buffer was added to the spin column and centrifuged (Biorad) at 12000 xg for 1 minute, and then the collection tube was emptied. 200µl of g-DNA Wash Buffer was added to the spin column and centrifuged (Biorad) at 12000 xg for 1 minute, and the collection tube and supernatant were discarded. The spin column was transferred into a new microcentrifuge tube. After transferring the spin column, 50µl of DNA Elution Buffer was added directly to the matrix. The microcentrifuge and spin column were incubated for 5 minutes at room temperature. After incubation, the microcentrifuge was centrifuged (Biorad) at a maximum speed for 1 minute to elute the DNA (Zymo Research Corp, California, USA).

### 3.8 DNA PURIFICATION USING THE ZYMO KIT PROTOCOL

Buffer was prepared by adding 24ml of 100% ethanol into 6ml of DNA wash buffer concentrate. 1.5ml tube was filled with 2-7 volumes of DNA Binding Buffer to a volume of each DNA sample. The tube was vortexed (Vortex-Genie 2 Vortex Mixer) for mixing. The mixture was transferred into a Collection Tube and placed within a Zymo-spin Column. It was then centrifuged (Biorad) for 30 seconds, and the flow through was discarded. After adding 200µl of DNA Wash Buffer to the column, the tube was centrifuged (Biorad) for 30 seconds- this step was repeated twice. 50µl of DNA Elution Buffer was added to the column matrix and incubated at room temperature for 1 minute. The tube was centrifuged (Biorad) for 30 seconds to elute (Zymo Research Corp, California, USA).

### 3.9 DNA QUANTIFICATION USING THE NANODROP

The DNA sample was quantified using the Nanodrop 8 software (according to the manufacturer's instructions). Briefly, the nanodrop was initialized, and the nanodrop was cleaned using DNA away, which was applied to the pedestal using a paper towel. 1µl of

the blank solution (Elution buffer) was loaded on the pedestal. The arm was closed and the baseline measurement was set. 1µl of the DNA sample was loaded onto the pedestal and then measured to obtain the absorbance readings. After each measurement, the pedestal was cleaned thoroughly using the DNA away and clean paper towel (NanoDrop™ Eight SciVault Compliance Software, Thermo Fischer, Waltham, Massachusetts, USA).

### 3.10 MOLECULAR IDENTIFICATION IN SPUTUM USING THE ALLPLEX/ANYPLEX

The *Mtb* was identified at Inqaba Biotec in Pretoria, South Africa. According to the manufacturer's instructions, *Mtb* was identified using the Allplex/Anyplex kit (Seegene, Seoul, South Korea). Briefly, a PCR reaction was prepared by combing the provided reagents, and then the extracted DNA was added to the mix. The mixture was then added to a real-time PCR instrument and the program was initialized according to the instructions.

### 3.11 ENZYME-LINKED IMMUNO-SORBENT ASSAY

In an ELISA, an analyte is found in a liquefied sample using a procedure that uses liquid reagents throughout the analysis. In this project, the DIAsource TNF-α – ELISA was used (according to the manufacturer's instructions). The assay uses monoclonal antibodies directed against distinct epitopes of TNF-α. Briefly, 50µl of incubation buffer was pipetted (Thermo Scientific) into the wells. Then 200µl of each calibrator, control, and sample was pipetted (Thermo Scientific) into appropriate wells. The plate was incubated for 2 hours at 18-25˚C on a horizontal shaker between 700 and 100 rpm. After incubation, the liquid was aspirated from each well, and then the plate was washed three times using the wash solution. 100µl of zero calibrator and 50µl of the anti-TNF-α HRP conjugate were added into each well. The plate was then incubated for 2 hours at 18-25˚C on a horizontal shaker set between 700 and 100 rpm. After incubation, the liquid from the wells was aspirated and the wells were washed three times using a wash solution. After washing, 100µl of the revelation solution was added into each well and the plate was incubated for 15 minutes on a horizontal shaker set between 700rpm-100rpm. After incubation, 100 µl of the stop solution was added to

each well. Therefore, absorbance was read at 450 nm and 490 nm (DIAsource ImmunoAssays® Diagnostics, Louvian-la-Neuve, Belgium).

### 3.12 MASSARRAY SEQUENCING AT INQABA BIOTEC

Briefly, the extracted DNA is amplified using PCR from which designed primers are used to target specific regions of interest. MassARRAY system uses the MALDI-TOF mass spectrometry as its foundation, from which SNP-sequence-specific extension primers are added to the PCR amplified product to extend 1 base at the SNP location. The chip's matrix and the produced sample analytes are subsequently co-crystallized. The crystal is inserted into the mass spectrometer's vacuum tube and immediately exposed to an instantaneous nanosecond laser for excitation. The energy that the radiation absorbs by the matrix molecules causes energy to build up and heat to be produced quickly, which sublimates the matrix crystals. The ions produced by the desorption of nucleic acid molecules are primarily single-charged ions that metastable. In the non-electric field drift zone, these individual charged ions are separated based on their mass-charge ratio and acquire the same kinetic energy in the accelerating field. The ion mass decreases with increasing detector speed (Inqaba Biotechnical Industries (Pty) Ltd, Menlo Park, South Africa).

#### 3.12.1 SNP Selection

Candidate SNPs for this study were selected according to the literature review of previous studies. They were selected if they were reported to be associated with diseases or predicted to affect the TNF gene's function. Additionally, other SNPs were selected from the PharrnGKB online database, even though they were not reported to be associated with TB. However, table 3.1 below only reports on the SNPs studied in various ethnic groups.

Table 3.1: Basic description of the selected SNPs

| SNP ID | SNP location | Polymorphism | Functional effects | Method | Genotyping platform |
|---|---|---|---|---|---|
| rs1800629 | Promoter region | G→A | Increased TNF-α expression | MassARRAY | Agena Bioscience MassARRAY System |

| | | | | | |
|---|---|---|---|---|---|
| rs1800630 | Promoter region | C→A | Influence transcriptional activity of the TNF-α gene | MassARRAY | Agena Bioscience MassARRAY System |
| rs1799724 | Intron 2 | C→T | Reduced TNF-α binding affinity to receptor | MassARRAY | Agena Bioscience MassARRAY System |
| rs1799964 | Intron 1 | C→T | Influence change in NTF-α levels | MassARRAY | Agena Bioscience MassARRAY System |
| rs361525 | Promoter region | G→A | Increased TNF-α production | MassARRAY | Agena Bioscience MassARRAY System |

### 3.13 STATISTICAL ANALYSIS

For this study, data analysis was carried out using the Jamovi for Windows, Version 2.5.3 (Jamovi Corp. New South Wales, Australia). The One-Sample t-test was used to compare the concentrations of TNF-α in TB participants and the p-values and t-values were computed. $\chi 2$ test of association was used to compare the distribution of cytokine gene polymorphisms between TB participants and TNF-α inflammation levels, whereas p-values $\leq 0.05$ were deemed significant. Odds ratios (OR) and their 95% confidence intervals (CI) were also computed.

# CHAPTER 4
# RESULTS

## 4.1 RESULTS

### 4.1.1. Factors associated with an increased risk of TB development

This study included 13 participants, with an average age of 48.44 years and a standard deviation of 17.88 years. In this study, 69% of the participants were male ages(30-76), frequency 9(69), and (31%) were females, ages (27-40 ) frequency 4(31), and about 62% (both) were ≤48 years of age, which may suggest that TB affects a relatively younger to middle-aged population 62% of the participants were unemployed and most (92%) stayed in modern houses. Most participants work in dusty open spaces (77%), which could contribute to respiratory issues and increased TB risk due to inhalation of dust and other particulates. About 54% of the participants were using public transport, which is a risk factor for TB transmission due to close contact with others. More than half of the enrolled individuals were co-infected with HIV (54%), which is a significant risk factor for developing active TB due to immune suppression. More of these risk factors are described in the table below.

Table 4.1: Risk factors associated with the development of TB

| VARIABLE | FREQUENCY |
|---|---|
| **Age** | |
| ≤48 | 8 (62) |
| >48 | 5 (38) |
| **Mean** | 48.38462 |
| **SD** | 19.0855 |
| **SE** | 5.293366 |
| **Gender** | |
| Male | 9 (69) |
| Female | 4 (31) |
| **Employment status** | |
| Employed | 5 (38) |
| Unemployed | 8 (62) |
| **House type** | |
| Modern house | 12 (92) |
| Squatter camp | 1 (8) |
| **Workspace** | |
| Dusty open space | 10 (77) |
| Not dusty but open | 3 (27) |
| **Transportation** | |

| | |
|---|---|
| Public Transport | 7 (54) |
| Walking | 6 (46) |
| **Family members with TB** | |
| Yes | 2 (15) |
| No | 11 (85) |
| **Smoking** | |
| Yes | 1 (8) |
| No | 12 (92) |
| **Drinking** | |
| Yes | 1 (8) |
| No | 12 (92) |
| **TB+HIV Co-infection** | |
| HIV | 7 (54) |
| None | 6 (46) |

### 4.1.2. Molecular identification of MTB

In this study, only 6 (46.15%) of the participants tested positive for *Mtb,* and the majority of those who tested positive were recently diagnosed with less than 3 months in treatment. However, the Cycle threshold (Ct) of all cycles was higher than the control, suggesting a low DNA target.

Table 2.2: The prevalence of MTB/NTM in participants

| **SAMPLE CODE** | **FAM** | | **Result Interpretation** |
|---|---|---|---|
| | **MTB** | **Ct** | |
| | | | |
| MD01 | - | N/A | - |
| MD02 | - | N/A | - |
| MD03 | + | 39.07 | MTB |
| MD04 | - | N/A | - |
| MD05 | - | N/A | - |
| MD06 | - | N/A | - |
| MD07 | - | N/A | - |
| MD08 | + | 38.59 | MTB |
| MD09 | - | N/A | - |

| | | | |
|---|---|---|---|
| MD10 | + | 41.36 | MTB |
| MD11 | + | 40.31 | MTB |
| MD12 | + | 40.23 | MTB |
| MD13 | + | 39.96 | MTB |
| PC | + | 18.57 | Positive Control |
| NC | - | N/A | Negative Control |

### 4.1.3 Plotting the calibrator curve

The sensitivity or the minimum detectable dose of TNF-α using this high-sensitivity DIAsource TNF-α ELISA kit was found to be 0.7pg/mL This was determined by adding two standard deviations above the average OD at zero binding when the zero standards were assayed 20 times. However, we considered 1.0pg/mL as the minimum value below which the cytokine levels were treated as undetectable to align with most of the moderately sensitive ELISA kits used in the literature. Below is a calibrator curve constructed by plotting the absorbance values of calibrator solutions with known concentrations. The resulting plot shows a linear relationship between the absorbance and concentration, described by the equation (y=0.002x-0.0056). This equation was used to calculate the unknown concentrations of the samples.

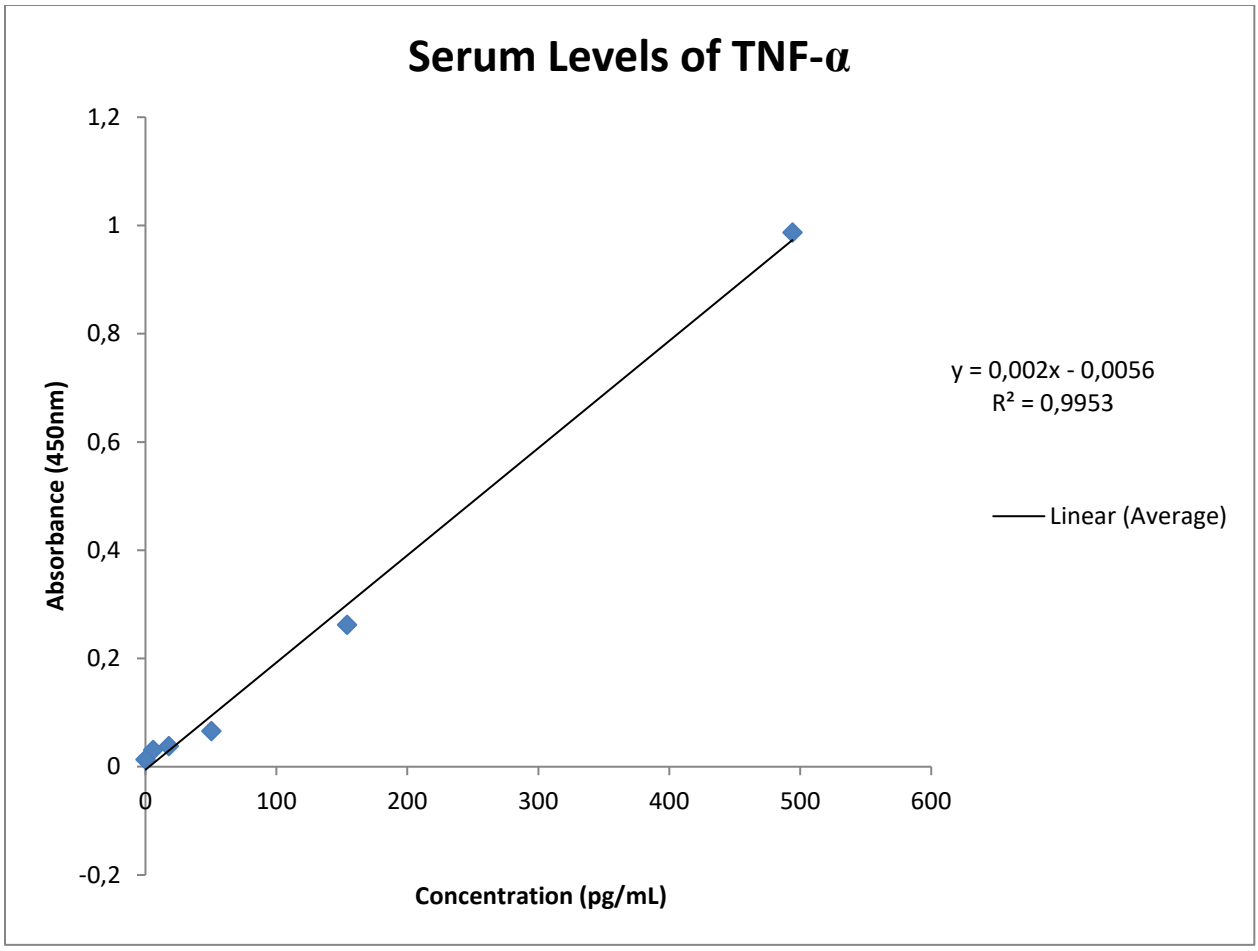


Figure 4.1: Calibrator curve illustrating the serum levels at different absorbance.

### 4.1.4 Comparison between serum TNF-α concentrations

There were varying mean concentrations across the samples. However, 7 (54%) samples showed significant ($p<0.05$) differences, suggesting that the observed mean concentrations are unlikely due to chance. In contrast, 6 (46%) samples showed no significant differences, indicating that the observed mean concentrations could be due to random variation. Table 4.3 below describes in detail the p-values for each sample.

Table 4.3: One sample statistic for TNF-α ELISA concentration obtained from TB-infected participants

| | Mean Conc. (pg/mL) | T-value | P-value |
|---|---|---|---|
| MD1 | 15.55 | 8.89 | 0.071 |
| MD 2 | 11.8 | 23.6 | 0.027 |

| | | | |
|---|---|---|---|
| MD 3 | 14.3 | - | - |
| MD 4 | 14.3 | 9.53 | 0.067 |
| MD 5 | 12.8 | 4.27 | 0.147 |
| MD 6 | 15.55 | 8.89 | 0.71 |
| MD 7 | 11.05 | 44.2 | 0.014 |
| MD 8 | 14.05 | 6.24 | 0.101 |
| MD 9 | 11.05 | 14.7 | 0.043 |
| MD 10 | 12.05 | 16.07 | 0.040 |
| MD 11 | 9.55 | 12.73 | 0.050 |
| MD 12 | 13.3 | 8.87 | 0.071 |
| MD 13 | 10.55 | 42.20 | 0.015 |
| Control 1 | 23.8 | - | - |
| Control 2 | 63.8 | - | - |

### 4.1.5 Reliability of the serum TNF-α mean average

The mean serum TNF-α level in TB positive participants was 12.76pg/mL while the mean serum TNF-α level in the given controls was 43.8pg/mL which suggests that the serum TNF-α level is higher in controls than in disease. The SD for the disease was 1.93 and the control was 28.28, meaning that there is more variation in the control than the disease. Also, the SE for the disease and control was small (0.55), suggesting that the mean for both disease and control have reliable estimates.

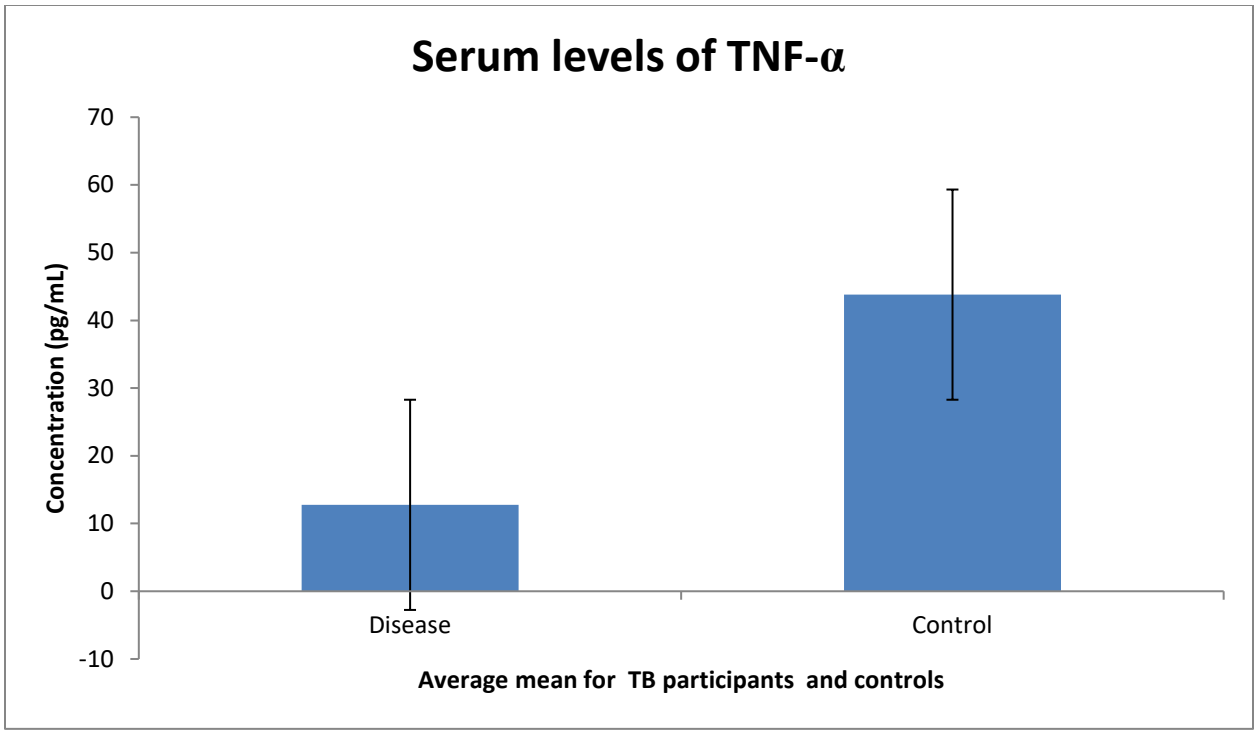


Figure 4.2: Average mean of TNF-α levels in participants and controls, with standard error bars

### 4.1.6 Association between risk factors for TB and TNF-α expression levels

We revealed that two variables (lifestyle changes and history of TB) were statistically significant ($p < 0.05$) concerning TNF-α expression levels, meaning that these risk factors might influence the TNF-α expression levels in this study. Age showed a p-value of 0.06, suggesting this risk factor might have some impact on the expression of TNF-α in this study, but more data would be needed to confirm this. The association is described in detail in the table below.

Table 4.4: $X^2$ test of association between TNF-α expression levels and TB risk factors

| Demographics | Variables | Moderate | Severe | $X^2$ | P-value |
|---|---|---|---|---|---|
| Age | ≥48 | 4 | 4 | | |
| | <48 | 5 | 0 | 3.61 | 0.06 |
| Gender | F | 2 | 2 | | |
| | M | 7 | 2 | 1.00 | 0.32 |

| | | | | | |
|---|---|---|---|---|---|
| Smoke | Yes | 1 | 0 | | |
| | No | 8 | 4 | 0.48 | 0.49 |
| Drink | Yes | 1 | 0 | | |
| | No | 8 | 4 | 0.48 | 0.49 |
| History of TB | Yes | 0 | 2 | | |
| | No | 9 | 2 | 5.32 | 0.02 |
| Lifestyle changes | Yes | 9 | 1 | | |
| | No | 0 | 3 | 5.31 | 0.02 |
| Duration period of Treatment intake | 1-4 months | 3 | 2 | | |
| | Above 4 months | 6 | 2 | 0.33 | 0.57 |
| TB+HIV | HIV | 4 | 3 | | |
| | None | 5 | 1 | 1.20 | 0.55 |
| MTB/NTM | MTB | 5 | 1 | | |
| | NONE | 4 | 4 | 1.04 | 0.31 |

### 4.1.7 Association between SERUM TNF-α levels and TNF-α SNPs

The $X^2$ test of association showed that there was a significant association between the TNF-α expression levels and TNF-α SNPs (rs1800629 and rs3093662). The study revealed that the carriers of genotype GG of the rs1800629 were associated with moderate/decreased levels of TNF-α. Additionally, the carriers of the genotype AA were associated with the moderate/decreased levels of TNF-α. The $X^2$ test of association for the SNPs is described in details below.

Table 4.5: Association between the TNF-α levels and TNF-α SNPs in TB participants

| **Variables** | **Frequency %** | **8.0-14.1pg/mL** | **14.1-16.0pg/mL** | **$X^2$** | **P-value** | **OR (95%CI)** |
|---|---|---|---|---|---|---|
| **Rs1524107** | | | | | | |
| **Genotype** | | | | | | |
| CC | 9 (69) | 7 | 2 | | | |
| CT | 4 (31) | 2 | 2 | 1.00 | 0.32 | 0.286 (0.023-3.52) |
| **Allele** | | | | | | |
| C | 22 (85) | 16 | 6 | | | |
| T | 4 (15) | 2 | 2 | 0.82 | 0.37 | 0.375 (0.043-3.29) |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Rs1799724** | | | | | | |
| **Genotype** | | | | | | |
| CC | 13 (100) | 9 | 4 | 0.0 | 1.0 | - |
| **Allele** | | | | | | |
| C | 26 (100) | 18 | 8 | 0.0 | 1.0 | - |
| **Rs1799964** | | | | | | |
| **Genotype** | | | | | | |
| TT+CT | 12 (92) | 8 | 4 | | | |
| CC | 1 (8) | 1 | 0 | 0.48 | 0.49 | 1.59 (0.0531-47.5) |
| **Allele** | | | | | | |
| T | 19 (73) | 14 | 5 | | | |
| C | 7 (27) | 4 | 3 | 0.657 | 0.42 | 2.10 (0.343-12.9) |
| **Rs1800629** | | | | | | |
| **Genotype** | | | | | | |
| GG | 10 (77) | 9 | 1 | | | |
| AG | 3 (23) | 0 | 3 | 8.77 | 0.003 | 0.023 ($7.33e^{-4}$-0.695) |
| **Allele** | | | | | | |
| G | 23 (88) | 18 | 5 | | | |
| A | 3 (12) | 0 | 3 | 7.63 | 0.006 | 23.5 (1.05-529) |
| **Rs1800630** | | | | | | |
| **Genotype** | | | | | | |
| CC | 11 (85) | 8 | 3 | | | |
| CA | 2 (15) | 1 | 1 | 0.41 | 0.52 | 0.375 (0.0174-8.10) |
| **Allele** | | | | | | |
| C | 24 (92) | 17 | 7 | | | |
| A | 2 (8) | 1 | 1 | 0.38 | 0.54 | 2.43 (0.133-44.5) |
| **Rs1800750** | | | | | | |
| **Genotype** | | | | | | |
| AA+AG | 3 (23) | 3 | 0 | | | |
| GG | 10 (77) | 6 | 4 | 1.73 | 0.19 | 4.85 (0.198-119) |
| **Allele** | | | | | | |
| G | 22 (85) | 14 | 8 | | | |
| A | 4 (15) | 4 | 0 | 2.10 | 0.147 | 0.190 (0.009-3.97) |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Rs3093662** | | | | | | |
| **Genotype** | | | | | | |
| GG+AG | 5 (38) | 5 | 0 | | | |
| AA | 8 (62) | 4 | 4 | 3.61 | 0.05 | 11.0 (0.459-264) |
| **Allele** | | | | | | |
| G | 7 (27) | 7 | 0 | | | |
| A | 19 (73) | 11 | 8 | 4.26 | 0.04 | 11.1 (0.554-222) |
| **Rs309376** | | | | | | |
| **Genotype** | | | | | | |
| AA+AG | 10 (77) | 7 | 3 | | | |
| GG | 3 (23) | 2 | 1 | 1.00 | 0.32 | 3.50 (0.284-43.2) |
| **Allele** | | | | | | |
| G | 13 (50) | 10 | 3 | | | |
| A | 13 (50) | 8 | 5 | 0.72 | 0.40 | 2.08 (0.378-11.5) |
| **Rs361525** | | | | | | |
| **Genotype** | | | | | | |
| AA | 13 (100) | 9 | 4 | 0.0 | 1.0 | - |
| **Allele** | | | | | | |
| A | 26 (100) | 18 | 8 | 0.0 | 1.0 | - |

# CHAPTER 5
## DISCUSSION

This study aimed to assess the production and gene polymorphism of TNF-α in patients with TB in the Vhembe district, Limpopo province. In this study, we assessed the risk factors (age, gender, history of TB, smoking, drinking, lifestyle changes, and co-infection) associated with TB and revealed that, while TB affects both males and females, the burden is disproportionally higher in males (69%) than in females (31%). Research has documented that social and environmental variables contribute to the high incidence of tuberculosis in males (Narasimhan et al. 2013). However, for this study it was not intentional but rather reflective of the actual patient demographic encountered during the study period. Factors such as clinic attendance patterns, availability during recruitment and willingness to participate may have contributed to this gender imbalance. We also found that over 77% of the participants were working in dusty environments, which could contribute to respiratory issues and increased TB risk due to inhalation of dust and other particulates. Our findings aligned with the study conducted by Narasimhan et al. (2013) which showed that indeed more demanding work situations, such as dusty and dirty conditions, could eventually affect one's health and make them more prone to infections.

Additionally, we reported on the association between smoking and drinking with TB development since it has been reported that drinking and smoking impair immunity, leaving people more vulnerable to diseases (Lin et al. 2008). When starting TB therapy, more than half of the trial participants who had previously smoked and drank quit. We found that, amid TB treatment, 92% of the participants were no longer on substance abuse (smoking and drinking). According to earlier research, drinking, and smoking are linked to the development of TB because drinking affects the immune system, and smoking damages the lungs (Bates et al. 2007; Lonnroth et al. 2009). Because the immune system will be weakened, this effect could potentially turn into a doorway for infections. According to a study done in Shanghai, the probability of smoking was 2.2 times higher for heavy smokers than for nonsmokers (Lin et al. 2008). The amount of smoking also appeared to have an impact on the risk; according to a U.S. study, those who had smoked for more than 30 years were at the highest risk.

The association between TB and co-infection in TB development was further examined in the study, and we found that co-infection was more common in males (57%) than in females (43%). This outcome aligns with the study conducted in Congo by Shah et al. (2021) which reported that TB/HIV coinfection was significantly higher in men (4.5%) than women (3.3%). Furthermore, Okonko et al (2018) reported that sex-related prevalence of HIV-TB coinfection within groups indicated significantly higher co-infection rates ($P < 0.05$) among males (23.1%) than females (9.6%). A weakened immune system is typically linked to the impact of co-infection. For example, TB is an opportunistic infection, and after HIV infection, a person's immune system deteriorates, making them more vulnerable to the infection. Lawn et al. (2005) carried out a study in which they showed that HIV patients on ART who subsequently acquired TB had different CD4 counts than those who did not develop TB.

According to a study done on mice, when the mice are exposed to *Mtb* for more than six months, their lung bacterial load increases because TNF-α neutralization occurs (Shang et al. 2011). This suggests that the development of mycobacteria and serum TNF-α levels are related pathophysiologically. For this reason, this study measured the serum levels of TNF-α in TB patients to evaluate its association with TB. As a result, we discovered that, in comparison to people over 48 years of age against those under 48 years, those under 48 years of age had fluctuating serum TNF-α levels. Additionally, the age group of ≤48 years old had higher TNF-α levels than the corresponding group, which is consistent with severe inflammation. According to the literature, age is associated with changes in serum levels of TNF-α. TNF-α levels often increase with age, which helps explain why systemic inflammation rises with age (Reichler et al. 2020). The buildup of senescent cells that release inflammatory cytokines, such as TNF-α, is partially to blame for this. People's immune systems become less effective as they age, and their states become more pro-inflammatory. This immunological dysregulation is associated with higher TNF-α levels, which make older persons more vulnerable to infections and have a slower rate of recuperation from illnesses. The study's findings showed no statistically significant relationship between TNF-α levels and age; however, the p-value was 0.06 which might suggest that age may indeed influence TNF-α expression levels. This outcome needs to be verified by a larger

sample size in this population. According to some research, the rise in TNF-α levels with aging may differ depending on an individual's gender. Normally women with post-menopause, occasionally exhibit higher levels, maybe as a result of hormonal changes that affect immune modulation (Beig et al. 2023; Reichler et al. 2020).

It has been documented that the secretion of TNF-α is one immunological function that is greatly impacted by gender hormones such as testosterone and estrogen (Klein and Flanagan, 2016). However, in this investigation, there was not statistically significant ($p = 1.00$) association between TNF-α levels and gender. Although within the moderate and severe TNF-α inflammatory categories males were more frequently distributed than females. On the contrary, it has been suggested that men often have higher baseline levels of TNF-α than women, particularly as they get older (Olawuyi & Arinola, 2021). This could be related to the ways that each gender reacts to infection and inflammation as well as variations in immunological control.

This study reported on the association between smoking and drinking with TB development since it has been reported that drinking and smoking impair immunity, leaving people more vulnerable to diseases (Lin et al. 2008). When starting TB therapy, more than half of the trial participants who had previously smoked and drank quit. We found that, amid TB treatment, 92% of the participants were no longer on substance abuse (smoking and drinking). Furthermore, this study evaluated the relationship between smoking and TNF-α levels in these TB participants, as studies have shown smoking to be a significant risk factor for the development of TB. However, there was no significant correlation ($p=0.49$) between smoking and serum TNF-α levels in TB participants from this study. These results differ from those of Beig et al. (2023), who found a substantial ($p<0.05$) correlation between smoking and TNF-α levels. When they tested for the influx of lung and splenic CD4 and CD8 effector and central memory T cells that produced TNF-α and IFN-$\gamma$, they discovered that when mice were exposed to cigarette smoke and infected with Mtb, they had a lower total number of CD4 cells that produce TNF-α. It has been shown that the reduction in T cells subsequently affects TNF-α levels and contributes to the likelihood of an elevated bacterial load in the host exposed to cigarette smoke (Shang et al. 2011). TNF-α and other pro-inflammatory

cytokines are released when these substances stimulate immune cells such as neutrophils, T-cells, and macrophages. As part of the immune system's defense, the body views toxins from smoking as dangerous and initiates an inflammatory reaction (Shang et al. 2011). According to Gajalakshmi et al. (1998), smoking cigarettes and bidis, which are common in the Indian subcontinent, carries a significant risk, and is associated with an absolute excess mortality from tuberculosis in individuals, aged 25 to 69. It is suggested that the immune system gets weakened during intoxication, which could potentially turn into a doorway for infections.

For this study, there was no association (p=0.49) between serum TNF-α levels and alcohol intake, which is contrary to a report by Beig et al (2023), showing a substantial correlation between TNF-α levels and alcohol intake. Similarly, Omidvari et al. (1998) found that nonsmokers with alcoholism had lower TNF-α levels. TNF-α and other pro-inflammatory cytokines can be directly stimulated by alcohol in immune cells, including macrophages and Kupffer cells (liver macrophages). Many organs, especially the gut and liver, experience inflammation as a consequence of this. Compared to heavy drinking or abstinence, some research indicates that moderate alcohol consumption—one drink per day, for example—may have anti-inflammatory benefits and lower TNF-α levels. This is believed to be because moderate alcohol use, especially red wine, which includes antioxidants like resveratrol, is good for the heart. However, the dangers of alcohol-related illnesses from excessive use exceed these possible advantages (Omidvari et al. 1998).

There was a statistically significant (p=0.02) correlation between TNF-α levels and participants' family members' history of TB. Our findings are in contrast with Beig et al., who found no significant correlation between TNF-α levels and a family history of TB (Beig et al. 2023). According to the literature, there are several ways in which a family member's history of TB might be linked to altered TNF-α levels, especially because of the stress of living with someone who has the disease and the direct and indirect consequences of *Mtb* exposure (Winthrop et al. 2006).

High and dysregulated TNF-α levels are closely linked to co-infection with HIV and TB (Nosik et al. 2021). However, we found no statistically significant (p=0.55) association

between TNF-α expression levels and co-infection in this study. Benjamin et al. (2013) found that the TNF- expression levels were significantly reduced in patients with TB/HIV, as compared to those without. HIV and TB both affect the immune system in ways that alter the production of TNF-α, which has important implications for the severity and progression of the disease. Co-infected people's elevated TNF-α indicates an immune system's attempt to counteract the increased pathogen load, but it might not be successful in stopping the development of active TB (Joshi et al. 2015).

For this study, there was no statistically significant (p=0.57) association between treatment and TNF-α expression levels. Cytokine responses in TB patients have been assessed in earlier research at different points in time following the start of treatment. According to most research, TNF-α levels are higher in TB patients than in control people soon after treatment starts, and they then drop to levels comparable to control subjects when the disease successfully resolves (Elhaj et al. 2013). TB patients who were in their fourth month of treatment or later had moderate levels of TNF-α, but there was no statistically significant (p =0.57) relationship between duration and TNF-α levels. The study by Shameem et al. (2015) investigated on the TNF-α levels in TB patients' serum wherein they reported no significant association (p =0.73), which is similar findings of this study. Olawuyi and Arinola (2021) investigated on the effects of medication on serum levels of TNF-α in MDR-TB patients and found that the case group had greater levels of TNF-α than the controls, with no significant correlation between treatment duration and serum levels (p>0.05). In many disease processes, including TB, elevated serum TNF-α has been associated with greater tissue damage (Richmond et al. 2012; Falkenstern-Ge et al. 2014). The importance of TNF-α in the pathogenesis and protective immunity against *Mtb* infection is demonstrated by the elevated level at diagnosis and the decreased level during therapy as compared to the control (Olawuyi and Arinola, 2021). However, following medication for two to three months, there have been discoveries with considerably lower TNF-α levels in TB patients, which demonstrated that therapy led to a considerable drop in serum TNF-α levels (Portales-Perez et al. 2002; Kawaguchi et al. 1996; Djoba et al. 2008).

The significance of TNF-α in TB patients has been demonstrated by other investigations (Tang et al. 2002; Portales-Perez et al. 2002; Kawakuchi et al. 1996). Serum TNF-α levels in patients with active pulmonary TB were compared to a control group by Nakaya et al. (1995), wherein, the TB patients' serum level of TNF-α were reported to considerably increase as compared to the control group. Serum levels of TNF-α in *Mtb* and non-tuberculous disorders were assessed in a different study (Taghari et al. 2010), where, the findings demonstrated no significant difference in the serum levels of TNF-α between TB patients and healthy controls. The results obtained by Taghari et al (2010) are consistent with the results of the current study, where p=0.57 demonstrates an insignificant association between duration of treatment and TNF-α levels which implies that duration of treatment has no influence on the levels of TNF-α in the context of this study. Another study found no significant correlation between the peripheral blood monocytes of Mycobacterium and control patients' serum levels of TNF-α (Shahemabadi et al. 2007).

There is growing interest in understanding the genetic components of TB. This study also assessed the relationship between the development of TB and SNPs and serum TNF-α cytokine levels. TT+CT and CC genotypes were more common in individuals with moderate TNF-α expression, according to the genotypic frequency of the rs1799964 TNF-α polymorphism. Participants with severe expression of TNF-α did not differ in the proportion of the genotypes for this SNP. Participants with moderate TNF-α levels were highly likely to have genotypes with the polymorphic allele T* (TT+CT). Nevertheless, this SNP was not statistically significant (p=0.49) about the levels of serum TNF-α. Consequently, there is a greater likelihood of TNF-α association; however, the SNP might not have a substantial impact on TNF-α levels in this cohort. On the other hand, a study in an Asian community confirmed that the rs1799964 TNF-α SNP with TB was significant (Wu et al. 2019) in the Tibetan population. The study further showed that rs1799964 (allele C and heterozygote CT) was a TB-preventive factor. Studies indicate that this SNP may regulate the course of TB disease by raising the RNA expression and cytokine levels of TNF-α, even though rs1799964 was not significant in this study (Wu et al. 2019). Furthermore, the immunopathology of TB, including tissue necrotizing reactions that are crucial for *Mtb* transmission, has been

linked to the upregulation of TNF-α (Gardam et al. 2003; Belay et al. 2015). In the Chinese Uygur community, rs1799964 was linked to TB drug-resistance but not to TB susceptibility, according to a published study (Li et al. 2017).

This study showed that participants with both moderate and severe TNF-α expression had a higher prevalence of the CC genotype, according to the TNF-α rs1799724 polymorphism. Among all, the C allele was the sole variant that was predominant. Consequently, when linked to serum TNF-α levels, this SNP was not statistically insignificant (p=1.00). In contrast, the Tibetan cohort's TNF-α rs1799724 SNP was linked to TB (Wu et al. 2019). Furthermore, other researchers reported that rs1799724 is significantly associated with TB (Ma et al. 2010; Wang et al. 2012; Li et al. 2017).

It was shown in this study that individuals with both moderate and severe TNF-α expression had a higher CC genotype, according to the genotypic frequency of the rs1800630 TNF-α polymorphism. Participants with moderate levels of TNF-α were highly likely to have genotypes with the polymorphic allele C* (CC and CA). Nevertheless, this SNP's association with serum TNF-α levels was not statistically insignificant (p=0.52). The findings of a study by Wu et al. (2019) were contradictory, in that they reported a strong correlation between TB and rs1800630 in TNF-α amongst the Tibetan community. TNF-α gene expression is elevated in response to *Mtb* infection, indicating a robust immunological response mediated by immunocytes. TNF-α contributes to the development of granulomas in addition to being essential for the immunological response to TB (Stein et al. 2005). The findings from a study by Wu et al. (2019) indicated that TNF-α was a causative gene for TB susceptibility and that rs1800630 (allele A and heterozygote CA) was a protective factor against TB. As previously reported rs1799964 and rs1800630 may enhance the expression of TNF RNA and the release of the TNF-α protein (Piosik et al. 2013; Rodriguez-Reyes et al. 2016; Nourian et al. 2017). According to a study by Li et al. (2017), the Chinese Uygur population has the rs1800630 AA+CA genotype which was identified as a TB risk factor.

In this study, participants with moderate and severe TNF-α expression had greater AA genotypes about the rs361525 TNF-α polymorphism, and the sole predominant allele among all subjects was A*. Therefore, when correlated with serum TNF-α levels, this

SNP was not statistically insignificant (p=1.00). The outcome of the current study is similar to a meta-analysis conducted on the association between TNF-α polymorphisms and susceptibility to pulmonary TB which has reported on no association between TB susceptibility and the TNF-α variant rs361525 in all study subjects (OR = 1.031, 95%CI = 0.741-1.436, P = 0.855) (Lee and Song 2015). Additionally, a study in China concerning Han and Tibetan populations likewise found no correlation between rs361525 and tuberculosis TB (Wu et al. 2019). In an Asian population, the meta-analysis revealed a strong correlation between one genetic model (GG+GA vs. AA) of TNF-α rs361525 (Yi et al. 2015). Studies have indicated that the frequency of genetic markers frequently showed high variation among various racial and ethnic groups (Ioannidis et al. 2004; Garte et al. 2001), which may help to explain the existence of variation among different ethnicities. In the meantime, gene-environment interactions may also contribute to the effect's complexities.

This study discovered a substantial correlation ($p \leq 0.05$) between TNF-α expression at both moderate and severe levels and TNF-α SNP rs1800629. The GG genotype was more prevalent in individuals with moderate TNF-α expression, according to the genotypic frequency of the rs1800629 TNF-α polymorphism. The polymorphic allele G*-containing genotypes (AG and GG) were very common in people with moderate and severe TNF-α levels. This shows that the likelihood of a TNF-α relationship is higher, and the SNP may have a major impact on the TNF-α levels in this cohort. According to studies done in Ethiopia (Adane et al. 2021) and Tibet (Wu et al. 2019), the wild-type genotype (GG) was linked to the onset of TB. All of these findings point to the possibility that the G* polymorphic allele plays a role in the emergence of disseminated TB (Sousa et al. 2023). In their evaluation of the TNF-α rs1800629 polymorphism, Sousa et al. (2023) found that genotypes with the polymorphic allele A* (GA + AA) had a higher frequency in the TB group, but with no statistical significance. Additionally, they reported a statistically significant difference about the allele frequency, with the A* allele having a higher prevalence in the TB group. Sinaga et al. (2021) found that the A* allele was strongly associated with the risk of pulmonary tuberculosis in an Indonesian population. The A* was also linked to osteoarticular TB and pulmonary TB and pneumoconiosis in coal workers in the Chinese population (Fan et al. 2010; Lv et al. 2016). These findings

imply that the TNF-α rs1800629 polymorphism might be a significant biomarker that initiates inflammatory processes in the lungs, particularly infectious ones. Impact of the TNF-α rs1800629 polymorphism in TB susceptibility in our community would therefore need to be confirmed by a bigger sample size investigation (Sinaga et al. 2021).

While the investigation of polymorphic genotypes and gene expression in Helicobacter pylori infection did not reveal a significant correlation, the evaluation of TNF-α rs1800629 genotypes with gene expression levels in the patient group did show a significant association (Zabaglia et al. 2015). Although other investigations have linked the minor allele A* to TB (Sinaga et al. 2021; Sousa et al. 2023), its prevalence was lower in this study. TB patients had greater frequencies of TNF-α allele G and genotype GG than the control group ($P = 0.00$ and $P < 0.001$, respectively) (Adane et al. 2021). Likewise, a study in the Lurs population discovered a strong ($p < 0.05$) correlation between the G* allele and TB (Iran) (Kurdistani et al. 2015). This could be because of the high levels of TNFα G allele and GG genotype, which can suppress adhesion molecule expression, migration, and the regulation of IFN-$\gamma$ production. Conversely, research in China, Brazil, Iran, and Korea showed that the frequency of the G* allele and GG genotype did not significantly differ between TB patients and healthy controls (Mira et al. 1999; Sandhya et al. 2013; Varahrarm et al. 2014). Sample sizes in various study areas and/or variations in genetic makeup could be the reason for this.

We also found a statistically significant ($p \leq 0.05$) association between the rs3093662 TNF-α SNP and TNF-α expression levels. The genotypic frequency AA of the SNP was more prevalent in both moderate and severe TNF-α expression levels in this study. The polymorphic allele A* was more frequent in both participants with moderate and severe TNF-α expression levels. The TNF-α variant rs3093662 was also studied in the Han population in China and the outcomes of the study revealed that the SNP had no significant association in relation to TB (Zhang et al. 2022). However, a study conducted in India indicated that this locus may be important in protection against TB as serum TNF-α level for AA genotype was significantly high as compared to GG genotype among healthy controls ($p < 0.05$). AA genotype being a higher producer of TNF-α might enjoy a certain degree of tolerance to tubercle bacilli. Abhimanyu et al (2011)

have also reported that there's no significant variation in the serum TNF-α levels in relation to the GG genotype though it was known as the lower producer of TNF-α in their study. Although there's not much information relating this SNP to TB, the variant was also studied in relation to the outcome of sepsis (Wurfel et al. 2008), in relation to CRF in cancer patients (Kühl et al. 2018), in donor kidneys with a higher risk of immediate graft loss (Poppelaars et al. 2022) and type 1 diabetes in south Croatian population (Boraska et al. 2009).

# CHAPTER 6
# CONCLUSION AND RECOMMENDATIONS

In the setting of infectious diseases like TB, TNF-α plays a critical role in the immune response. This cytokine plays a major role in *Mtb* confinement within the granulomas, causing the granulomatous inflammation seen in TB. Thus, the cytokine's synthesis and signalling are essential for the containment and elimination of *Mtb*, underscoring the need for a precisely balanced cytokine activity to ensure efficient immunization while avoiding excessive inflammation and tissue damage. Numerous studies have shown that these cytokines are linked to several illnesses, including cancer and TB. Research has demonstrated that elevated TNF-α levels are typically linked to the illness's onset or severity. Moreover, they have documented that genetic variants, risk factors, and environmental factors all influence TNF-α expression levels, which are then connected to a disease. To gain insight into how TNF-α can be utilized as a biomarker for tuberculosis, it was necessary to evaluate the production and genetic polymorphisms of the cytokine in TB patients and look into the risk factors linked to TB and TNF-α. We evaluated the TNF-α production and genetic variants in TB patients in the Vhembe region of the South African province of Limpopo.

Our findings demonstrated that TNF-α expression levels and TB risk variables are unrelated. TNF-α levels, however, have been linked to TB risk factors and have been shown to influence the onset and severity of TB cases. Because TNF-α levels were not monitored before therapy began to determine whether there was a correlation, the study's results might be a consequence of a small sample size. Additionally, 29% of the patients had severe TNF-α expression levels, while 71% of the patients had moderate levels. Additionally, we discussed the genetic variations of TNF-α. One SNP (rs1800629) showed an association with TNF-α expression levels, whereas the others did not. The allele that causes the relationship varies from study to study, even though this SNP has been linked to both TB and TNF-α levels in numerous investigations. This can be the result of ethnic differences.

Several shortcomings have been noted in this investigation. There were no controls in the study, the sample size was small, and because samples were only taken from the

Venda tribe, it was difficult to evaluate the variety of individuals in the Vhembe district. If the participant's TNF-α had been tracked at the beginning of treatment, it would have been easier to determine how TNF-α expression levels related to treatment duration. Studies that were carried out decades ago were linked to the ELISA kit that was used in this investigation.

Therefore, a big sample size should be given top priority when making recommendations in the future. This indicates that more case-control studies are still required in the Vhembe district to properly evaluate the connection between TB and the genetic variants that affect TNF-α production. The use of ELISA kits that have been suggested in the literature is advised in order to achieve accurate findings.

In conclusion, the limited sample size and high participant attrition raise substantial concerns regarding the clinical applicability of the findings. Given the small overall sample size, even the slight variations in participation rates had noticeable impact on the gender distribution. The small cohort not only restricts the ability to draw reliable conclusions but also undermines the statistical power needed to support clinically meaningful interpretations. Furthermore, while Jamovi is a robust and user-friendly statistical tool, its effectiveness is inherently dependent on the quality and quantity of input data. In this case, the small sample size significantly compromises the reliability of the statistical outputs, warranting cautious interpretation and emphasizing the need for larger, more rigorously controlled studies to validate these results.

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# Appendices

## Appendix 1: Ethical clearance

ETHICS APPROVAL CERTIFICATE

RESEARCH AND INNOVATION
OFFICE OF THE DIRECTOR

NAME OF RESEARCHER/INVESTIGATOR:
Prof AN Traore

STAFF NO:
3854

PROJECT TITLE: ADME polymorphism in tuberculosis: Pharmacogenetic analysis of samples from patients in hospitals in the Vhembe district of Limpopo, South Africa.

PROJECT NO: SMNS/20/MBY/13/2104

SUPERVISORS/ CO-RESEARCHERS/ CO-INVESTIGATORS

| NAME | INSTITUTION & DEPARTMENT | ROLE |
|---|---|---|
| Prof AN Traore | UNIVEN, Biochemistry and Microbiology | Principal Investigator Staff |
| Dr NE Madala | UNIVEN, Biochemistry and Microbiology | Co- investigator |
| Prof N Potgieter | UNIVEN, Biochemistry and Microbiology | Co- investigator |
| Prof KS Heysel | University of Virginia | Co- investigator |
| Dr D van Der Westhuizen | Inqaba Biotechnology | Co- investigator |
| Musoliwa R (11628331) | UNIVEN, Biochemistry and Microbiology | PHD Student Co- investigator |
| Mashilo MS (11640422) | UNIVEN, Biochemistry and Microbiology | PHD Student Co- investigator |
| Banda NT (16013629) | UNIVEN, Biochemistry and Microbiology | PHD Student Co- investigator |
| Molepo MS (17006631) | UNIVEN, Biochemistry and Microbiology | Master's Student Co- investigator |
| Ndou LF (20006782) | UNIVEN, Biochemistry and Microbiology | BSc HONS Student Co- investigator |
| Mambane T (20019199) | UNIVEN, Biochemistry and Microbiology | BSc HONS Student Co- Investigator |

Type: **Staff Research**
Risk: **Risk to humans, animals, environment, or a sensitive research area (Category 3)**
Approval Period: **August 2024 – August 2025**

The Human and Clinical Trails Research Ethics Committee hereby approves Recertification and Amendments on your project as indicated above.

General Conditions
While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following.

- The project leader (principal investigator) must report in the prescribed format to the REC:
  - Biannually ( or when requested) on the progress of the project, and upon completion of the project
  - Within 48hrs in case of any adverse event (or any matter that interrupts sound ethical principles) during the course of the project.
  - Annually a number of projects may be randomly selected for an external audit.
- The approval applies strictly to the protocol as stipulated in the application form. Would any changes to the protocol be deemed necessary during the course of the project, the project leader must apply for approval of these changes at the REC. Would there be deviated from the project protocol without the necessary approval of such changes, the ethics approval is immediately and automatically forfeited.
- The date of approval indicates the first date that the project may be started. Would the project have to continue after the expiry date; a new application must be made to the REC and new approval received before or on the expiry date.
- In the interest of ethical responsibility, the REC retains the right to:
  - Request access to any information or data at any time during the course or after completion of the project,
  - To ask further questions; Seek additional information; Require further modification or monitor the conduct of your research or the informed consent process.
  - withdraw or postpone approval if:
  - Any unethical principles or practices of the project are revealed or suspected.
  - It becomes apparent that any relevant information was withheld from the REC or that information has been false or misrepresented.
  - The required annual report and reporting of adverse events was not done timely and accurately.
  - New institutional rules, national legislation or international conventions deem it necessary

ISSUED BY:
UNIVERSITY OF VENDA, RESEARCH ETHICS COMMITTEE
Date Considered: August 2024

Name of the Chairperson of the Committee: Prof NS Mashau

Signature:

UNIVERSITY OF VENDA
OFFICE OF THE DIRECTOR
RESEARCH AND INNOVATION
2024 -08- 12
Private Bag X5050
Thohoyandou 0950

University of Venda
PRIVATE BAG X5050, THOHOYANDOU, 0950, LIMPOPO PROVINCE, SOUTH AFRICA
TELEPHONE (015) 962 8504/8313 FAX (015) 962 9080
"A quality driven financially sustainable, rural-based Comprehensive University"

## Appendix 2: Approval from Limpopo Department of Health

LIMPOPO
PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF
HEALTH

Ref : LP_2023-05-013
Enquires : Dr Ramalivhana NJ
Tel : 015-293 6028
Email : Phoebe.Mahlokwane@dhsd.limpopo.gov.za

**Prof Traore AN et al**

**PERMISSION TO CONDUCT RESEARCH IN DEPARTMENTAL FACILITIES**

Your Study Topic as indicated below;

**ADME POLYMORPHISM IN TUBERCULOSIS: PHARMACOGENETIC AND PHARMACOKINETICS ANALYSIS IN TB PATIENTS FROM HEALTHCARE FACILITIES IN THE VHEMBE DISTRICT OF LIMPOPO, SOUTH AFRICA**

1. Permission to extend your research study as per your research proposal is hereby Granted.
2. Kindly note the following:
    a. Present this letter of permission to the Office of District Executive Manager a week before the study is conducted.
    b. In the course of your study, there should be no action that disrupts the routine services or incur any cost on the Department.
    c. After completion of study, it is mandatory that the findings should be submitted to the Department to serve as a resource.
    d. The researcher should be prepared to assist in the interpretation and implementation of the study recommendation where possible.
    **e. The approval is only valid for a 1-year period.**
    f. If the proposal has been amended, a new approval should be sought from the Department of Health
    g. Kindly note that, the Department can withdraw the approval at any time.

Your cooperation will be highly appreciated.

Ramalivhana

14/07/2023

pp **Head of Department** **Date**

**Private Bag X9302, Polokwane 0700**
**Fidel Castro Ruz House, 18 College Street, Polokwane 0700**
**Tel: 015 293 6000. Fax: 015 293 6211. Website: www.doh.limpopo.gov.za**

**The heartland of Southern Africa** - *development is about people!*

## Appendix 3: Approval from the Department of Health Vhembe district

LIMPOPO
PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF
HEALTH
VHEMBE DISTRICT

**Ref: S8/4/2/3**

**Eng: Gertrude Baloyi**

**Date: 29 February 2024**

**TO: Prof Traore AN et al**
**University Of Venda**

**SUBJECT: REQUEST TO CONDUCT A STUDY (RESEARCH) AT HEALTH FACILITIES IN VHEMBE DISTRICT**

**Your study topic: ADME POLYMORPHISM IN TUBERCULOSIS: PHARMACOGENETIC AND PHARMACOKINETICS ANALYSIS IN TB PATIENTSFROM HEALTH CARE FACILITIES IN THE VHEMBE DISTRICT, LIMPOPO PROVINCE, SOUTH AFRICA**

1. The above matter has reference

2. The Department of Health has acknowledged your communiqué received on the 29 February 2024 for the above mentioned. Kindly be informed that permission has been granted to conduct a research at **Health Care Facilities in Vhembe District from 04 February 2024-04 July 2024.**

3. You are also advised to comply or adhere with the Departmental Policies, rules and regulations during your operations.

Hoping that you will find this in order.

.....
**CHIEF DIRECTOR: HEALTH SERVICES**

29/2/2024
.....
**DATE**

VHEMBE DISTRICT, Private Bag X5009 THOHOYANDOU 0950
Old Parliamentary Building Tel: (015) 962 1848, (015) 962 1852, (015) 962 1754, (015) 962 1001/2/3/4/5/6 Fax (015) 962 2373.

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