ADDIS ABABA UNIVERSITY, COLLEGE OF HEALTH SCIENCE, SCHOOL OF GRADUATE STUDIES

BLOOD NEUTROPHIL COUNT, SPUTUM MYCOBACTERIAL LOAD AND MYCOBACTERIAL LINEAGES IN HIV POSITIVE INDIVIDUALS SUSPECTED WITH PULMONARY TUBERCULOSIS, ADDIS ABABA, ETHIOPIA.

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Blood neutrophil count, sputum mycobacterial load and mycobacterial lineages in HIV positive individuals suspected with pulmonary tuberculosis, Addis Ababa, Ethiopia.

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List of Abbreviations

AFB	Acid Fast Bacilli
ANC	Absolute Neutrophil Count
ALIPB	Akililu Lemma Institute of Pathobiology
ART	Antiretroviral Therapy
AIDS	Acquired Immune Deficiency Syndrome
BD	Becton Dickinson
BMI	Body Mass Index
CBC	Complete Blood Count
CCL	Chemokine ligand
CD4	Cluster of Differentiation four
CD8	Cluster of Differentiation eight
CMI	Cell-mediated immunity
DC	Dendritic Cell
DERC	Departmental Ethical Review and Research Committee
DMIP	Department of Medical Microbiology, Immunology and Parasitology
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra Acetic Acid
G-CSF	Granulocyte Colony Stimulating Factor
HClO	Hypochlorous acid
HIV	Human Immunodeficiency Virus
IFN	Interferon
IL	Interleukin
IQR	Inter Quartile Range
LJ	Lowenstein-Jensen
LPS	Lipopolysacharide
LSP	Large Sequence Polymorphisms
MALT	Mucosa-associated lymphoid tissue
MHC	Major Histocompatibility complex
MDR	Multi-drug resistance

MIP	Macrophage Inflammatory Protein
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MTC	*Mycobacterium tuberculosis* Complex
MPO	Myeloperoxidase
NaOH	Sodium hydroxide
NETs	Neutrophils extracellular traps
NOS2	Nitric oxide synthase
PAMPs	Pathogen-associated molecular patterns
PLHIV	People Living with Human Immunodeficiency Virus
PPD	Purified protein derivative
PMNs	Polymorphnuclears
PTB	Pulmonary tuberculosis
PZA	Pyrazinamide
ROS	Reactive Oxygen Species
rt-PCR	Real Time Polymerase Chain Reaction
SNP	Single Nucleotide Sequencing
SOD	Superoxide dismutases
SPSS	Statistical Package for Social Sciences
TB	Tuberculosis
TCH	Thiophen-2-Carboxylic acid Hydrazide
TGF	Tumor Growth Factor
TLR	Toll Like Receptors
TNF-α	Tumournecrosis factor-alpha
WHO	World Health Organization
ZN	Ziehl-Neelsen

Abstract

Background: The protective and pathologic response of host to *M.tuberculosis* is complex and multifaceted, involving many components of the immune system. There are evidences which suggest that neutrophils play a role in the host response to *M.tuberculosis.* In patients with established tuberculosis (TB) disease, higher peripheral blood neutrophil counts are associated with delayed mycobacterial clearance from sputum and worse clinical prognosis.

Objective: To determine neutrophil counts in peripheral blood of HIV positive individuals with suspected pulmonary tuberculosis(PTB) and to find out the association with mycobacterial load and mycobacterial lineage in Zewoditu-Memorial Hospital and Federal Police Hospital, Addis Ababa, Ethiopia.

Methods: A cross-sectional study was conducted from January 2014 to July 2014. EDTA (Ethylene Diamine Tetra Acetic Acid) anti-coagulated venous blood and sputum samples were obtained. Blood absolute neutrophil counts (ANC) were determined using Cell Dyn 1800 automated hematology analyzer. Sputum samples were decontaminated using sodium hydroxide, which were then concentrated by centrifugation. Smears were prepared from the pellet and stained with ZN (Ziehl-Neelsen) stain for microscopy. The remaining sputum pellets were then used for culture, using LJ (Lowenstein-Jensen) medium. Isolates were also heat killed for molecular genotyping and characterized using spoligotyping. Statistical analyses were done using SPSS and statistical tests were significant at $p<0.05$.

Result: Among 117 participants, the median blood neutrophil count was $2.70x10^3$cells/µl (IQR, 2.1–3.7). PTB was confirmed in 28 out of 117 participants. Patients with PTB had a median blood neutrophil count of $4.06x10^3$/µl (IQR, 3.22–5.91) compared to $2.56x10^3$ cells /µl (IQR, 2.1–3.1) among those who were PTB negative ($p<0.05$). Participants with low mycobacterial load had median ANC values of $3.5x10^3$cells/µl (IQR, 2.6–4.2) and those participants with high mycobacterial burdens had ANC values of $5.5x10^3$cells/µl (IQR, 3.9–7.4) ($p<0.05$). Participants infected with modern mycobacterial lineage had higher blood ANC than those infected with ancient lineage ($p=0.41$).

Conclusions: Increased blood neutrophil counts were observed among PTB positive individuals and among individuals with high sputum mycobacterial load.

Keywords: Neutrophils count, HIV, Pulmonary tuberculosis, sputum mycobacterial load, mycobacterial lineage.

CHAPTER ONE

1. INTRODUCTION

1.1. Background

Tuberculosis (TB) remains a major global health problem, responsible for ill health among millions of people each year. TB ranks as the second leading cause of death from an infectious disease worldwide, after the human immunodeficiency virus (HIV). Every year, more than 1.5 million people die of TB (13% HIV positive), and 9 million new cases are reported. Ethiopia is among the countries most heavily affected by the HIV and TB. The World Health Organization has classified Ethiopia 11th among the 22 high burden countries with TB and HIV infection in the world (WHO., 2014).

TB disease is the most frequent co-infection in HIV infected patients, thought to have caused a third to a half of all acquired immune deficiency, particularly in sub-Saharan Africa and South East Asia, areas of world where HIV infection is expanding most rapidly (WHO., 2014). The risk of developing disease is greatly increased by acquired immunodeficiency Syndrome (AIDS) and other immune-compromising conditions, indicating that protective immunity works in the majority of TB-infected individuals to suppress the infection. The progressive immune compromise associated with HIV infection and AIDS results in reactivation of TB disease in latently infected individuals, and an increase in primary TB and secondary TB infection (Toossi et al., 2007, UNAIDS, 2007).

The protective and pathologic response of host to *M. tuberculosis* is complex and many-sided, involving many components of the immune system. In most individuals, immunity is able to inhibit growth of the pathogen, leading to latent infection with persistent and dormant bacteria. Generally the immune response against *M. tuberculosis* can be an innate and adaptive response in any immunocompetent individuals (Van Crevel et al., 2002). Adaptive immune response is dependent on the acquisition of CD4 + T-cell-mediated immunity and is characterized by granuloma formation involving epithelioid macrophages and multinucleated giant cells. In this regard, since HIV causes a depletion of CD4 T cells, which is important in the control of TB its

contribution to the susceptibility of co-infected persons to TB is high (Ulrichs and Kaufmann, 2006).

The early innate immune host response to *M. tuberculosis* infection is characterized by a flood of phagocytic cells (Zhang et al., 1995, Law et al., 1996). The response is considered to be in the main reconciled by mononuclear leukocytes, but there were evidences coming out, in suggesting that neutrophils may also play a role (Burg and Pillinger, 2001, De Larco et al., 2004, Kumar and Sharma, 2010, Martineau et al., 2007). Also study had shown that neutrophil was the most commonly infected phagocytic cell in sputum samples in the airways of patients with active TB (Eum, 2010, Lowe et al., 2012).

In general, findings suggest that neutrophils may play an important role as part of the innate host response to mycobacteria and contribute to the early control of *M. tuberculosis* infection. On the other hand, however, in patients with established TB disease, higher peripheral neutrophil counts are associated with delayed mycobacterial clearance from sputum and worse clinical prognosis (Martineau et al., 2011). Hence, neutrophils might have disagreeing roles in the response to *M. tuberculosis* that may be raised from different factors.

Molecular typing techniques have been extensively used to speciate strains of *M. tuberculosis* involved in TB infections, studying molecular epidemiology of Mtb, providing insights into dissemination dynamics, evolutionary genetics, and detection of suspected outbreaks and person-to-person transmission(Brosch et al., 2002). Although the members of the *Mycobacterium tuberculosis* Complex (MTC) show a high degree of sequence similarity at the genome level the combination of different forms of genotyping such as Single Nucleotide Sequencing (SNP) and Large Sequence Polymorphisms (LSP) have shown that some lineages are associated with increased transmissibility, whereas others induce stronger host inflammatory responses (Nahid et al., 2010).

1.2. Statement of the problem

Of the many cell types present in the naive human lung that may reconcile control of *M. tuberculosis*, most investigators have focused on alveolar macrophages or monocyte-derived macrophages. However the ability of human macrophages to kill *M. tuberculosis* in culture has never been convincing and consistent, suggesting that other cell types present at the site may also play important roles in innate resistance. Neutrophils are the poorly ranked components of host defence in case of TB. The reason behind this low profile chapter of neutrophils is because of inherent difficulties in working with these cells (Lowe et al., 2012).

Studies confirmed that neutrophils actively participate in both recruitment of different cells to the site of infection and granuloma formation. Additionally they are the most commonly infected phagocytic cell in sputum samples in the airways of patients with active TB (Eum, 2010, Kumar and Sharma, 2010, Ley et al., 2006, Martineau et al., 2007). Also it has been shown that, during direct contacts of patients with proven TB-disease, the risk of acquiring TB infection was increasing as the peripheral blood neutrophils count decrease (inversely related) (Yousefi et al., 2009). Moreover neutrophil come into view to not only have a phagocytic role but they also produce antimicrobial peptides, which has direct activity against *M. tuberculosis* (Martineau et al., 2007). In concert, findings suggest that neutrophils may play an important role as part of the innate host response to mycobacteria and contribute to the early control of *M. tuberculosis* infection.

On the other hand, however, in patients with established TB disease, higher peripheral neutrophil counts are associated with delayed mycobacterial clearance from sputum and worse clinical prognosis (Martineau et al., 2011). Hence, neutrophils might have disagreeing roles in the response to *M. tuberculosis* that may be raised from different factors. We therefore, want to know whether blood neutrophil count has an association with mycobacterial load and mycobacterial lineage (to see Mycobacterium-specific factor) among patients enrolling in the study. Finally we also assess the prognostic value of neutrophil among all those testing positive for TB.

1.3. Significance of the study

HIV and TB co-infected individuals present a diagnostic challenge as they often have atypical clinical and radiological features, as well as paucibacillary disease with negative microbiological tests (Naidoo et al., 2011). For these reason beside diagnostic tools that rely on the direct detection of mycobacteria, alternative markers those can predict and tell the prognoses of the disease are good candidates for better control of the disease and more effective diagnosis.

Neutrophil count can be one of the markers to be proposed and is measured at the point-of-care with results available within a few minutes. Nevertheless, this marker has not been adopted into routine use because of its supposedly poor correlation with disease progression. Therefore, studying whether an association exists between blood neutrophil count and HIV-associated TB is very essential. This association may be used as clue diagnosis for HIV-associated TB and to know the clinical prognosis of the TB disease. Additionally this observation might supports the future literature regarding the potential role for neutrophils in the host response to TB.

1.4. Literature Review

1.4.1. *Mycobacterium Tuberculosis* Complex (MTC)

The MTC refers to group of species (*M.tuberculosis*, *M.canettii*, *M.africanum*, *M.microti*, *M. bovis*, *M.caprae* and *M.pinnipedii*) that are 99.9% genetically similar (Huard, R. C. et al., 2003). From those species, *M.tuberculosis* is the most well known member, infecting human population and it is also able to infect animals that have contact with humans. *M.canettii* and *M. africanum*, closely related to *M.tuberculosis*, can also cause human TB and are usually isolated from West African patients. *M. bovis* displays the broadest spectrum of host infection, affecting humans, bovines and goats. *M.caprae* has been isolated only from goats. *M. microti* is a rodent pathogen, usually isolated from voles that can also cause disease in immunocompromized human patients and *M. pinnipedii* infects seals (Brosch et al., 2002, Niemann et al., 2000).

It has been suggested that MTC members have evolved from a common ancestor via successive DNA deletions/insertions resulting in the present Mycobacterium speciation and their differences in pathogenicity. Genomic analysis has been fundamental for these studies and helped to identify 14 regions (known as regions of difference or RD1–14). These regions, present in the reference laboratory strain *M. tuberculosis* H37Rv, are absent from the vaccine strain *M. bovis* BCG; thus, helping to pinpoint chromosomal genes related to pathogenicity. In parallel, six regions, known as H37Rv deletion 1 to 5 (RvD1–5) and *M.tuberculosis* specific deletion 1 (TbD1), are absent from the *M. tuberculosis* H37Rv genome relative to other members. By contrast, *M.canettii* contains all of the RD, RvD and TbD1 regions and it is believed that this is the most closely related genome to that of the bacilli's ancestor. *M. africanum* strains mainly isolated from West Africa lack the RD9 region. *M. microti* lacks a specific region, RD7, RD8, RD9 and RD10. The most common *M. bovis* strains, "classical *M. bovis*," isolated from bovines as well as from humans, showed the greatest number of RD deletions, lacking regions RD4, RD5, RD6, RD7, RD8, RD9, RD10, RD12 and RD13. *M.caprae* is closely related to *M. bovis* except that it contains several nucleotide substitutions in the gyrB gene that are not found in other members of the MTC. *M. pinnipedii,* as *M. bovis* they have deletion in RD7, RD8, RD9 and RD10 regions but have intact RD4, RD5 and RD6 regions (Brosch et al., 2002, Rastogi et al., 2001, Sreevatsan et al., 1997).

1.4.2. Pathogenesis of tuberculosis

The tubercle bacilli establish infection in the lungs after they are carried in droplets small enough (5 to 10 microns) to arrive at the alveolar spaces. If the defense system of the host fails to eliminate the infection, the bacilli proliferate inside alveolar macrophages and eventually kill the cells (Korf et al., 2006). The infected macrophages produce cytokines and chemokines that attract other phagocytic cells, including monocytes, other alveolar macrophages and neutrophils, which finally form a nodular granulomatous structure called the tubercle. If the bacterial replication is not controlled, the tubercle enlarges and the bacilli enter local draining lymph nodes which leads to lymphadenopathy, a characteristic clinical manifestation of primary TB (Van Crevel et al., 2002). The bacilli continue to proliferate until an effective cell-mediated immune (CMI) response develops, usually two to six weeks after infection. Failure by the host to mount an effective CMI response and tissue repair leads to progressive destruction of the lung. Tumournecrosis factor-alpha (TNF-α), reactive oxygen and nitrogen intermediates and the contents of cytotoxic cells (granzymes, perforin) may all contribute to the development of caseating necrosis that characterizes a tuberculous lesion (Dheda et al., 2010a).

Unchecked bacterial growth may lead to haematogenous spread of bacilli to produce disseminated TB. Bacilli can also spread by erosion of the caseating lesions into the lung airways -and the host becomes infectious to others (Bloom and Cese, 1992). Reactivation of TB results from proliferation of a previously dormant bacterium seeded at the time of the primary infection. Among individuals with latent infection and no underlying medical problems, reactivation disease occurs in 5 to 10% (Comstock, 1982). Immunosuppression is associated with reactivation TB, and the disease process in reactivation TB tends to be localized (in contrast to primary disease): there is little regional lymph node involvement and less caseation. The lesion typically occurs at the lung apices, and disseminated disease is unusual unless the host is severely immunosuppressed. It is generally believed that successfully contained latent TB confers protection against subsequent TB exposure (Frieden et al., 2003).

1.4.3. Clinical manifestation of tuberculosis

When a patient progresses to active TB, early signs and symptoms are often non-specific. Manifestations often include progressive fatigue, malaise, weight loss, and a low-grade fever accompanied by chills and night sweats (Center of Disease Control and Prevention, 2008). Wasting, a classic feature of TB is due to the lack of appetite and the altered metabolism associated with the inflammatory and immune responses (Paton et al., 2004). A cough finally develops in most patients. Although the cough may firstly be non-productive, it proceeds to a productive cough of purulent sputum. The sputum may also be streaked with blood, due to destruction of blood vessel located in the wall of the cavity. The inflamed parenchyma may cause pleuritic chest pain. Extensive disease may lead to dyspnea because the increased interstitial volume leads to a decrease in lung diffusion capacity. Although many patients with active disease have few physical findings, rales may be detected over involved areas during inspiration, particularly after a cough. Hematologic studies might reveal anemia, which is the cause of the weakness and fatigue. Leukocytosis may also occur in response to the infection (American Thoracic Society and Centers for Disease Control and Prevention, 2000).

1.4.4. Tuberculosis diagnosis

Mantoux test

A PPD (Purified protein derivative) of Mycobacterium is used to distinguish TB-infected patients from those who have never been infected, based on the size of reaction through intra-dermal injection. However, the test does not show favoritism between patients with previous vaccination, silent infection or active infection. Its false positivity compromises the value of the test and incentivizes the search for sensitive and specific laboratory tests (Thomas et al., 2003).

Interferon gamma release assay (IGRA)

IGRA is relatively new tool introduced for the diagnosis of TB infection, based on the ability of the Mtb antigens, early secretory antigen target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) to stimulate host production of IFN-γ. It utilizes a quantitative in vitro diagnostic assay, using a single-step enzyme-linked immunosorbent assayed after over-night incubation of plasma derived from undiluted *M. tuberculosis* antigen stimulated whole blood. The measurability of interferon

via an enzyme colorimetric assay by antibody and its low false positivity make it preferable to tuberculin skin test (Mazurek et al., 2001).

Microscopy

Acid-Fast staining remains the initial step for evaluation of TB using direct microscopic examination of the AFB in a smear. Because it is cheap and fairly rapid, it is the only diagnostic test for TB, particularly in developing countries (Truffot-Pernot et al., 2006). Among the three types of staining procedures: ZN, fluorochrome and Kinyoun, ZN (carbolfuchsin) stain is preferable for organisms recovered from culture for its enhanced visualization of the morphologic features of the organism and greater specificity for identification of *M. tuberculosis*. The bright fluorescence of stained bacteria under UV microscopy increases the sensitivity of detection at relatively low microscopic power by fluorochrome dye rhodamine (or rhodamine-auramine) staining and is useful for screening. Kinyoun stains the bacilli without heating (Swaminathan et al., 2010).

Culture

Culture on LJ medium is still the gold standards for the diagnosis of active TB although many new molecular diagnostic methods have been developed. For resource limited countries, culture and ZN staining are used to confirm TB in patients with a clinical presumption of active disease. Six weeks or longer on solid media and 7-21days with liquid culture media will take the organism to grow. It is also important to test drug susceptibility (Rieder et al., 2007).

Biochemical test

The differentiation of MTC by biochemical study includes colony morphology, niacin accumulation test, growth in the presence of thiophen-2-carboxylic acid hydrazide (TCH; 2µg/ml), nitrate reduction on modified Dubos broth, and growth characteristics on Lebek medium and on bromcresol purple medium (induction of a pH-dependent change of color from blue to yellow). Oxygen preference in Mycobacterium isolates on Lebek (a semisolid medium) can be described as aerophilic (growth on the surface) and microaerophilic (growth below the surface) (Normung, 1986). Nitrate reduction and niacin accumulation are the characteristic of *M. tuberculosis*. *M.bovis* is intrinsically resistant to PZA, major criterion for differentiation. Biochemical tests have now

been replaced with molecular techniques for the identification and classification of MTC (Niemann et al., 2000, Wayne et al., 1991).

Region of difference based analysis

A series of classical tests based upon growth, phenotypic, and biochemical properties have been traditionally used to set aside members of the MTC. However, together these tests can be slow, burdensome, imprecise, non-reproducible, and time-consuming, and they may not give clear result in every case and many not be performed by every laboratory. In recent times, comparative genomics studied employing several different genetic hybridization strategies revealed regions of difference (RD) representing the loss of genetic material in *Mycobacterium bovis* BCG compared to *Mycobacterium tuberculosis* H37Rv (Gordon et al., 1999) and these have been used in other studies to delineate species of the MTC (Huard, et al., 2006). Regions of difference are now used to differentiate between the species with in MTC.

Genus typing-Multiplex PCR

The genus typing (multiplex PCR protocol) uses six different primers. Firstly, it targets a sequence region within the 16S rRNA gene specific for the Mycobacterium genus. The two primers MYCGEN-F and MYCGEN-R are designed to amplify a specific PCR product from genomic DNA of all known mycobacteria. Secondly, the PCR mix also includes primers that are specific for a hyper variable region of the 16S rRNA gene of *M.intracellulare* (MYCINT-F) and *M.avium* (MYCAV-R), giving one additional PCR product if the DNA template is any of these two species. Thirdly, species from the MTC can also be identified due to the two primers (TB-F, TB-R) that target the MPB70 gene, specific for MTC (Katoch, 2004).

Spoligotyping

Spoligotyping is the simplest, rapid and most cost effective PCR-based technique used to differentiate sub-species of *M. tuberculosis* strains (Groenen et al., 1993). MTC strains contain different chromosomal region with multiple direct repeats (DRs) of 36-bp interspersed by 35 to 41 bp DNA sequences of unique spacer. Spoligotyping is based on amplification of the DR region and subsequent differential hybridization of the amplified products with membrane bound oligonucleotides complementary to the variable spacer regions localized between the DRs. This typing method relies on determination of binary result (the presence or absence) of spacers in the

in vitro-amplified DNA by hybridizing with labeled PCR-amplified DR locus of the tested strain to multiple membranes spotted 43 synthetic spacer oligonucleotide covalently bound to a filter (Kamerbeek et al., 1997). Results can be detected by chemiluminescence, and interpreted by computerized database.

Insertion sequence

Due to the lack of clearly documented lateral gene transfer among members of the MTC, it has become generally accepted within the scientific community that the MTC species do not undergo genetic exchange (Frost et al., 2005). The development of genetic tools, facilitated by the genomic rearrangement of bacteria, is crucial to know the mechanisms of genetic variety of chromosomes. Specific DNA and protein mostly involved in genomic rearrangement. The induction of homologous recombination, which takes place between repeated DNA sequences, is abundantly by insertion sequences (ISs) (Reif and Saedler, 1975). A repetitive and IS-like element, IS6110, from Mycobacterium library was isolated. It has similarity with IS3 family and is specific to members of MTC. The copy numbers of IS6110 varies from 1to 25 between species and strains of MTC (Thierry et al., 1990).

Histopathological examination

Microscopically, after sectioning and staining with eosin-hemathoxyline staining, the inflammation produced with TB infection is granulomatous, with epithelioid macrophages and Langhans giant cells along with lymphocytes, plasma cells, maybe a few PMN's, fibroblasts with collagen, and characteristic caseous necrosis in the center are characteristic feature. TB bacilli can also be seen by ZN staining of biopsy (Pulimood et al., 2008).

GeneXpert MTB/RIF

Xpert MTB/RIF is the newly developed assay overcomes a number of limitations which are seen in other molecular assay. It utilizes rt-PCR technology to both diagnose TB and detect rifampicin resistance along with using unprocessed clinical specimens, regardless of their smear status. The assay is conducted within a simple, almost fully automated cartridge-based system. The simplicity for the user makes this an assay that could feasibly be widely implemented outside centralized laboratories and potentially impact on TB control (Lawn and Nicol, 2011, Nicod, 2007).

1.4.5. Treatment and prevention

TB is completely curable if the correct drugs are taken for the correct length of time. Several antibiotics need to be taken over a number of months to prevent resistance developing to the TB drugs (Dheda et al., 2010b). Standard regimens for new TB patients, known, to have drug-susceptible TB for the intensive phase treatment includes isoniazid, rifampicin, pyrazinamide and ethambutol (HRZE), and HR are recommended for the continuation phase for 4 months. TB bacteria grow very slowly and divide only occasionally when the antibiotics start to kill them, so treatment usually has to be continued for six months to ensure all active and dormant bacteria are killed and the person with TB is cured. People with respiratory TB are usually not infectious after two weeks of treatment. Dosing frequency for new TB patients daily with acceptable alternative provided that the patient is receiving directly observed therapy (DOT) (WHO, 2009).

The current challenge with the treatment and prevention of TB is the development of multi-drug resistant TB (MDR-TB) and extensively-drug resistant TB (XDR-TB). An isolate that is resistant to at least the two main first-line TB drugs rifampicin (RIF) and isoniazid (INH) is said to be MDR-TB whereas an MDR isolate which is further resistant to fluoroquinolone (FQ) and at least one of the second-line injectable agents: amikacin (AMK), kanamycin (kan) or capreomycin (CAP) is XDR-TB (WHO, 2009).

In latent tuberculosis there are many thousand times fewer TB bacteria than in active tuberculosis. Treatment with a single drug for six months, or two drugs for a shorter time, is sufficient to kill the latent bacteria, preventing the person developing active tuberculosis later in their life. Following TB treatment, the disease can return (relapse) in a small number of people, because not all bacteria have been killed. This is obviously much more likely if the course of treatment has been interrupted, not completed or otherwise not followed. However, it is also possible to catch TB a second time, unlike some other infectious diseases (National Collaborating Centre for Chronic Conditions, 2011). As prevention BCG is a vaccine used in many countries to protect children against severe forms of TB disease. However, its efficacy in preventing TB in adults is variable and controversial (Behr, 2002).

1.4.6. Immune response to TB

1.4.6.1. Innate immune response

Neutrophils

Neutrophils are the most plentiful (40% to 70%) type of white blood cells in mammals and form an essential part of the innate immune system. They are formed from stem cells in the bone marrow and are short-lived and highly motile. They form part of the polymorph nuclear cell family (PMNs) together with basophils and eosinophils (Kolaczkowska and Kubes, 2013, Witko-Sarsat et al., 2000). The name neutrophil derives from staining characteristics on hematoxylin and eosin (H & E) histological or cytological preparations. Neutrophils have an average diameter of 12-15μm in peripheral blood smears and stain a neutral pink. Neutrophils will show increasing segmentation (many segments of nucleus) as they mature. Normally, neutrophils contain a nucleus divided into 2–5 lobes. Hyper segmentation is not normal, and occurs in some disorders, most particularly Vitamin B-12 deficiency (Zucker-Franklin et al., 1988). Approximately 10^{11} neutrophils produced daily, the stated normal range for human blood counts varies between laboratories, but a neutrophil count of 2.5–7.5 x 10^{9}/L is a standard normal range. People of African and Middle Eastern descent may have lower counts, which are still normal (Edwards, 1994).

Neutrophil chemotaxis

Neutrophils are a type of phagocyte and are normally found in the blood stream. During the acute phase of inflammation, particularly as a result of bacterial infection (Jacobs et al., 2010) and some cancers, (De Larco et al., 2004, Waugh and Wilson, 2008) neutrophils are one of the first-responders of inflammatory cells to migrate towards the site of inflammation. They migrate through the blood vessels, then through interstitial tissue, following chemical signals. Cell surface receptors allow neutrophils to detect chemical gradients of molecules such as IL-8, IFN-γ, C3a, C5a and leukotriene, which these cells use to direct the path of their migration. Neutrophils have a variety of specific receptors, including complement receptors, cytokine receptors for interleukins and IFN-γ, receptors for chemokines, receptors to detect and adhere to endothelium, receptors for lectins and proteins, and Fc receptors for opsonin (Serhan et al., 2010).

Lifespan of neutrophil

The average lifespan of neutrophils in the circulation has been reported by different approaches to be between 5 and 90 hours (Tak et al., 2013). Upon activation, they migrate and undergo selectin-dependent capture followed by integrin-dependent adhesion in most cases, after which they migrate into tissues, where they survive for 1–2 days (Wheater and Stevens, 2002). The short lifetime of neutrophils minimizes propagation of those pathogens that parasitize phagocytes. Neutrophils will often be phagocytozed by macrophages after digestion of pathogens. Also, because neutrophil antimicrobial products can also damage host tissues, their short life limits damage to the host during inflammation (Kolaczkowska and Kubes, 2013).

Anti-microbial mechanism of neutrophil

Being highly motile, neutrophils quickly come together at a focus of infection, attracted by cytokines expressed by activated endothelium, mast cells, and macrophages. Neutrophils express and release cytokines, which in turn amplify inflammatory reactions by several other cell types (Ear and Mcdonald, 2008). In addition to recruiting and activating other cells of the immune system, neutrophils play a key role in the front-line defence against invading pathogens. Neutrophils have three methods for directly attacking micro-organisms: phagocytosis, release of soluble anti-microbials (including granule proteins) and generation of NETs (Neutrophil extracellular traps) (Hickey and Kubes, 2009).

Neutrophils are phagocytes, capable of ingesting microorganisms. They can internalize and kill many microbes, each phagocytic event resulting in the formation of a phagosome into which reactive oxygen species and hydrolytic enzymes are secreted. The consumption of oxygen during the generation of reactive oxygen species has been termed the “respiratory burst”. The respiratory burst involves the activation of the enzyme NADPH oxidase, which produces large quantities of superoxide, a reactive oxygen species. Superoxide decays spontaneously or is broken down via enzymes known as superoxide dismutases (SOD), to hydrogen peroxide, which is then converted to hypochlorous acid (HClO), by enzyme myeloperoxidase. It is thought that the bactericidal properties of HClO are enough to kill bacteria phagocytozed by the neutrophil, but this may instead be a step necessary for the activation of proteases. Neutrophils also release an

assortment of proteins in granules by a process called degranulation, the contents have antimicrobial properties, and help to combat infection (Segal, 2005).

In 2004, Brinkmann and colleagues described a striking observation that activation of neutrophils causes the release of web-like structures of DNA, which represents a third mechanism for killing bacteria (Brinkmann et al., 2004). These NETs comprise a web of fibers composed of chromatin and serine proteases that trap and kill microbes extracellularly. It is suggested that NETs provide a high local concentration of antimicrobial components and bind, and kill microbes independent of phagocytic uptake. In addition to their possible antimicrobial properties, NETs may serve as a physical barrier that prevents further spread of pathogens (Clark et al., 2007).

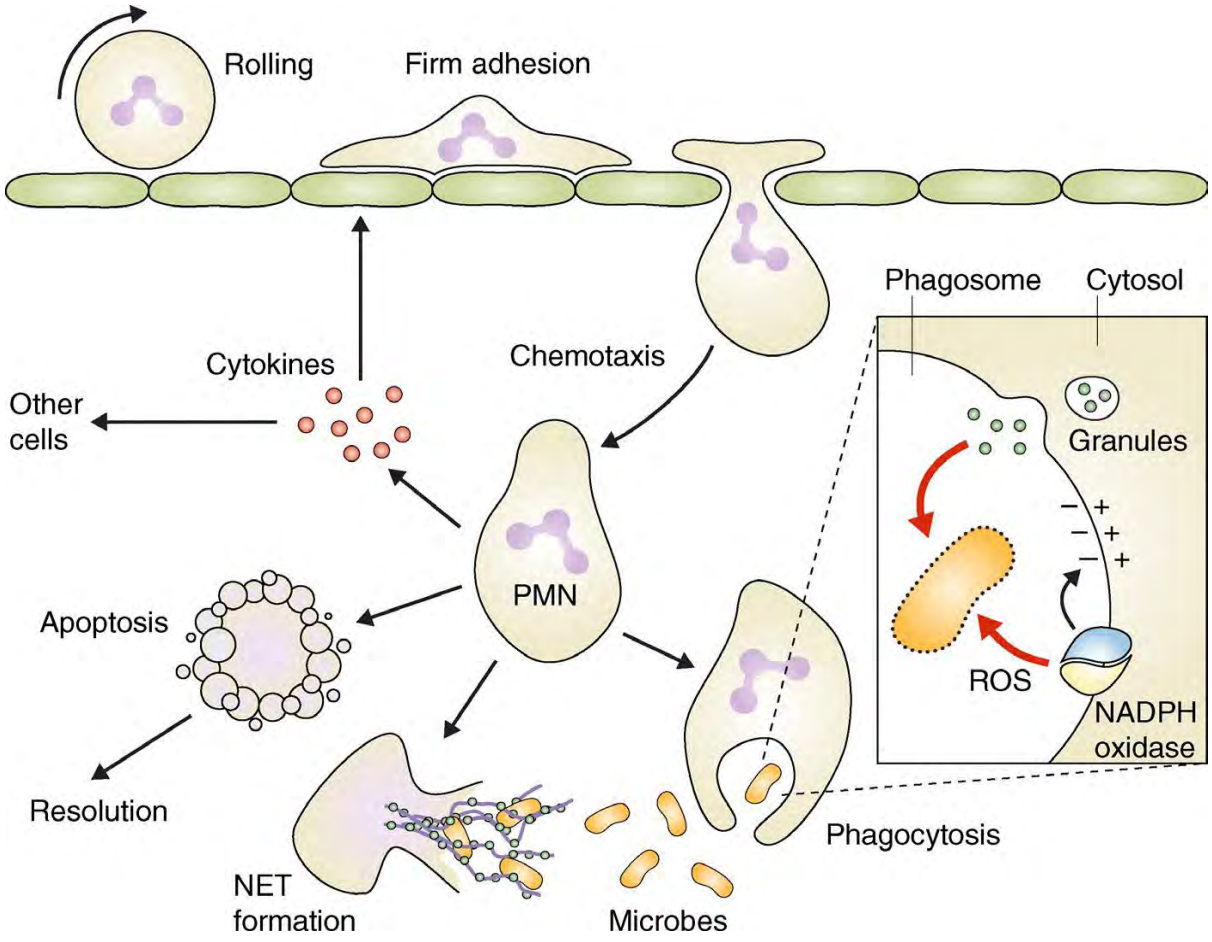

Figure 1.4.6.1. Functions of neutrophil (Mócsai, 2013).

Cellular crosstalk of neutrophil

The first evidence that neutrophils can cooperate with is DCs, induces the maturation of DCs in vitro, as well as their production of IL-12. As a result, DCs that are matured by neutrophils acquire the potential to induce T cell proliferation and polarization towards a TH1 cell phenotype (Megiovanni et al., 2006). Incontrast ectosomes released by human neutrophils either following stimulation in vitro or at the site of inflammation in vivo (Hess et al., 1999) inhibit the maturation of both DCs and monocyte-derived macrophages, by increasing their production of the immunosuppressive cytokine transforming growth factor-β1 (TGFβ1) (Eken et al., 2008).

Human neutrophils can also modulate the activation status of NK cells, either by themselves or in cooperation with other cell types. In the steady state, neutrophils are required for the maturation and function of NK cells, both in humans and mice. In vitro, neutrophils can modulate NK cell survival, proliferation, and cytotoxic activity and IFN-γ production via the generation of ROI and prostaglandins and/or the release of granule components (Costantini and Cassatella, 2011).

Human neutrophils can also crosstalk with B cells. Studies have shown that human neutrophils are a major source of cytokines that are crucial for the survival, maturation and differentiation of B cells (Scapini et al., 2008). Remarkably, neutrophils in the inflamed synovial fluid of patients with rheumatoid arthritis, in inflamed mucosa-associated lymphoid tissue (MALT) or in various B cell malignancies and solid tumours express and secrete high levels of APRIL. APRIL promotes the survival and proliferation of normal and malignant B cells. Hence, neutrophil-derived APRIL could sustain autoantibody production, as in rheumatoid arthritis, or malignant growth and progression, as in B cell lymphoma (Huard, B. et al., 2008, Roosnek et al., 2009).

The other cross talk is with T cells. A first level of interaction between T cells and neutrophils is related to the ability of these cells to modulate each other's recruitment to inflamed tissues. It has been recently shown that activated neutrophils can attract TH1 and TH17 cells to sites of inflammation via the release of CCL2 and CCL20, respectively (Pelletier et al., 2010). In addition, activated T cells can recruit neutrophils, and the mechanism used by individual T cell subsets differs (Himmel et al., 2011, Pelletier et al., 2010).

A second level of interaction between neutrophils and T cells is related to the ability of these cells to modulate each other's functions. Indeed, activated CD4+ and CD8+ T cells, including TH17 cells, produce cytokines (such as IFN-γ, GM-CSF and TNF) that modulate neutrophil survival and expression of activation markers in vitro culture systems (Davey et al., 2011, Pelletier et al., 2010). In addition, IL-17 released by TH17 cells stimulate epithelial cells to secrete granulopoietic factors (such as G-CSF and stem cell factor), as well as neutrophil chemoattractants (such as CXCL1, CXCL2, CXCL5 and CXCL8), which thus amplify neutrophil recruitment and activation (Abi Abdallah et al., 2011).

Functional heterogeneity of neutrophils

Evidence supports the existence of distinct neutrophil subsets that have diverse roles in infection, inflammation and cancer immunology (Fridlender et al., 2009, Tsuda et al., 2004). However, it is still an unlocked issue whether these subsets represent truly dissimilar lineages or instead develop from a single plastic neutrophil precursor. During infection of mice with methicillin-resistant *Staphylococcus aureus* (MRSA), distinctive types of neutrophils were identified and associated with resistance or susceptibility of mice to MRSA. In addition, the neutrophil populations isolated from the MRSA-resistant or MRSA-susceptible mice were also distinct from the neutrophils isolated from naive mice. Each of the three distinct neutrophil populations showed a unique pattern of cytokine and chemokine production and differed in their expression of Toll-like receptors (TLRs) and their surface expression of CD49d/ CD11b integrins (Tsuda et al., 2004).

Differential subsets of neutrophils were also shown to be present in the circulation of human volunteers who received lipopolysaccharide (LPS) compared with untreated controls (Kamp et al., 2012, Pillay et al., 2012), and again this may simply have reflected different states of maturity of the same cell type rather than the existence of distinct neutrophil lineages. Certainly, neutrophils show plasticity and can show marked phenotypical changes. In models of chronic autoimmunity, neutrophils changed their surface phenotypes to express a distinct array of chemokine receptors and adhesion molecules (Johnston et al., 1999). Likewise, some pathogens can induce a change in neutrophil phenotype. In mice, Trypanosoma cruzi induced neutrophils to become anti-inflammatory, make IL-10 and inhibit T cell proliferation and IFN-γ production (Boari et al., 2012).

Role of neutrophils in tuberculosis

Analysis of healthy human individuals who have been in contact with TB patients revealed strong inverse correlation between the risk of TB infection and peripheral blood neutrophil counts (Yousefi et al., 2009). The authors also showed that in vitro growth of mycobacteria in whole blood was dramatically increased upon neutrophil depletion, while neutrophil-derived antimicrobial peptides inhibited mycobacterial growth (Wartha et al., 2007). This study suggested that neutrophils actively fight against mycobacteria and may provide protection from infection.

In addition, it was shown that NETs can trap mycobacteria (Fuchs et al., 2007). Despite the ability to bind mycobacteria, NETs were unable to kill *M. tuberculosis*. As an alternative, Mtb-induced NETs were able to kill *L.monocytogenes* which confirm their antimicrobial effect (Remijsen et al., 2011), and therefore *M. tuberculosis* is resistant to the microbicidal activity of NETs. It looks as if the molecular composition and structural features of the mycobacterial cell wall confer an effective permeability barrier. Since NETs trap but do not kill *M. tuberculosis*, the role of NETs in vivo could be relevant in maintaining the infectious focus localized, as a result preventing mycobacterial spreading and at the same time locating the basis for granuloma formation (Sandilands et al., 2005).

In resting neutrophils, MHC class I molecules are expressed, while MHC class II and costimulatory molecules are not detected on the cell surface (Tian et al., 2005). However, these surface molecules exist intracellularly and some studies indicate that human neutrophils express MHC Class II molecules on the cell surface, following in vitro activation, with IFN-γ (Blomgran and Ernst, 2011). Since neutrophils have a short life-span and are highly susceptible to apoptosis, their role in antigen presentation has been questioned.

Neutrophil also has role in the regulation of the immune response and the interaction with other cellular elements. A cellular element that can interact with neutrophils is the dendritic cells (DCs), which represent an essential element for the generation of T cell responses, during mycobacterial infections (Seiler et al., 2000). DCs are present as immature cells in different tissues, but when these cells sense a microorganisms or an inflammatory response they fully mature and migrate to draining lymph nodes, where they are responsible for the selection and activation of antigen specific naïve T cells. A more recent work showed that in vivo neutrophils are important for DCs

migration from the lung to mediastinal lymhnodes facilitating the inducton of CD4+ response. It was suggested that neutrophils deliver *M. tuberculosis* to DCs and this process promotes the migration of DC, making this more efficient and favoring the T cell response (Ashitani, 2002).

Likewise neutrophils participate in the tissue damage of *M. tuberculosis* infection in which, besides necrotic tissue with macrophage and lymphocytes infiltration, there is an increased neutrophils influx tuberculous lesion site. The consistent presence of neutrophils in the necrotic areas could be mediated by IL-17. In fact, IFN-γ is able to regulate the IL-17 response during BCG infection and in IFN-γ absence in TB granuloma there is an increase in neutrophils. Thus, IL-17 could overcome the apparent IFN-γ mediated regulation and participate in immunopathology (Bisweswar and Behar, 2011).

Study showed that, over expression of IL-17 and IL23 has been related with neutrophils influx in necrotic pulmonary lesions. IL-17 induces the production of the chemokine MIP-2 α which is an efficient neutrophils chemo attractant molecule (Ulrichs and Kaufmann, 2006). Also it has been recently demonstrated neutrophils can be protective during early TB infection, but when exposed to excess of IL-23 or IL-17, their function is altered and they become more able to mediate tissue damage (Desvignes and Ernst, 2009). Undeniably, neutrophils are abundant in the sputum and bronchoalveolar lavage of patients with active TB (Eum, 2010).

Neutrophil counts are increased among patients with active-TB disease in the context of a poorly functioning acquired immune response and having high sputum mycobacterial burden is a greatest risk for having increased neutrophil counts in peripheral blood. Even though granuloma formation typically involves the interaction of macrophages and lymphocytes, neutrophils are also observed at the site of active mycobacterial disease (Eum, 2010, Martineau et al., 2011, Kerkhoff et al., 2013).

Macrophages

Macrophages are important effector cells in immunity to intracellular bacteria but at the same time are subjugated as host cells by a number of microorganisms such as *M. tuberculosis*. Macrophage cell death is thought to play a major role in TB pathogenesis (Lee et al., 2009). As *M. tuberculosis* is a facultative intracellular parasite of macrophages, in order to establish infection, it must first enter into alveolar macrophages following the inhalation of infectious aerosols.

Macrophages are able to engulf inhaled bacteria by using a variety of phagocytic receptors such as complement, toll-like and mannose receptors (Schlesinger, 1993).

After phagocytosis, nonpathogenic *M. tuberculosis* is degraded by the acidification of the phagosomal compartment that contains hydrolases. Keys to the virulence of *M. tuberculosis* are its capacity to prevent the incorporation of the ATP/proton pump into the phagosome membrane and to restrict the fusion of this vacuole with lysosomes (Rohde et al., 2007). In this line it has been shown that, in resting macrophages, pathogenic *M. tuberculosis* blocks phagosome maturation to assure intracellular survival and replication (De Chastellier, 2009).

However, in activated macrophages by IFN-γ, the maturation of phagosome was blocked, possibly through induction of and intersection with the autophagic pathway. Within the phagolysosome, *M. tuberculosis* is deprived of essential nutrients such as iron and exposed to microbicidal effectors generated by IFN-γ-activated macrophages such as antimicrobial peptides (AMPs) and reactive oxygen or nitrogen intermediates, the products of NADPH oxidase and nitric oxide synthase (NOS2), respectively (Purdy and Russell, 2007). As well, it has been shown that macrophages may respond by undergoing TNF-α-mediated apoptosis and possibly through other cell-death pathways (Park et al., 2006). Apoptosis contributes to host defense by removing the Mtb growth, by direct antimicrobial effects, and by packaging *M. tuberculosis* bacilli and antigens in apoptotic bodies. The subsequent engulfment of these apoptotic bodies by newly recruited macrophages and DCs promotes the eradication of infection and the induction of the adaptive immune response (Lee et al., 2009)

Dendritic Cells

DCs are most potent APCs of the innate immune system that have the ability to stimulate inactive, naive, or memory T lymphocytes (Banchereau and Steinman, 1998). DCs exist at various stages of development, activation, and maturation that are defined by distinct phenotypic and functional modalities. After inhalation, *M. tuberculosis* is phagocytosed by alveolar macrophages and DCs resident in the alveolar space of lungs. It has shown that DCs are able to efficiently travel to local lymph nodes and successfully present antigen to T cells, which generates effective cell mediated immunity (Tian et al., 2005). Translocation of *M. tuberculosis* to the lymph node by infected DCs is an important precursor to T-cell activation. Upon exposure of DCs to Mtb, IL-12 and IL-23 are

induced which are playing critical role in the pathogenesis of TB. Besides, it has been shown that DCs undergo cell death after infection with *M. tuberculosis* in vitro, just as macrophages, and could help by this way the protection of host against TB (Ryan et al., 2011).

1.4.6.2. Adaptive immune response

After approximately two weeks of *M. tuberculosis* infection of an in-bred mouse, the adaptive immune response has been mounted and this is accompanied by a drop in bacterial replication (Dheda et al., 2010a). Infected DC and macrophages present Mtb-antigens to T lymphocytes through MHC class I, to CD8+ cytotoxic T lymphocytes and through MHC class II, to CD4+ T helper cells, leading to the activation and proliferation of the lymphocytes. Additionally, CD1-restricted T cells can be activated through presentation of glycolipid antigens by DC, and γδ T cells through presentation of phospholigands, and these contribute to protective immunity against TB by producing IFN-γ or exerting cytotoxic activity (Dheda et al., 2010a).

The infected macrophages and DC secrete cytokines including IL-12, IL-23, IL-7, IL-15 and TNF-α, leading to attraction of more leukocytes to the infection site. Depending on the cytokine environment, the CD4+ T cells can mount a Th1 response (IL-12, IL-18, IFN-γ), or a Th2 response (IL-4, IL-5, IL-13). A Th1 response leads to the release of pro-inflammatory cytokines including IFN-γ, which is thought to enhance killing of intra macrophage mycobacteria through NO and ROS production (Dheda et al., 2010a, Flesch and Kaufmann, 1987). A Th2 response, on the other hand, leads to release of IL-4, IL-5, IL-10 and IL-13, promoting B lymphocyte activation leading to an antibody response, and promoting an anti-inflammatory macrophage response (Dheda et al., 2010a).

Specific activation of CD8+ cytotoxic T cells can lead to killing of *M. tuberculosis* through a perforin and granulysin-mediated pathway by which the infected macrophage undergoes cell death, or by induction of apoptosis through the extrinsic pathway via Fas ligand (Woodworth and Behar, 2006). It is known that TB patients display a defect in the killing capacity of their cytotoxic T cells. Thus, aTh1/Th17 response, but also activation of cytotoxic T cells, is thought to be important aspects of the adaptive immune response to Mtb infection (Dheda et al., 2010a).

The role of B lymphocytes and a humoral response in protection against TB is unclear, and researchers have long dismissed their importance because of the intracellular localization of

M. tuberculosis (Raja, 2004). However, evidence from experimentally infected animals suggests that an antibody response can have an immunomodulating effect on cellular immunity through cytokine signaling, as well as a protective role against infection by inhibiting bacterial replication, neutralizing bacterial products, triggering of the complement system, and promoting antibody-dependent cellular cytotoxicity. Perhaps most importantly, an antibody response results in opsonization of extracellular bacilli with IgG leading to phagocytosis by macrophages and DC through FcγR (De Valliere et al., 2005).

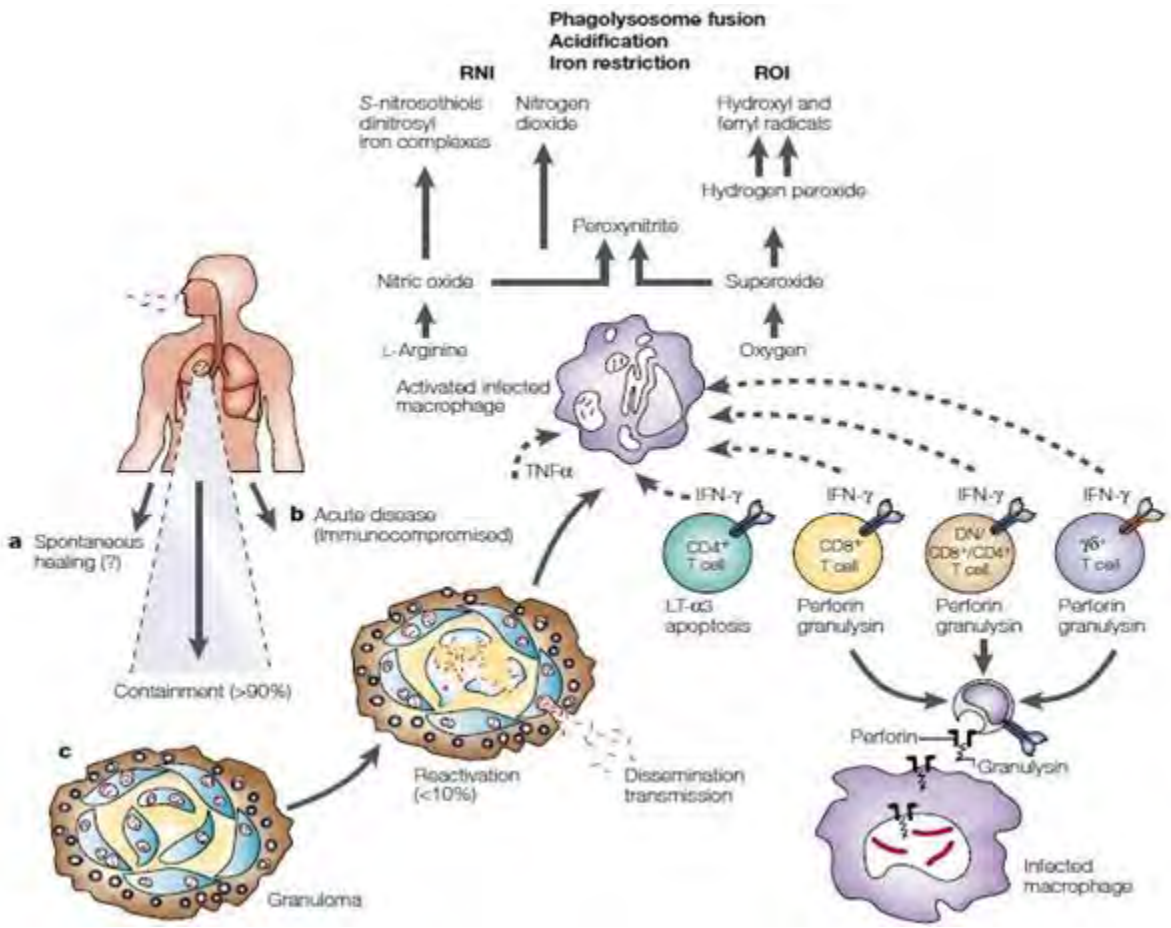

Figure: 1.4.6.2. Components of immune system involved in TB immunology (Kaufmann, 2002).

1.5. Objectives

1.5.1. General objectives

- To determine neutrophil counts in peripheral blood of HIV positive individuals suspected with PTB and to find out the association with mycobacterial load and mycobacterial lineage.

1.5.2. Specific objectives

- To compare blood neutrophils count among TB positive and TB negative HIV patients.
- To find out the association of blood neutrophils count with sputum mycobacterial load.
- To determine the association of blood neutrophil count with mycobacterial lineage.
- To assess the prognostic value of neutrophil among all those testing positive for PTB.

CHAPTER TWO

2. MATERIALS AND METHODS

2.1 Study area

The study area is Addis Ababa city, the capital city of Ethiopia covering an area of 540 sq. km. The study was conducted at Zewoditu-Memorial Hospital and Federal Police Hospital, Addis Ababa, Ethiopia. These hospitals are conveniently selected and are good in their ART service, a great multitude of people from any corner of the country visiting these hospitals for different medical reason. However, patient from Addis Ababa and nearby are more commonly served.

2.2. Study design and period

A cross-sectional study design was employed during data collection period, January to July 2014.

2.3. Source and study populations

The source population was all HIV positive individuals who visited the selected health facilities (Zewoditu-Memorial Hospital and Police Hospital) during the data collection period. The study populations were HIV positive individuals suspected with PTB.

2.4. Sample size and sampling techniques

2.4.1. Sample size

The estimated sample size was 117 PLHIV assuming 7.5% prevalence of TB among PLHIV in North Ethiopia (Wondimeneh et al., 2012). Sample size derived using the following formula:

$$N = \frac{Z^2_{\alpha/2} PQ}{d^2}$$

- Where: N = sample size; $Z_{\alpha/2}$ = standard normal distribution abscissa corresponding to 95% confidence interval (1.96); P = prevalence of TB in HIV positive individuals in study noted above (7.5%); Q = (1-P); and d = desired level of precision (5%).
- **Note**:- Contingency of 10% were added on calculated sample size

2.4.2. Sampling technique

Convenient sampling technique was used, all volunteer individuals consecutively recruited until the calculated sample size reached.

2.5. Selection criteria

2.5.1. Inclusion criteria

HIV positive individuals with sign and symptom of PTB, ART naïve, age above 18 years and volunteers.

2.5.2. Exclusion criteria

Pregnant females, co-morbid individuals, who couldn't produce sputum, who are on isonized preventive therapy and who are on anti-TB treatment.

2.6. Data collection methods and laboratory diagnosis

2.6.1. Data collection methods

After briefly describing of the purpose, benefit and discomfort of the study to the study participants, the consent of every individual was taken. Questionnaires were used to collect information about socio-demographic and clinical feature of the study participants. Morning and spot sputum sample was collected using clean, leak proof sputum cup and transported as soon as possible in an icebox at a temperature of 4°C to laboratory and were processed accordingly. Four milliliter blood sample was collected to EDTA tube (anti- coagulated) and blood neutrophils count was done immediately. The selection of participant was based on the inclusion criteria.

2.6.2. Laboratory diagnosis

Sputum sample processing

In the laboratory both samples (morning and spot) were mixed and homogenized. The homogenate was then decontaminated using 4% NaOH for 15 min and centrifuged at 3000 rpm for another 15 min. Two drops of phenol red indicator was added to the sediment after the supernatant was discarded and 2N HCl was added to neutralize. Neutralization was deemed to be achieved when

the color of the solution was changed from purple to yellow. Then the sediment was inoculated immediately onto prepared L-J culture medium.

Blood neutrophils count

Four-milliliter (4 ml) blood sample for blood neutrophils count was collected from any visible vein to EDTA vacutainer tube after appropriate disinfection of vein puncture site by 70% alcohol. The sample collector and transporter took all necessary safety precautions. The Cell-Dyn 1800 Hematology analyzer was used to perform a blood neutrophils count. This instrument utilizes impedance-based principle. ANC normal range is $2.0 – 7.5x10^3$ cells/µl. Neutrophilia was defined as an ANC>$7.5x10^3$ cells/µl, the upper limit of the normal reference range (Edwards, 1994).

Smear microscopy

After specimen processing and culture inoculation, smear was prepared, using Ziehl-Neelsen staining technique, and graded according to WHO recommendations. The actual number of AFB observed on smear was documented for all positive smears. Smears were then documented and reported using the following scale: negative (no AFB seen per 100 fields), + 1 (10–99 AFB per 100 fields), +2 (1– 10 AFB per field in at least 50 fields), and +3 (10 AFB in at least 20 fields).

Culturing and identification of Mycobacterium

Two sets of Lowenstein-Jensen slants; one supplemented with 0.4% Sodium pyruvate (L-J pyruvate) and the other with glycerol (standard L-J) were prepared. After appropriate labeling and inoculation of the culture with 0.2-0.4ml (2-4 drops) of the centrifuged sediment, it was incubated aerobically at 37°C. The tubes were put in slant position for 5-7 days and then in upright position. All cultures were examined 72 hours after inoculation to check that liquid has completely evaporated, to tighten caps in order to prevent drying out of media, and to detect contaminants. The incubation period lasts for at least eight weeks, with weekly observation for discernible growth. Identification was based on morphology, color, rate of growth, and the acid-fastness (confirmed by Ziehl-Neelsen (ZN) staining). Thereafter, isolates from the positive cultures were preserved with freezing media while at the same time heat killed in water bath at 80°C for 1 hour. The frozen and heat killed isolates were stored at -20°C for further mycobacteriology and molecular typing analysis.

Region of deference based deletion typing (PCR)

Heat killed isolates were investigated by PCR for the presence or absence of RD9 using specific primers (Qiagen, United Kingdom). The PCR amplification mixture used for RD9 typing was as follows: the HotStarTaq Master Mix (Qiagen, United Kingdom) was used for PCR, with primers described below (Table 2.7). The reaction mixture contained 10 µl of HotStarTaq Master Mix, 0.3 µl x 3 of each primer (flankR, F and Int), 2 µ DNA template and 7 µl distilled water to a final volume of 20 µl. Known *M. tuberculosis* was used as positive control while Qiagen water was used as negative control. The mixture was heated in Programmed Thermal Controller (Eppendorf, Hamburg, Germany) using an initial hot start of 95°C for 10 minutes followed by 35 cycles of 95°C for 1 minute; 55°C for 1 minute; and 72°C for 1 minute; a final extension step of 72°C for 10 minutes to complete the cycle. PCR products were electrophoresed in 1.5% agarose gel in 1XTAE running buffer. Ethidium bromide at ratio of 1: 10, 100bp DNA ladder and blue 6X loading dye at a ratio of 1:5 were used in electrophoresis. The gel was visualized in Multi–image UV light cabinet (EPi Chemi II Darkroom). The result was interpreted as *M. tuberculosis* (RD9 present) when a band of 396bp was observed comparing to commercially available ladder, divided by 100bp (Gordon et al., 1999).

Table 2.7: Oligonucleotide primers used for RD9 typing of Mycobacterium isolates and sizes of the expected PCR products (Qiagen, United Kingdom)

Locus	Primer name	Primer sequence	present	Absent
RD9	RD9_FlankF	AACACGGTCACGTTGTCGTG	396	575
	RD9_FlankR	CAAACCAGCAGCTGTCGTTG		
	RD9_InternalF	TTGCTTCCCCGGTTCGTCTG		

Spoligotyping

Spoligotyping was carried out using the commercially available kit from Ocimum Biosolutions, India, according to the manufacturer's instructions. Briefly, the direct-repeat (DR) region was amplified with primers DRa (biotinylated at the 5' end) and DRb, and the amplified DNA was hybridized to inter-DR spacer oligonucleotides covalently bound to a membrane. DNA from *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis* H37Rv were used as positive controls, whereas autoclaved ultrapure water was used as a negative control. The amplified DNA was subsequently hybridized to a set of 43 oligonucleotide probes by reverse line blotting. The presence of spacers was visualized on film as black squares after incubation with streptavidin-peroxidase and detected with the enhanced chemoluminescence system detection liquid (Amersham, Little Chalfont, United Kingdom)

The spoligotyping results were prepared in octal and binary formats into Microsoft Excel spreadsheets; spoligotype patterns were designated as 43-character-long strings consisting of white squares and hyphen representing the presence or the absence of an individual spacer, respectively. The spoligopatterns which were prepared in binary and octal were entered and determined by comparing the spoligotyping results with already existing designations in the international spoligotyping database, SpolDB4.0 (Brudey et al., 2006). In this database, two or more patient isolates sharing identical spoligotype patterns are defined as SIT (spoligotype international type) whilst single spoligopatterns are defined as "orphan" isolates. Patterns that were not found in SpolDB4.0 were assigned to families and subfamilies using the SpotClust program, which was built on the SpolDB3 database (SPOTCLUST) (Kamerbeek et al., 1997).

The global population structure of *M. tuberculosis* is defined by six phylogeographical lineages: Indo-Oceanic lineage, East Asian lineage, East African Indian lineage, Euro-American lineage, West African lineage I and West African lineage II (Gagneux and Small, 2007). The Indo-Oceanic lineage (lineage I), West African lineage I (lineage 5), and West African lineage II (lineage 6) are belonging to ancient lineages whereas the East Asian lineage (lineage 2), East African-Indian lineage (lineage3) and Euro-American lineage (lineage 4) are belonging to the modern lineage (Gagneux et al., 2006).

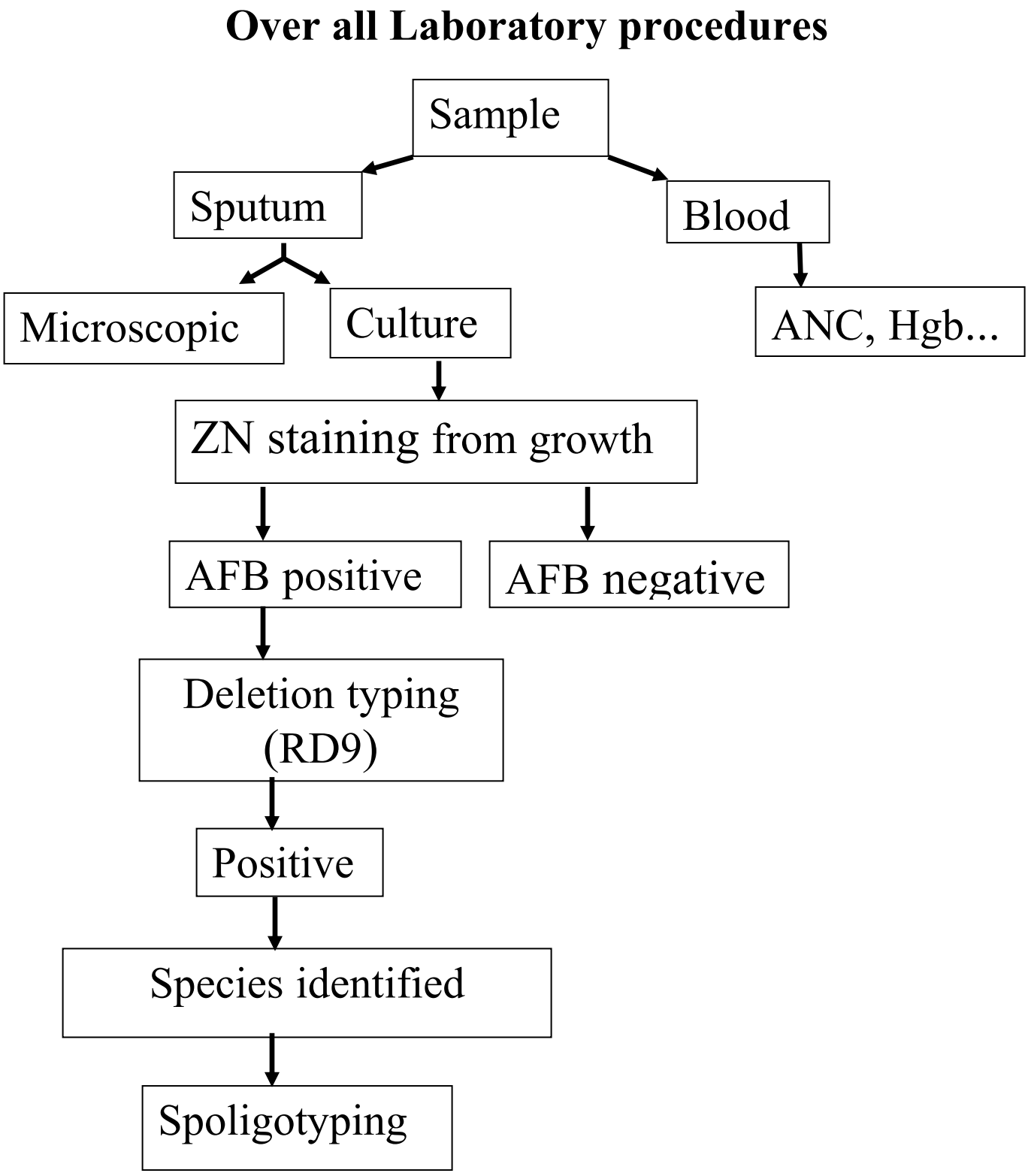

Fig.2.6 Chart showing overall procedures of the laboratory

2.7. Operational definitions

Some of the definitions might not be standard; they were defined according to the situation of this thesis.

- Absolute neutrophil count (ANC): the real number of white blood cell in the blood that is neutrophil (normal range is 2.0 – 7.5x10^3 cells/µl).
- High absolute neutrophil count: absolute neutrophil in the blood ≥2.70x10^3cells/µl
- Neutrophilia: defined as an ANC>7.5x10^3 cells/µl, the upper limit of the normal reference range.
- Anemia: a medical condition in which the hemoglobin concentration is less than normal (<12g/dl and <13g/dl for female and male respectively).
- Severe anemia: an anemia case when hemoglobin concentration <8g/dl.
- Pulmonary tuberculosis suspect: any person who presents with symptoms or signs suggestive of tuberculosis (mainly coughs for more than two weeks).
- Pulmonary tuberculosis case: a patient with *M.tuberculosis* identified from sputum specimen by culture.
- Pulmonary tuberculosis: a case of tuberculosis (as defined above) that involves the lung.
- Smear positive pulmonary tuberculosis: a condition when AFB bacilli are detected in sputum sample by smear microscopy.
- Smear negative pulmonary tuberculosis: a condition when AFB bacilli are not detected in sputum sample by smear microscopy but detected by sputum culture.
- High sputum bacterial load: a condition when sputum sample is examined to be positive for AFB bacilli by smear microscopy.
- Low sputum bacterial load: Conditions when sputum sample is examined to be negative for AFB bacilli by smear microscopy and tested positive by sputum culture.

2.8. Quality assurance

Care was taken in preparing LJ medium, and then it was put at 37°C for 48 hours to check for contamination. ZN staining reagent was cheeked; with known positive and negative control slides. All reagents, media and other accessories were prepared, used and stored in accordance with SOPs. The quality control for neutrophils count was performed according to the recommendations

of the kit manufacturers. Quality control was done every other day when the instrument is turned on and whenever new lot of reagent is opened.

2.9. Statistical analysis

Statistical analyses were performed with SPSS Software version 20. The blood neutrophil count and characteristics of patients with and without TB were compared. Logistic regression was used to identify factors associated with neutrophil count values $\geq 2.70 \times 10^3$cells/µl. To explore the prognostic value of neutrophil, the characteristics of TB patients stratified by neutrophil count$\geq 2.70 \times 10^3$cells/µl and $<2.70 \times 10^3$cells/µl (a close approximation to the median value) were defined and compared. The statistical tests were significant at $p<0.05$.

2.10. Ethical considerations

Before starting the study, ethical clearance was obtained from Departmental Ethical Review and Research Committee (DERC) of department of Medical Microbiology, Immunology and Parasitology (DMIP), College of Health Science, Addis Ababa University. Official letter of co-operation was written to respective hospitals and to ALIPB, for sample collection and sample processing respectively. The confidentiality of the information collected was maintained by using code numbers for participants. In addition, the clinical specimens collected during the study period were used for the stated objectives. All participants who were positive for PTB treated with first line anti TB drugs through their physician. Health information was given to prevent spread of the infection to others.

2.11. Dissemination of the result

The findings of this study were presented to department of Medical Microbiology, Immunology and Parasitology, College of Health Science, Addis Ababa University. The findings will also be disseminated to different organizations (governmental and non-governmental) that will have a contribution to improve and preventing the wide spread of *M. tuberculosis*. Findings will also be presented on different seminars and workshops and it may also be submitted for possible publication to scientific journals.

CHAPTER THREE

3. RESULTS

3.1. Socio-demographic background of the Study participants

A total of 117 tuberculosis suspected HIV patients were included in the study. Out of the total 117 participants 48(41%) were males and 69 (59%) were females with male to female ratio of 0.7:1. The participants' median age was 34year IQR (28-38years). In terms of residence, 92(78.6%) of the patients were living in urban and 25 (21.4%) in rural areas. Majority of the patients were married 84 (71.8%) with high school educational status 63(53.8%).

Table 3.1. Socio-demographic background of the study participants, January to July 2014, Addis Ababa, Ethiopia.

Variables		Number	%
Sex	Male	48	41
	Female	69	59
Address	Urban	92	78.6
	Rural	25	21.4
Marital status	Single	16	13.7
	Married	84	71.8
	Divorce	6	5.1
	Widowed	11	14.4
Educational status	Non educated	11	9.4
	Elementary school	30	25.6
	High school	63	53.8
	Higher education	13	11.1
Occupation	Employee	62	53
	House wife	23	19.7
	Daily laborer	25	21.4
	Merchant	5	4.3
	Farmer	2	1.7

3.2. Clinical back ground of study participants

Participants' medical charts were reviewed for further clinical data. As shown in Table 3.2, the major complaint of the study participants was cough ≥ 2 weeks. Among participants with confirmed TB cases (n=28) the median CD4, BMI and hemoglobin were 151 cells/µl (IQR, 99-196), 18.4 kg/m^2 (IQR, 18-20) and 11 g/dl (IQR, 10-13), respectively. From those PTB confirmed patient (n=28), 16 (57.1%) had a low sputum mycobacterial load (smear negative and culture positive) and 12 (42.9%) had a high sputum mycobacterial load (both smear-positive and culture positive). Patients with higher levels of sputum mycobacterial burden had shorter days of culture positivity than patient with lower mycobacterial load.

Table: 3.2. Clinical background of the study participants, January to July 2014, Addis Ababa, Ethiopia.

Clinical back ground		PTB negative (n=89)	PTB positive(n=28)	Low bacterial load (n=16)*	High bacterial load (n=12)**
Cough ≥ 2wk, no. (%)		89(100)	28(100)	16(100)	12(100)
Fever, no. (%)		68(76.4)	28(100)	16(100)	12(100)
Night sweat, no. (%)		81(91)	28(100)	16(100)	12(100)
Weight loss, no. (%)		52(58.4)	28(100)	16(100)	12(100)
HIV stage, no. (%)	I or II	59(66.3)	12(42.9)	8(50)	8(66.6)
	III or IV	30(33.7)	16(57.1)	8(50)	4(33.4)
CD4, median(IQR)		187(112-300)	151(99-196)	159(85-266)	149(129-423)
BMI, median(IQR)		20.4(19-22)	18.4(18-20)	18.7(18.0-21.7)	18.6(18.2-20.2)
Hgb, median(IQR)		13(11.5-14)	11(10-13)	12(11-13)	10.5(10-13)
CCT, median(IQR)		--	36 (31-40)	40(38-45)	29(25-33)

Note: "*"= culture positive, smear negative "**"= culture positive, smear positive and "CCT"=culture conversion time, in days

3.3. Sputum examination result

A total of 117 study subjects were included in the study and all of them produced (morning and spot) sputum sample for PTB diagnosis. The samples were processed and used for culture and smear microscopy. Positive PTB diagnosed by culture was 28(23.9%) and by smear microscopy were 12(10.3%) as shown in Table 3.3. In combination, culture and smear microscopy diagnosed about 28(23.9%) of PTB positive individuals from 117 participants. The remainder were sputum culture-negative (n=89). Out 12 smear positive PTB, seven (58.3%) had +2 AFB grading, three (25%) had +1 AFB grading and two (16.7%) had +3 AFB grading microscopically.

Table 3.3: Detection of Mtb with culture and smear microscopy diagnostic methods (n=117), from January to July, 2014, Addis Ababa, Ethiopia.

Assays	**Positive results (%)**
Culture	28(23.9)
Smear microscopy	12(10.3)
Combination	28(23.9)

3.4. Identification and characterization of Mycobacterium, species and strain

3.4.1. Region of difference based species identification

Culture isolates were confirmed for acid fastness by ZN stain and then additional investigations carried out to differentiate the species among MTC by presence or absence of RD9. The presence of RD9 was confirmed in all of 28 isolates, indicating that they were *M. tuberculosis* (figure 3.4.1).

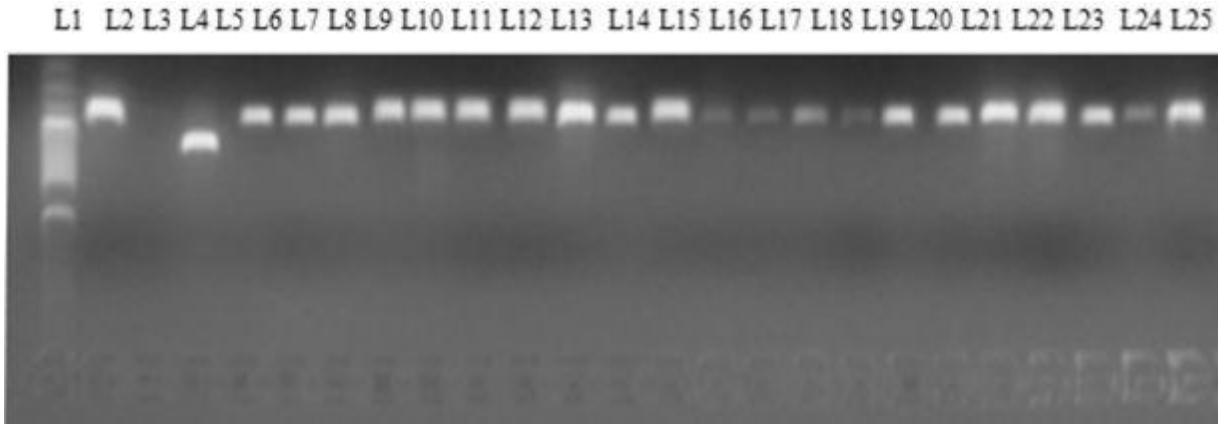

Figure3.4.1. Gel picture showing Mycobacterium species identification with RD9 Primer from sputum culture isolates. Description; Lane1= ladder (100bp), Lane2= H37Rv (*M.tuberculosis* +ve control), Lane3=Qiagen H_2O (-ve control), Lane4= *M.bovis* (+ve control). Lanes 5 through 25 are clinical samples from sputum culture.

3.4.2. Spoligotyping result

A total of 13 dissimilar spoligotype patterns were identified from the 28 isolates analyzed using spoligotyping (Figure 3.4.2). Twenty (71.4%) isolates were grouped in 5 clusters of spoligotype patterns. The largest cluster identified in the present study, SIT523, consisted of 8 isolates; the second largest cluster was SIT54, comprising 5 isolates. SIT2731 and SIT336 comprised respectively 3 and 2 isolates. However, 8 (28.6%) clinical isolates were represented by a unique (non-clustered) spoligotype pattern. From a total of 13 distinct spoligotype patterns there were 4 previously unreported spoligopatterns, new to SpolDB4.

Genotype assignment revealed that the most predominant family in our study were the family33 21(75.5%) followed by T1 family and X1 family each of them consisting of 2(7%) isolates. Other families found were CAS family 1(3.5%), H37Rv family 1(3.5%) and the Beijing family 1(3.5%). Classification of the strains by lineage showed three different *M. tuberculosis* lineages; the most prevalent were Indo-Oceanic (20 isolates) and second most prevalent were Euro-American (5 isolates), followed by unknown lineage (2) and Beijing (1).

webdings format	Octal format	SIT	Family	Lineage	No. of strain	% of strain
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□□□	777777777777771	523	Family33	Indo-Oceanic	8	28.5
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Figure 3.4.2: Spoligotype pattern of clustered *M. tuberculosis* strains. The hyphen and white boxes indicate the absence and presence, respectively, of the specific spacer at position 1 to 43 in the DR locus.

3.5. Neutrophil counts among culture positive and culture negative patients

The median ANC among the total participants (n =117) was $2.70x10^3$ cells/µl (IQR, 2.1–3.7). Nevertheless those with PTB positive had higher blood ANC than those without PTB (Figure.3.5). The median ANC of those PTB positive (*M. tuberculosis* identified from sputum specimen by culture) was $4.05x10^3$ cells /µl (IQR, 3.2–5.9) whereas those PTB negative (*M. tuberculosis* not identified from sputum specimen by culture) had median ANC of $2.50x10^3$ cells/µl (IQR, 1.9–3.1). There is a significant association between increased blood neutrophil count and PTB ($P<0.05$). In addition, 28.5% of HIV positive individual with PTB had neutrophilia compared to 2.2% of those without PTB. Also, significant association was observed between PTB and neutrophilia ($p<0.05$).

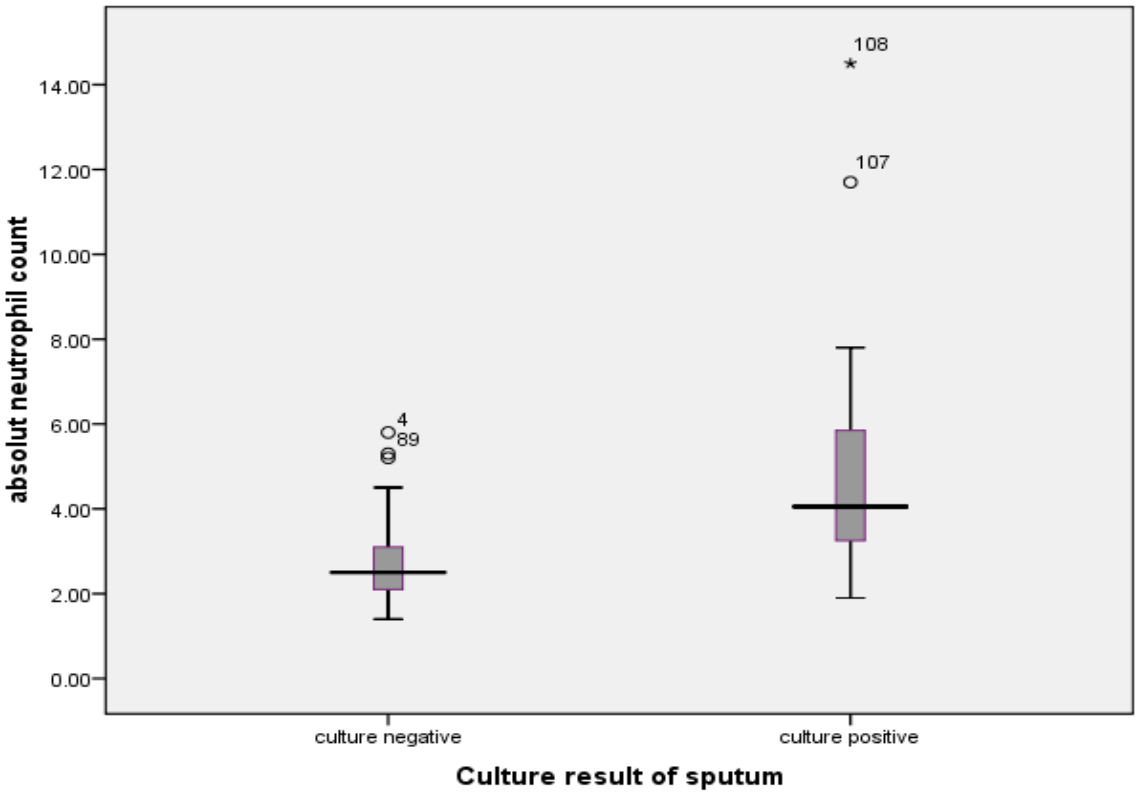

Figure: 3.5. Box and whisker diagram of absolute neutrophil count among those with and without PTB. The middle of the box represents the median value. The outer box edges represent the 25th percentiles and 75th percentiles. The ends of the whiskers represent the lower and upper adjacent values. The dots above the upper adjacent values were outliers.

3.6. Neutrophil counts among low and high sputum mycobacterial load

By using the results of culture and sputum smear microscopy, we classified patients into low mycobacterial load and high mycobacterial load. Both reflect different TB disease severity. In this study there was higher blood neutrophil counts among those patients with higher sputum mycobacterial load (Figure 3.6). The median ANC values of culture negative participants (n=89) were 2.5x10^3/µl (IQR, 2.1–3.1). Participants with low mycobacterial load (those with smear negative and culture positive PTB) had median ANC values of 3.5x10^3 cells/µl (IQR, 2.6–4.2) and those participants with high mycobacterial load (with smear positive and culture positive PTB) had ANC values of 5.5x10^3 cells/µl (IQR, 3.9–7.4). The absolute neutrophil count was significantly different between high and low mycobacterial burden ($p<0.05$). Just about 41% of

patients with high sputum mycobacterial burden and 18.7% of patient with low sputum mycobacterial burden had neutrophilia (ANC>7.50x10^3cells/µl).

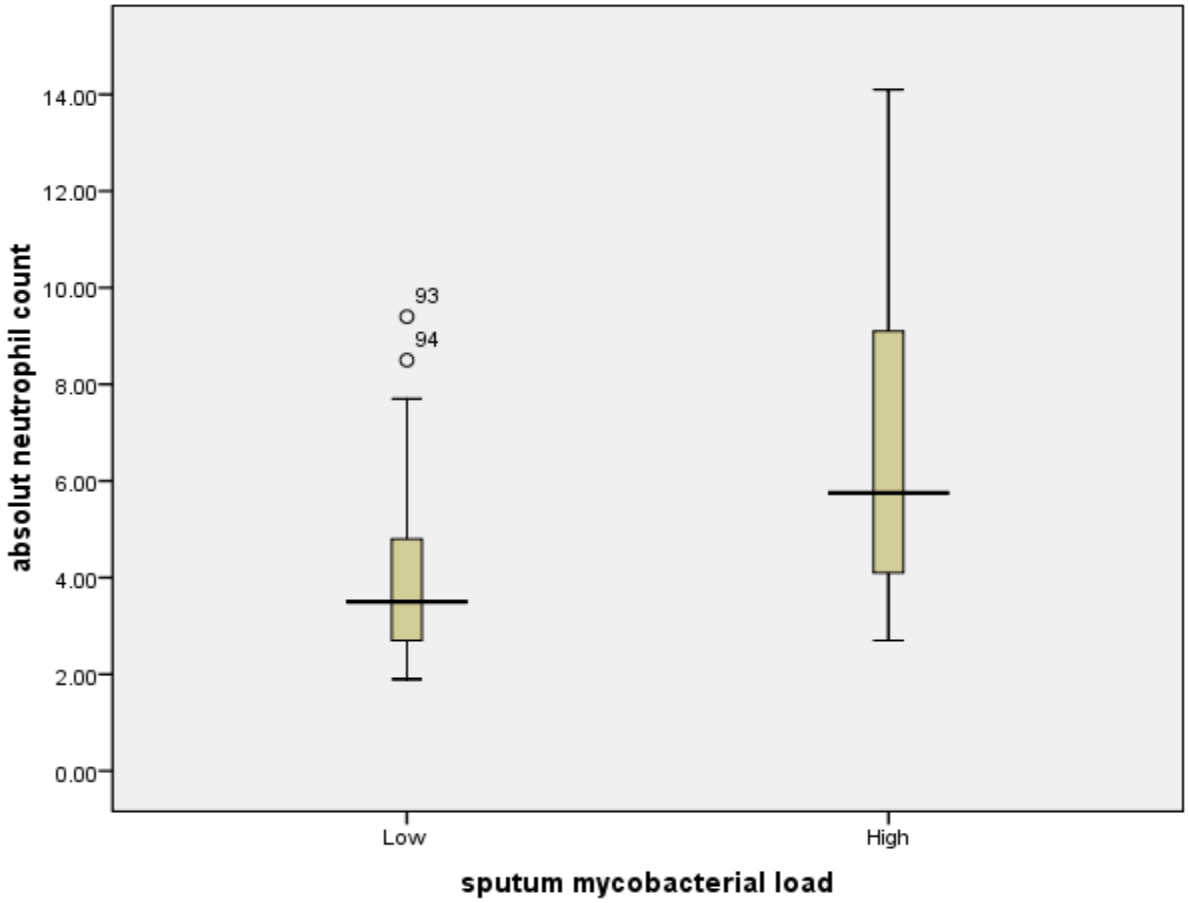

Figure 3.6: Box and whisker diagram of absolute neutrophil count with sputum mycobacterial load. The middle of the box represents the median value and the outer box edges represent the 25th and 75th percentiles (IQR). The ends of the whiskers represent the lower and upper adjacent values. The dots above the upper adjacent values were outliers.

3.7. Neutrophil counts in patients grouped by mycobacterial lineage

Of the 28 patients confirmed with tuberculosis case, 21(75%) were infected with ancient lineage and 7(25%) were infected with modern lineage. The median ANC among those infected with modern lineage was higher than those infected with ancient lineage (Figure.3.7). Those infected with modern lineage had median ANC of $6.50x10^3$ cells /µl (IQR, 3.50–8.20) compared to $3.90x10^3$ cells/µl (IQR, 3.05–6.05), of those infected with ancient lineage, in multivariable analysis the association is not statistically significant (p=0.41). Fifty seven percent of patient infected with modern lineage and 13% of patients infected with ancient lineage had neutrophilia.

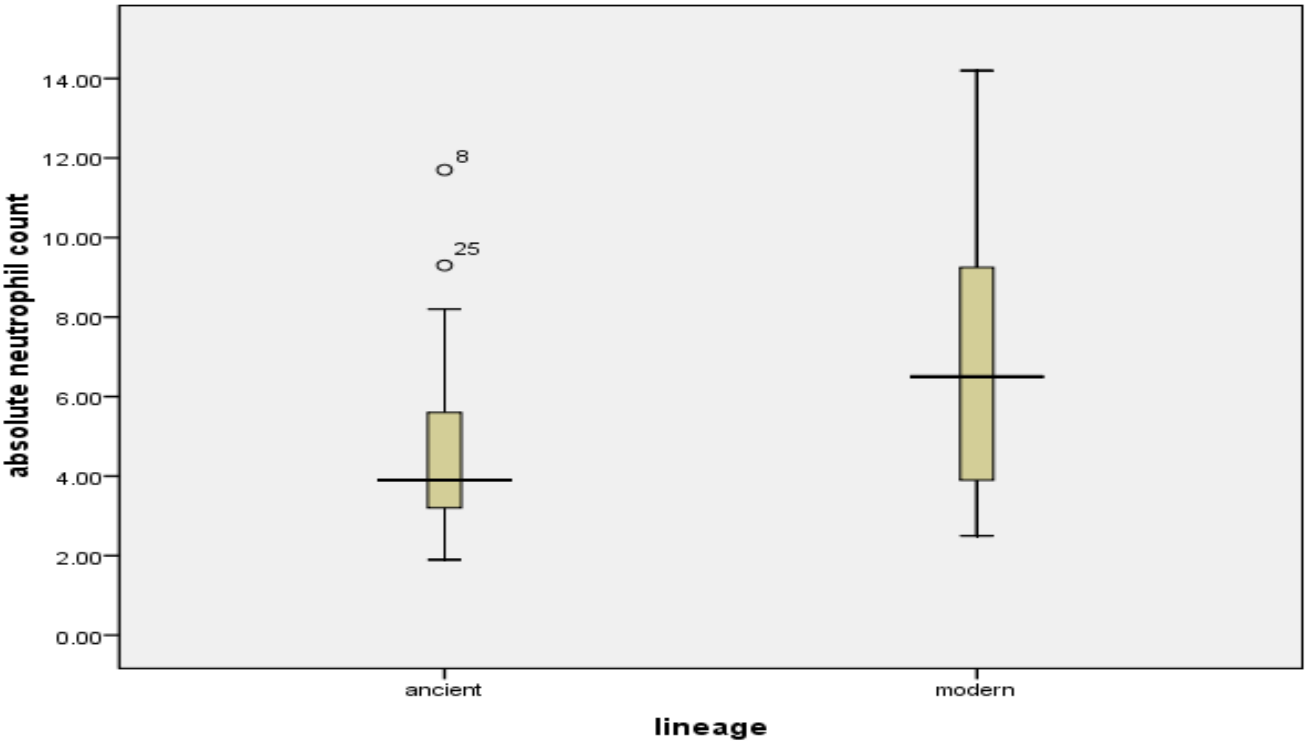

Figure 3.7: Box and whisker diagram of absolute neutrophil count among mycobacterial lineage. The middle of the box represents the median value and the outer box edges represent the 25th and 75th percentiles (IQR). The ends of the whiskers represent the lower and upper adjacent values. The dots above the upper adjacent values were outliers.

3.8. Prognostic value of neutrophil

We compared the characteristics of patients with high and low neutrophil counts, using a cut-off $2.70x10^3$cells/µl, which approximated to the median value. Patients with high neutrophil had worse prognostic characteristics, such as lower BMI, lower hemoglobin, lower blood CD4 cell count and more advanced WHO HIV clinical stage (Table 3.8). To explore the relationship between neutrophil count and mycobacterial load, we compared the results of blood neutrophil count in the high and low mycobacterial load groups (figure 3.6). It was striking that sputum samples from the high neutrophil group were far more likely to test positive by smear microscopy. The time to culture positivity of sputum was also significantly shorter. Taken together these data, we strongly suggested that patients with higher neutrophil counts had higher mycobacterial load, which has the tendency to show poor prognosis.

Table 3.8. Characteristics of patients with tuberculosis and either high or low blood neutrophil count.

Variable		All patient with PTB (n=28)	High ANC ($\geq 2.70x10^3$) (n=22)	Low ANC ($<2.70x10^3$) (n=6)
CD4, median IQR		151(99-196)	138(63-205)	187(173-201)
BMI, median IQR		20.1 (18.0-22.9)	18.4(18-20)	21.2 (20.9-21.9)
Hgb, median IQR		11(10-13)	9.1 (8-11)	11.8 (10.9-12.2)
HIV stage	I or II	15(21.4%)	6(27.3%)	4(66.7%)
	III or IV	13(32.1%)	16(72.7%)	2(33.3%)

CHAPTER FOUR

4. DISCUSSION

Neutrophils are the most plentiful circulating leukocytes; even so their full spectrum of biological functions in immunity is just beginning to emerge generally. Studies published in the last few years suggest that neutrophils play a much more diverse role than previously appreciated in TB immunity (Kumar and Sharma, 2010, Martineau et al., 2007, Nicod, 2007, Segal, 2005, Korbel et al., 2008).

In this study, we assessed blood neutrophil counts during routine screening of patients for HIV-associated TB prior to starting ART in Zewoditu-Memorial Hospital and Federal Police Hospital, Addis Ababa, Ethiopia. Using a rigorous culture-based gold standard for TB diagnosis, we found that PTB disease was associated with increased blood neutrophil counts (blood neutrophil count $\geq 2.70 \times 10^3$cells/µl). The median ANC in present participants (n=117) was 2.70×10^3 cells/µl (IQR, 2.1–3.7), which was 4.05×10^3 cells /µl (IQR, 3.2–5.9) and 2.50×10^3 cells/µl (IQR, 1.9–3.1) in those with PTB and in those without PTB respectively ($P<0.05$). This showed that blood neutrophil count was high among HIV positive individual with PTB. This may be due to the fact that TB develops years after the establishment of latent or subclinical infection by *M. tuberculosis* (Ulrichs and Kaufmann, 2006). Since T cell immunity modulates both innate and adaptive immunity during chronic infection and inflammation caused by *M. tuberculosis* through IFN-γ production (Bisweswar and Behar, 2011), the development of these clinical symptoms of TB is a sign of failed immunity as in HIV positive individuals. Experimental study showed that, in the absence of IFN-γ response, the accumulation of neutrophils in the lungs of *M. tuberculosis* infected mice increased (Cruz et al., 2010, Desvignes and Ernst, 2009). This means IFN-γ directly inhibits neutrophil accumulation in the lung. It is possible that if T cell immunity fails for any reason or IFN-γ responsiveness is reduced; the recruitment of neutrophil to the lung is increased, followed by immunological loss of controlling the infection and worsening the outcome of infection. Consequently this can cause increased neutrophil counts in peripheral blood.

In other explanation the clinical presentation of TB depended on the stage of HIV infection and associated degree of immunodeficiency. As shown in (Table 3.2) those PTB positive participants

had relatively low CD4 count, low BMI, low hgb concentration and additionally most of them were in WHO HIV stage III or IV. This implies that PTB positive individuals were more immunodeficient than those PTB negative individuals. In PTB positive individuals since they were immunodeficient, the ability of T cell immunity to amend the inflammation caused by *M. tuberculosis* is failed.

We also choose to study two different indicators of TB disease severity that reflected degree of sputum mycobacterial load. These two groups, differing in their sputum mycobacterial load. Culture and AFB microscopy results were used in combination to stratify patients in to these two groups, because both have different sensitivity for *M. tuberculosis* detection (Lawn and Nicol, 2011). The groups were; those with low sputum mycobacterial load (smear negative and culture positive cases) and those with high sputum mycobacterial load (both smear positive and culture positive cases). This was also supported by the finding that sputum sample with low mycobacterial load had longer culture positivity time and sputum sample with high mycobacterial load had shorter culture positivity time (Kerkhoff et al., 2013), in our case 40 days IQR (38-45) and 29 days IQR (25-33) respectively.

In our study, generally there was higher blood neutrophil counts among those patients with higher sputum mycobacterial load. Those with low mycobacterial burden had median ANC values of $3.5x10^3$cells/µl (IQR, 2.6–4.2) and those patients with high mycobacterial loads had ANC values of $5.5x10^3$/µl (IQR, 3.9–7.4). Because immunological loss of controlling mycobacterial replication in HIV case (the problem with T cells) (Bisweswar and Behar, 2011, Desvignes and Ernst, 2009), HIV positive individuals appeared to have high sputum mycobacterial load in their sputum which also reflected by having high neutrophil counts in their peripheral blood. Therefore, we suggested that high sputum mycobacterial load is indicative of poorly controlled pulmonary mycobacterial replication in HIV positive individuals.

In the other way, the immune response triggered, against *M. tuberculosis* is not uniform in all exposed people. Though the possibility of clinical manifestation incident at any stage of life in patients who cannot control the infection is there, in the huge majority of humans no disease develops at any time (Bloom and Cese, 1992). So, the course of the infection and its epidemiological consequences depend upon the influence of the genetic diversity of the infecting organisms (Lopez et al., 2003). In our study patients infected with isolates belonging to the modern

lineage had higher blood ANC than those infected with ancient lineage with median count of $6.50x10^3$ cells /µl (IQR, 3.50–8.20) and $3.90x10^3$ cells/µl (IQR, 3.05–6.05) respectively(P=0.41).

Experimental study showed that in comparison with animals infected with ancient lineage, those infected with modern lineage shows a delayed and fainter expression of IFN-γ, and an earlier and augmented expression of TNF-α in their lungs (Lopez et al., 2003). This retarded IFN-γ expression leading to the progression of TB disease, result in high degree of inflammation which may be explained by high number of neutrophil count in peripheral blood. Interestingly, this study might show that isolates belonging to the modern lineage induced higher levels of neutrophil counts though the association is not significant ($p>0.05$).

We next explored the helpfulness of neutrophil as a prognostic marker in those patients with culture confirmed TB (n=28). Among confirmed TB cases, high ANC values ($\geq 2.70x10^3$cells/µl) were strongly associated with poor prognostic clinical features and higher mycobacterial load. Though there is strong correlation between presence of TB in HIV infected individuals and high blood neutrophil count, its likely utility for either rule-in or rule-out TB in HIV-infected patients has been questioned. Nonetheless, we found that at least neutrophil had a prognostic value among HIV-associated TB. There was a strong relation between high blood neutrophil count and poor prognostic features. Neutrophil synthesis in the bone marrow is immunologically mediated via granulocyte colony stimulating factor (G-CSF) production (Zeidler et al., 2009). Thus, theoretically, high blood neutrophil count could arise from strong immune response, regardless of pathogen load or alternatively might correlate with high mycobacterial load. As we have tried to touch in the above discussion, by assessing the results of both smear microscopy and culture test done on sputum samples, it was striking that high neutrophil count correlated with much more frequent and rapid detection of *M. tuberculosis* in clinical samples. These parameters, in turn, reflect mycobacterial load. A total of 28 patients had direct proof of TB, with *M. tuberculosis* bacilli being detected in sputum sample using culture and smear microscopy. Of these, 22 (78.6%) had a blood neutrophil count $\geq 2.70x10^3$cells/µl.

Thus, we understood that the prognostic value of neutrophil reflects, at least in fraction, mycobacterial load. It is reasonable that higher numbers of bacilli activate greater numbers of macrophages and, in turn, increase secretion of IL-23 thereby upregulating neutrophil synthesis. This is because of the fact that neutrophils are continuously generated in the bone marrow from

myeloid precursors under the control of G-CSF (Zeidler et al., 2009), produced in response to interleukin-17 (IL-17). Release of IL-17 by T cells is in turn under the control of IL-23 originating from tissue-resident macrophages and dendritic cells (Ley et al., 2006). During inflammation the number of neutrophils in blood increases, and with time the cells die apoptotically and are removed by macrophages and dendritic cells. This process results in downregulation of IL-23 synthesis by those cells and therefore reduces G-CSF release (Ley et al., 2006, Stark et al., 2005).

4.1. Limitation of the study

The limitation of this study were confounding may be present including sepsis. Additionally, patient sputum mycobacterial burdens were not directly quantified using colony counts. Also, as this is an observational study, only associations were reported and immunological mechanisms cannot be explored. Taking neutrophilia as ANC>$7.5x10^3$ cells/µl might not be the right for African population generally. Finally, we only assessed the prognostic value of neutrophil at a single time-point and it may be better if measured serially during empiric TB treatment.

4.2. Conclusions and recommendation

In this study, we assessed blood neutrophil counts during routine screening of patients for HIV-associated TB prior to starting ART. Using a rigorous culture-based gold standard for TB diagnosis, we found that PTB disease was associated with increased blood neutrophil counts (ANC$\geq$2.70x10^3cells/µl). Though there is strong correlation between presence of TB in HIV infected individuals and high blood neutrophil count, its likely utility for either rule-in or rule-out TB in HIV-infected patients has been questioned.

The clinical presentation of TB depended on the stage of HIV infection and associated degree of immunodeficiency. PTB positive participants had relatively low CD4 count, low BMI, low hgb concentration and advance WHO HIV stage. Sputum sample with low mycobacterial load had longer culture positivity time and sputum sample with high mycobacterial load had shorter culture positivity time.

In our present study there are higher bloods neutrophil counts among those patients with greater sputum mycobacterial load. To explore the relationship between neutrophil count and mycobacterial load, we compared the results of blood neutrophil count in the high and low mycobacterial load groups. It was striking that sputum samples from the high neutrophil group were far more likely to test positive by smear microscopy. The time to culture positivity of sputum was also significantly shorter. Taken together these data, we strongly suggested that patients with higher neutrophil counts had higher mycobacterial load, which has the tendency to show poor prognosis.

Thus, we suppose that the prognostic value of neutrophil reflects, at least in fraction, mycobacterial load. Since the course of the infection and its epidemiological consequences depend upon the influence of the genetic diversity of the infecting organisms, we can't exclude the contribution of mycobacterial lineage also.

In accordance with these, the following recommendations were forwarded.

1. As this observation supports the role of neutrophils in the host response to HIV-associated TB, similar studies are needed.
2. Studies are needed to understand the role of neutrophils in TB disease with and without concomitant HIV-disease.
3. To better understand the role of mycobacterial lineages on high neutrophil count, studying on high sample size is needed.
4. Using laboratory tests are needed in knowing co-morbid individuals, to reduce confounding factor.
5. Establishing normal range for ANC in our setting is needed, to avoid false low count.

REFRENCES

Abi Abdallah, D. S., Egan, C. E., Butcher, B. A. and Denkers, E. Y. (2011) Mouse neutrophils are professional antigen-presenting cells programmed to instruct Th1 and Th17 T-cell differentiation. *International immunology,* 23**:** 317-326.

American Thoracic Society and Centers for Disease Control and Prevention. (2000) Diagnostic standards and classification of tuberculosis in adults and children. *American Journal of Respiratory and Critical Care Medicine,* 161 (4)**:** 1376-1395.

Ashitani, J. I. (2002) Elevated Levels of α-Defensins in Plasma and BAL Fluid of Patients with Active Pulmonary Tuberculosis. *Chest,* 121**:** 519-526.

Banchereau, J. and Steinman, R. M. (1998) Dendritic cells and the control of immunity. *Nature,* 392(6673)**:** 245- 252.

Behr, M. A. (2002) Different strains, different vaccines? *Lancet of Infectious Diseases,* 2**:** 86-92.

Bisweswar, N. and Behar, S. M. (2011) Regulation of neutrophils by interferon-ϒ limits lung inflammation during tuberculosis infection. *Journal of Expermental Medicine,* 208 (11)**:** 2256-2257.

Blomgran, R. and Ernst, J. D. (2011) Lung Neutrophils Facilitate Activation of Naive Antigen-Specific CD4+ T cells during Mycobacterium tuberculosis Infection. *Journal of Immunology,* 186(12)**:** 7110-7119.

Bloom, B. R. and Cese, M. (1992) Tuberculosis commentary on a reemergent killer. *Science,* 257**:** 1055-64.

Boari, J. T., Vesely, M. A., Bermejo, D. A., Ramello, M. C., Montes, C. L., Cejas, H., et al. (2012) IL-17RA signaling reduces inflammation and mortality during Trypanosoma cruzi infection by recruiting suppressive IL-10-producing neutrophils. *PLoS pathogens, 8*(4)**:** e1002658-e1002658.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D., Weinrauch, Y. and Zychlinsky, A. (2004) Neutrophil Extracellular Traps Kill bacteria. *Science,* 303(5663)**:** 1532-1535.

Brosch, R., Gordon, S. V., Marmiesse, M., Brodin, P., Buchrieser, C., Eiglmeier, K., et al. (2002) A new evolutionary scenario for the Mycobacterium tuberculosis complex. *Proceedings of the National Academy of Sciences,* 99**:** 3684-3689.

Brudey, K., Driscoll, J. R., Rigouts, L., Prodinger, W. M., Gori, A., Al-Hajoj, S. A., et al. (2006) Mycobacterium tuberculosis complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. *BMC microbiology,* 6**:** 23.

Burg, N. D. and Pillinger, M. H. (2001) The neutrophil: function and regulation in innate and humoral immunity. *Clinical Immunology,* 99(1)**:** 7-17.

Center of Disease Control and Prevention. (2008) TB elimination: the difference between latent TB infection and active TB disease.

Clark, S. R., Ma, A. C., Tavener, A. S., Mcdonald, B., Goodarzi, Z., Kelly, M. M., et al. (2007) Platelet Toll-Like Receptor-4 Activates Neutrophil Extracellular Traps to Ensnare Bacteria in Endotoxemic and Septic Blood. *Nature Medicine* 13 (4)**:** 463-9.

Comstock, G. W. (1982) Epidemiology of tuberculosis. *American Review of Respiratory Diseases,* 125**:** 8.

Costantini, C. and Cassatella, M. A. (2011) The defensive alliance between neutrophils and NK cells as a novel arm of innate immunity. *Journal of Leukocyte Biology,* 89**:** 221-233

Cruz, A., Fraga, A. G., Fountain, J. J., Rangel-Moreno, J., Torrado, E., Saraiva, M., et al. (2010) Pathological role of interleukin 17 in mice subjected to repeated BCG vaccination after infection with Mycobacterium tuberculosis. *The Journal of experimental medicine,* 207**:** 1609-1616.

Davey, M. S., Lin, C. Y., Roberts, G. W., Heuston, S., Brown, A. C., Chess, J. A., et al. (2011) Human neutrophil clearance of bacterial pathogens triggers anti-microbial γδ T cell responses in early infection. *PLoS pathogens,* 7**:** e1002040.

De Chastellier, C. (2009) The many niches and strategies used by pathogenic mycobacteria for survival within host macrophages *Immunobiology,* 214(7)**:** 526-542.

De Larco, J. E., Wuertz, B. R. and Furcht, L. T. (2004) The Potential Role of Neutrophils in Promoting the Metastatic Phenotype of Tumors Releasing Interleukin-8. *Clinical Cancer Research,* 10 (15)**:** 4895-900.

De Valliere, S., Abate, G., Blazevic, A., Heuertz, R. M. and Hoft, D. F. (2005) Enhancement of innate and cell-mediated immunity by antimycobacterial antibodies. *Infection and Immunity* 73(10)**:** 6711-20.

Desvignes, L. and Ernst, J. D. (2009) Interferon-gamma-responsive nonhematopoietic cells regulate the immune response to *Mycobacterium tuberculosis*. *Immunity,* 31**:** 974-985.

Dheda, K., Schwander, S. K., Zhu, B., Van Zyl-Smit, R. N. and Zhang, Y. (2010a) The immunology of tuberculosis: From bench to bedside. *Respirology,* 15(3)**:** 433-450.

Dheda, K., Shean, K., Zumla, A., Badri, M., Streicher, E. M., Page-Shipp, L., et al. (2010b) Early treatment outcomes and HIV status of patients with extensively drug-resistant tuberculosis in South Africa: a retrospective cohort study. *The Lancet,* 375**:** 1798-1807.

Ear, T. and Mcdonald, P. P. (2008) Cytokine generation, promoter activation, and oxidant-independent NF-kappaB activation in a transfectable human neutrophilic cellular model *BMC Immunol,* 9**:** 14.

Edwards, S. W. (1994) Biochemistry and physiology of the neutrophil. *Cambridge University Press,* 6**:** ISBN 0-521-41698-1

Eken, C., Gasser, O., Zenhaeusern, G., Oehri, I., Hess, C. and Schifferli, J. A. (2008) Polymorphonuclear neutrophil-derived ectosomes interfere with the maturation of monocyte-derived dendritic cells. *The Journal of Immunology,* 180(2)**:** 817-824.

Eum, S. Y. (2010) Neutrophils Are the Predominant Infected Phagocytic Cells in the Airways of Patients With Active Pulmonary TB. *Chest,* 137**:** 122.

Flesch, I. and Kaufmann, S. H. (1987) Mycobacterial growth inhibition by interferongamma-activated bone marrow macrophages and differential susceptibility among strains of *Mycobacterium tuberculosis*. *Journal of Immunology,* 138(12)**:** 4408-13.

Fridlender, Z. G., Sun, J., Kim, S., Kapoor, V., Cheng, G., Ling, L., et al. (2009) Polarization of tumor-associated neutrophil phenotype by TGF-β:"N1" versus "N2" TAN. *Cancer cell,* 16(3)**:** 183-194.

Frieden, T. R., Sterling, T. R., Munsiff, S. S., Watt, C. J. and Dye, C. (2003) Tuberculosis. *Lancet of Infectious Diseases,* 362**:** 887-899.

Frost, L. S., Leplae, R., Summers, A. O. and Toussaint, A. (2005) Mobile genetic elements: the agents of open source evolution. *Nature Review Microbiology,* 3**:** 722-732.

Fuchs Ta, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, Weinrauch Y, Brinkmann V and Zychlinsky A. (2007) Novel cell death program leads to neutrophils extracellular traps. *The Journal of Cell Biology,* 176(2)**:** 231-241.

Gagneux, S., Deriemer, K., Van, T., Kato-Maeda, M., De Jong, B. C., Narayanan, S. and Pm., S. (2006) Variable host-pathogen compatibility in Mycobacterium tuberculosis. *Proceedings of the National academy of Sciences of the United States of America,* 103(8)**:** 2869-2873.

Gagneux, S. and Small, P. M. (2007) Global phylogeography of *Mycobacterium tuberculosis* and implications for tuberculosis product development. *The Lancet Infectious Diseases,* 7(5)**:** 328-337.

Gordon, S., Brosch, R., Billaut, T., Garnier, K. and Eiglmeier, S. T. (1999) Identification of variable regions in the genomes of tubercle bacilli using bacterial artificial chromosome arrays. *Molecular Microbiology,* 32**:** 643-655.

Groenen, P. M., Bunschoten, A. E., Van Soolingen, D. and Van Embden, J. D. (1993) Nature of DNA polymorphism in the direct repeat cluster of Mycobacterium tuberculosis; application for strain differentiation by a novel typing method. *Journal of Molecular Microbiology,* 10**:** 1057-1065.

Hess, C., Sadallah, S., Hefti, A., Landmann, R. and Schifferli, J. A. (1999) Ectosomes released by human neutrophils are specialized functional units. *Journal of Immunology,* 163**:** 4564-4573.

Hickey, M. J. and Kubes, P. (2009) Intravascular immunity: the host-pathogen encounter in blood vessels. *Nature Reviews Immunology,* 9 (5)**:** 364-75.

Himmel, M. E., Crome, S. Q., Ivison, S., Piccirillo, C., Steiner, T. S. and Levings, M. K. (2011) Human CD4+ FOXP3+ regulatory T cells produce CXCL8 and recruit neutrophils. *European journal of immunology, 41*(2)**:** 306-312.

Huard, B., Mckee, T., Bosshard, C., Durual, S., Matthes, T., Myit, S., et al. (2008) APRIL secreted by neutrophils binds to heparan sulfate proteoglycans to create plasma cell niches in human mucosa. *The Journal of clinical investigation, 18*(8)**:** 2887.

Huard, R. C., Fabre, M., De Haas, P., Lazzarini, L. C. O., Van Soolingen, D., Cousins, D. and John, L. (2006) Novel genetic polymorphisms that further delineate the phylogeny of the Mycobacterium tuberculosis complex. *Journal of Bacteriology,* 188**:** 4271-4287.

Huard, R. C., Lazzarini, L. C., Butler, W. R., Van Soolingen, D. and Ho, J. L. (2003) PCR-based method to differentiate the subspecies of the Mycobacterium tuberculosis complex on the basis of genomic deletions. *Journal of Clinical Microbiology,* 41**:** 1637-1650.

Jacobs, L., Nawrot, T. S., De Geus, B., Meeusen, R., Degraeuwe, B., Bernard, A., et al. (2010) Subclinical responses in healthy cyclists briefly exposed to traffic-related air pollution *Environmental Health,* 9(64)**:** 64.

Johnston, B., Burns, A. R., Suematsu, M., Issekutz, T. B., Woodman, R. C. and Kubes, P. (1999) Chronic inflammation upregulates chemokine receptors and induces neutrophil migration to monocyte chemoattractant protein-1. *Journal of Clinical Investigation, 103*(9)**:** 1269.

Kamerbeek, J., Schouls, L., Kolk, A., Van Agterveld, M., Van Soolingen, D., Kuijper, S., et al. (1997) Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology. *Journal of Clinical Microbiology,* 35**:** 907-914.

Kamp, V. M., Pillay, J., Lammers, J. W. J., Pickkers, P., Ulfman, L. H. and Koenderman, L. (2012) Human suppressive neutrophils CD16bright/CD62Ldim exhibit decreased adhesion. *Journal of leukocyte biology,* 92(5)**:** 1011-1020.

Katoch, M. (2004) Infections due to non-tuberculous mycobacteria. *Indian Journal of Medical research,* 120**:** 290-304.

Kaufmann, S. (2002) Protection against tuberculosis: cytokines, T cells, and macrophages. *Annals of the Rheumatic Disease,* 61**:** 54-58.

Kerkhoff, A. D., Wood, R., Lowe, D. M., Vogt, M. and Lawn, S. D. (2013) Blood Neutrophil Counts in HIV-Infected Patients with Pulmonary Tuberculosis: Association with Sputum Mycobacterial Load. *PloS ONE,* 8(7)**:** e67956.

Kolaczkowska, E. and Kubes, P. (2013) Neutrophil recruitment and function in health and inflammation *Nature Reviews Immunology,* 13**:** 159-175.

Korbel, D. S., Schneider, B. E. and Schaible, U. E. (2008) Innate immunity in tuberculosis: myths and truth. *Microbes and Infection* 10**:** 995-1004.

Korf, J. E., Pynaert, G. and Tournoy, K. (2006) Macrophage reprogramming by mycolic acid promotes a tolerogenic response in experimental asthma. *American journal of respiratory and critical care medicine,* 174(2)**:** 152-160.

Kumar, V. and Sharma, A. (2010) Neutrophils: Cinderella of innate immune system. *International Immunopharmacology,* 10(11)**:** 1330-32.

Law, K. F., Jagirdar, J., Weiden, M. D., Bodkin, M. and Rom, W. N. (1996) Tuberculosis in HIV-positive patients: cellular response and immune activation in the lung. *American journal of respiratory and critical care medicine,* 153 (4)**:** 1377 - 1384.

Lawn, S. D. and Nicol, M. P. (2011) Xpert MTB/RIF assay: development, evaluation and implementation of a new rapid molecular diagnostic for tuberculosis and rifampicin resistance. *Future Microbiology,* 6(9)**:** 1067-1082.

Lee, J., Hartman, M. and Kornfeld, H. (2009) Macrophage apoptosis in tuberculosis. *Yonsei Medical Journal,* 50**:** 1-11.

Ley, K., Smith, E. and Stark, M. A. (2006) IL-17A-producing neutrophil-regulatory Tn lymphocytes. *Immunology Research,* 34**:** 229-242.

Lopez, B., Aguilar, D., Orozco, H., Burger, M., Espitia, C., Ritacco, V. and Dego., V. S. (2003) A marked difference in pathogenesis and immune response induced by different Mycobacterium tuberculosis genotypes. *Clinical and Experimental Immunology,* 133(1)**:** 30-37.

Lowe, D. M., Redford, P. S., Wilkinson, R. J., O'garra, A. and Martineau, A. R. (2012) Neutrophils in tuberculosis: friend or foe? *Trends in Immunology,* 33.

Martineau, A. R., Newton, S. M., Wilkinson, K. A., Kampmann, B., Hall, B. M., Nawroly, N. and Rj, W. (2007) Neutrophil-mediated innate immune resistance to mycobacteria. *Journal of Clinical Investigation,* 117(7)**:** 1988.

Martineau, A. R., Timms, P. M., Bothamley, G. H., Hanifa, Y., Islam, K., Claxton, A. P., et al. (2011) High-dose vitamin D 3 during intensive-phase antimicrobial treatment of pulmonary tuberculosis: a double-blind randomised controlled trial. *The Lancet,* 377**:** 242-250.

Mazurek, G. H., Lobue, P. A. and Daley, C. L. (2001) Comparison of a whole blood interferon gamma assay with tuberculin skin testing for detecting latent Mycobacterium tuberculosis infection. *Journal of American Medical Association,* 286**:** 1740-1747.

Megiovanni, A. M., Sanchez, F., Robledo-Sarmiento, M., Morel, C., Gluckman, J. C. and Boudaly, S. (2006) Polymorphonuclear neutrophils deliver activation signals and antigenic molecules to dendritic cells: a new link between leukocytes upstream of T lymphocytes. *Journal of leukocyte biology,* 79(5)**:** 977-988.

Mócsai, A. (2013) Diverse novel functions of neutrophils in immunity, inflammation, and beyond. *Journal of Expermental Medicine,* 210(7) 1283-1299.

Nahid, P., Bliven, E. E., Kim, E. Y., Mac Kenzie, W. R., Stout, J. E., Diem, L., et al. (2010) Influence of M. tuberculosis lineage variability within a clinical trial for pulmonary tuberculosis. *PLoS One,* 5**:** e10753.

Naidoo, K., Naidoo, K., Padayatchi, N. and Karim, Q. A. (2011) HIV-associated tuberculosis. *Clinical and Developmental Immunology,* 2011**:** 8.

National Collaborating Centre for Chronic Conditions. (2011) Clinical diagnosis and management of tuberculosis, and measures for its prevention and control. *TB clinical guideline*

Nicod, L. P. (2007) Immunology of tuberculosis. *Swiss Medical Weekly,* 137 (25-26)**:** 357-362.

Niemann, S., Richter, E. and Rusch-Gerdes, S. (2000) Differentiation among Members of the Mycobacterium tuberculosis Complex by Molecular and Biochemical Features: Evidence for Two Pyrazinamide-Susceptible Subtypes of M. bovis. *Journal of Clinical Microbiology,* 38**:** 152-157.

Normung, D. I. F. (1986) Medical Microbiology: Diagnosis of Tuberculosis. Part 9: Minimum Requirements for the Identification of the Tubercle Bacilli. *DIN, Beuth Verlag, Berlin, Germany***:** 58943-58949.

Park, J. S., Tamayo, M. H., Gonzalez-Juarrero, M., Orme, I. M. and Ordway, D. J. (2006) Virulent clinical isolates of*Mycobacterium tuberculosis* grow rapidly and induce cellular necrosis but minimal apoptosis in murine macrophages *Journal of Leukocyte Biology,* 79(1)**:** 80-86.

Paton, N. I., Chua, Y. K., Earnest, A. and Chee, C. B. (2004) Randomized controlled trial of nutritional supplementation in patients with newly diagnosed tuberculosis and wasting. *American Journal of Clinical Nutrition,* 80**:** 450-465.

Pelletier, M., Maggi, L., Micheletti, A., Lazzeri, E., Tamassia, N., Costantini, C., et al. (2010) Evidence for a cross-talk between human neutrophils and Th17 cells. *Blood,* 115(2)**:** 335-343.

Pillay, J., Kamp, V. M., Van Hoffen, E., Visser, T., Tak, T., Lammers, J. W., et al. (2012) A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. . *The Journal of clinical investigation,* 122(1)**:** 327.

Pulimood, A. B., Shajan, P., Rook, G. W. A. and Donoghue, H. D. (2008) In Situ PCR for Mycobacterium tuberculosis in Endoscopic Mucosal Biopsy Specimens of Intestinal Tuberculosis and Crohn Disease. *American Journal of Clinical Pathology,* 129**:** 846-851.

Purdy, G. E. and Russell, D. G. (2007) Lysosomal ubiquitin and the demise of*Mycobacterium tuberculosis Cellular Microbiology,* 9(12)**:** 2768-2774.

Raja, A. (2004) Immunology of tuberculosis. *Indian Journal of Medical Research,* 120(4)**:** 213-32.

Rastogi, N., Legrand, E. and Sola, C. (2001) The mycobacteria: an introduction to nomenclature and pathogenesis. *Review scientific technique Office international Epizooties,* 20**:** 21-54.

Reif, H. J. and Saedler, H. (1975) IS1 is involved in deletion formation in the gal region of E. coli K12. *Molecular and General Genetics,* 137**:** 17-28.

Remijsen, Q., Tw', K., Wirawan, E., Lippens, S., Vandenabeele, P. R. and Berghe, T. V. (2011) Dying for a cause: NETosis, mechanisms behind an antimicrobial cell death modality. *Cell Death and Differentiation,* 18**:** 581-588.

Rieder, H. L., Kam, K. M., Kim, S. J. and Chonde, T. M. (2007) Priorities for tuberculosis bacteriology services in low-income countries: International Union Against Tuberculosis and Lung Disease. *Paris, France*.

Rohde, K., Yates, R. M., Purdy, G. E. and Russell, D. G. (2007) *Mycobacterium tuberculosis* and the environment within the phagosome. *Immunological Reviews,* 219(1)**:** 37-54.

Roosnek, E., Burjanadze, M., Dietrich, P. Y., Matthes, T., Passweg, J. and Huard, B. (2009) Tumors that look for their springtime in APRIL. *Critical reviews in oncology/hematology,* 72(2)**:** 91-97.

Ryan, R. C. M., O'sullivan, M. P. and Keane, J. (2011) *Mycobacterium tuberculosis* infection induces non-apoptotic cell death of human dendritic cells *BMC Microbiology,* 11(1)**:** 237.

Sandilands, G. P., Ahmed, Z., Perry, N., Davison, M., Lupton, A. and Young, B. (2005) Cross linking of neutrophil CD11b results in rapid cell surface expression of molecules required for antigen presentation and T-cell activation. *Immunology,* 114**:** 354-68.

Scapini, P., Bazzoni, F. and Cassatella, M. A. (2008) Regulation of B-cell-activating factor (BAFF)/B lymphocyte stimulator (BLyS) expression in human neutrophils. *Immunology Letters,* 116**:** 1-6

Schlesinger, L. S. (1993) Macrophage phagocytosis of virulent but not attenuated strains of *Mycobacterium tuberculosis* is mediated by mannose receptors in addition to complement receptors. *Journal of Immunology,* 150(7)**:** 2920-2930.

Segal, A. W. (2005) How neutrophils kill microbes. *Annual Reviews Immunology,* 23**:** 197-203.

Seiler, P., Aichele, P., Raupach, B., Odermatt, B., Steinhoff, U. and Kaufman, S. (2000) Rapid neutrophil response controls fast-replicating intracellular bacteria but not slow replicating Mycobacterium tuberculosis. *The Journal of Infectious Diseases,* 181(2)**:** 671-680.

Serhan, C. N., Ward, P. A. and Gilroy, D. W. (2010) Fundamentals of Inflammation. *Cambridge University Press***:** p53-54.

Sreevatsan, S., Pan, X. I., Stockbauer, K. E., Connell, N. D., Kreiswirth, B. N., Whittam, T. S. and Musser, J. M. (1997) Restricted structural gene polymorphism in the Mycobacterium tuberculosis complex indicates evolutionarily recent global dissemination. *Proceedings of the National Academy of Sciences,* 94**:** 9869-9874.

Stark, M. A., Huo, Y., Burcin, T. L., Morris M. A., Olson, T. S. and Ley, K. (2005) Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. *Immunity,* 22**:** 285-294.

Swaminathan, S., Padmapriyadarsini, C. and Narendran, G. (2010) HIV-Associated Tuberculosis: Clinical Update. *Clinical infectious diseases,* 50**:** 1377-1386.

Tak, T., Tesselaar, K., Pillay, J., Borghans, J. A. and Koenderman, L. (2013) What's your age again? Determination of human neutrophil half-lives revisited. *Journal of Leukocyte Biology,* 94 (4)**:** 595-601.

Thierry, D., Brisson-Noel, A., Vincent-Levy-Frebault, V., Nguyen, S., Guesdon, J. and Gicquel, B. (1990) Characterization of *Mycobacterium tuberculosis* Insertion Sequence, IS6110, and Its Application in Diagnosis. *Journal of Clinical Microbiology,* 28**:** 2668-2673.

Thomas, R. F., Sterling, T. R., Munsiff, S. S., Watt, C. J. and Dye, C. (2003) Tuberculosis. *The Lancet,* 362**:** 887-899.

Tian, T., Woodworth, J., Sk¨Old, M. and Behar, S. M. (2005) In vivo depletion of CD11c+ cells delays the CD4+ T cell response to *Mycobacterium tuberculosis* and exacerbates the outcome of infection. *Journal of Immunology,* 175(5)**:** 3268- 3272.

Toossi, Z., Mayanja-Kizza, H., Lawn, S. D., Hirsch, C. S., Lupo, L. D. and Butera, S. T. (2007) Dynamic variation in the cellular origin of HIV type 1 during treatment of tuberculosis in dually infected subjects. *AIDS research and human retroviruses,* 23**:** 93-100.

Truffot-Pernot, C., Veziris, N. and Sougakoff, W. (2006) Modern diagnosis of tuberculosis. *Presse Med,* 35**:** 1739-1746.

Tsuda, Y., Takahashi, H., Kobayashi, M., Hanafusa, T., Herndon, D. N. and Suzuki, F. (2004) Three different neutrophil subsets exhibited in mice with different susceptibilities to infection by methicillin-resistant Staphylococcus aureus. *Immunity,* 21(2)**:** 215-226.

Ulrichs, T. and Kaufmann, S. H. E. (2006) New insights into the function of granulomas in human tuberculosis. *Journal of Pathology,* 208**:** 261-269.

Unaids. (2007) TB-HIV: Networking for Policy Change. *A Participant's Guide*.

Van Crevel, R., Ottenhoff, T. H. M. and Van Der Meer, J. W. M. (2002) Innate immunity to Mycobacterium tuberculosis. *Clinical Microbiology Review,* 15 (2)**:** 294 - 309.

Wartha, F., Beiter, K., Albiger, B., Fernebro, J., Zychlinsky, A., Normark, S. and Henriques-Normark, B. (2007) Capsule and d-alanylated lipoteichoic acids protect Streptococcus pneumoniae against neutrophil extracellular traps. *Cellular microbiology,* 9(5)**:** 1162-1171.

Waugh, D. J. and Wilson, C. (2008) The interleukin-8 pathway in cancer. *Clinical Cancer Research,* 14 (21)**:** 6735-41.

Wayne, L. G., Good, R. C., Krichevsky, R. C., Blacklock, Z., David, H. L., Dawson, D., et al. (1991) Fourth report of the cooperative, open-ended study of slowly growing mycobacteria by the International Working Group on Mycobacterial Taxonomy. *International Journal of Systemic Bacteriology,* 41**:** 463-472.

Wheater, P. R. and Stevens, A. (2002) Wheater's basic histopathology: a colour atlas and text. *Edinburgh: Churchill Livingstone***:** ISBN 0-443-07001-6.

WHO. (2009) Guidelines for surveillance of drug resistance in tuberculosis. *WHO/HTM/TB***:** 422.

WHO. (2014) Global Tuberculosis Control. *WHO Report*.

Witko-Sarsat, V., Rieu, P., Descamps-Latscha, B., Lesavre, P. and Halbwachs-Mecarelli, L. (2000) Neutrophils: molecules, functions and pathophysiological aspects. *Laboratory Investigation,* 80 (5)**:** 617-53.

Wondimeneh, Y., Muluye, D. and Belyhun, Y. (2012) Prevalence of Pulmonary tuberculosis and immunological profile of HIV co-infected patients in Northwest Ethiopia. *BMC Research Notes,* 5**:** 331.

Woodworth, J. S. and Behar, S. M. (2006) *Mycobacterium tuberculosis*-specific CD8+ T cells and their role in immunity. *Critical Review Immunology,* 26(4)**:** 317-52.

Yousefi, S., Mihalache, C., Kozlowski, E., Schmid, I. and Simon, H. U. (2009) Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death and Differentiation,* 16(11)**:** 1438-1444.

Zeidler, C., Germeshausen, M., Klein, C. and Welte, K. (2009) Clinical implications of ELA2-, HAX1-, and G-CSF-receptor (CSF3R) mutations in severe congenital neutropenia. *Brtain Journal of Haematology,* 144**:** 459-467.

Zhang, Y., Broser, M. and Cohen, H. (1995) Enhanced interleukin-8 release and gene expression in macrophages after exposure to Mycobacterium tuberculosis and its components. *Journal of Clinical Investigation,* 95 (2)**:** 586 - 592.

Zucker-Franklin, D., Greaves, M. F., Grossi, C. E. and Marmont, A. M. (1988) *"Neutrophils". Atlas of Blood Cells: Function and Pathology 1 (2nd ed.).* Philadelphia Lea & Ferbiger.

ANNEXS

Annex 1: Participant Information Sheet

Date ----------

Title: Blood neutrophil count in HIV positive individuals with suspected pulmonary tuberculosis and; its association with sputum mycobacterial burden and mycobacterial lineages.

Background: Tuberculosis is an infectious disease that causes considerable morbidity and mortality. Globally, 9 million people are estimated to develop TB each year, and yearly 1.5 million deaths result from the disease. Ethiopia ranks 11th among the world's 22 high-burden TB countries. TB is common infectious disease associated with HIV.

Objective of the study: To determine neutrophil counts in peripheral blood of HIV positive individuals with suspected pulmonary tuberculosis and to find out the association with mycobacterial burden and mycobacterial lineage.

Organizations: The study will be conducted by Addis Ababa University, School of Graduate studies. Laboratory procedure is to be carried out at ALIHB laboratory.

Procedures: Expert (in the area) laboratory technologist will collect the sample and laboratory investigation will be performed by principal investigation. In case if you are unable to produce enough samples you will be excluded from the study.

Participation: The procedure is to be carried out after getting your willingness to participate. All eligible volunteer HIV patients with sign and symptom of TB will be included.

Risks associated with sample collection: There is no expected pain or problem during sputum sample collection, there may be mild pain during blood collection from your vein.

Benefit: As different study shows, most of the time, HIV is a risk factor for TB, so you will be benefited from the study; because it will be part of your diagnosis and might be a key to your current and/or your future problem if it will come up with positive result.

Compensation: You will receive your result through your physician. You will get treatment for free if you become positive for TB. Fees for transport and elapsed time will also be compensated.

Confidentiality: From medical ethics point of view and research ethics, every part of your personal information will be kept confidentially. Information to be collected and variables expressing your identity will be coded secretly. The only responsible person to link your variables

(important for your follow up and treatment) with the code number is the principal investigator. However, other researchers can see your clinical information, which is without your identity. Your result and information will be used only for the mentioned purpose.

Sharing the Result: Eventually, the result, devoid of your identity, will be reported through publication or by other means. Have no suspicion on the confidentiality of your information, even at this time. We request your permission to use the result for reporting.

Right to refuse or withdraw: Thus, it is your right to agree or to refuse to participate in the study. Withdrawal from the study is also possible, at any time. Withdraw or refusing to participate will not have any impact on your normal diagnosis or medical follow up. You can address your problem or question through the addresses given bellow.

Contact Addresses: Negeri Debela, Address: Addis Ababa University, College of Health Science, department of Medical Microbiology, Immunology and Parasitology.

Mobile: +251912656272

E-mail – negideb@gmail.com

Thank you for your patience and kindness!

Annex 2: Informed Consent Form

Name ----------, Age ----------, Sex -------

Identification No ----------, lab No ----------

I read and/or well informed about the nature of the study, entitled **"**Blood neutrophil count in HIV positive individuals with suspected pulmonary tuberculosis and its association with sputum mycobacterial burden and mycobacterial lineages.**"**

Finally, she/he told me that this will be certain if I agree on the following points and signed bellow.

a) I understood the objective of the study

b) I understood that the sample won't be used for farther study, and after completion of the whole procedure, the leftover sample will be discarded safely

c) I am aware of any information describing my identity, collected using questionnaire and, won't be disclosed

d) I understood report of my result won't include my name

e) I understood that I won't get money for being part of the study except compensation for transportation and elapsed time if only the result become positive.

f) I clearly informed as I have the right to refuse to participate and withdraw (if I change my idea) from the study at any time

g) I understood that my refusal to take part in this study won't have impact to the normal diagnosis and to my future medical follow up.

I have had enough time to think over it freely and I understood it well. I found it would have positive impact in the investigation of my case. My agreement to this consent is without any external enforcement, and will be confirmed by my signature, below.

The information sheet was given/ explained by: ----------, signature ---------, phone ----------

Name of participant: ----------, signature ----------, phone ------

Annex 3. Questionnaire

Part 1: Socio-demographic characteristics of PTB suspected HIV patients in Zewoditu-memorial hospital and Federal police hospital, Addis Ababa**,** Ethiopia from January to July 2014			
Variables	Coding categories		Remarks
1.1. Identification number	_____		
1.2. Age (years)			
1.3. Sex(don't ask)	1. Male 2. Female		
1.4. Marital status	1. Single 2. Married 3. Divorced 4. Widowed		
1.5. Education level	1. Illiterate 2. Elementary school 3. Secondary school 4. Higher education		
1.6. Occupation	1. Employed 2. House wife 3. Farmer 4. Merchant 5. Laborer		
Part 2: Clinical data of tuberculosis suspected HIV patients in Zewoditu-memorial hospital and Federal police hospital, Addis Ababa**,** Ethiopia from January to July 2014			
Variables	Categories		Remarks
2.1.TB constitutional symptoms	cough >2 weeks duration	1.yes 2.no	
	fever	1.yes 2.no	
	night sweat	1.yes 2.no	
	weight loss	1.yes 2.no	
2.2. WHO HIV stage	I		
	II		
	III		
	IV		
2.3. CD4 count (cells/µl)	____		
2.4. Body mass index(kg/m^2)	____		

DECLARATION

I, the undersigned, declare that this MSc research thesis is my original work. It has not been for a degree in any other university. False statements cause the invalidation of this research thesis and may lead to other administrative or legal actions.

Principal Investigator:

Negeri Debela (BSc) Address: E-mail – negideb@gmail.com

Signature: _____

Advisors:

Tamrat Abebe (MSc, PhD) Signature: _____

AAU, College of health Science, department of Medical Microbiology and Immunology

Adane Mihiret (DVM, PhD) Signature: _____

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