# Assessment of Specific Immune Responses to Tuberculosis in HIV Infected Patients before and during Highly Active Antiretroviral Therapy (HAART)


**By**

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## ABBREVIATIONS

| | |
|---|---|
| 3TC | Lamivudine |
| AAU | Addis Ababa University |
| ABC | Abacavir |
| AHRI | Armauer Hansen Research Institute |
| AIDS | Acquired Immunodeficiency Syndrome |
| ALERT | All African Leprosy, Tuberculosis Rehabilitation, Research and Training center |
| ALT | Alanine amino transferase |
| AMV-RT | Avian Myeloblastosis Virus Reverse Transcriptase |
| AP | Alkaline phosphatase |
| APCs | Antigen presenting cells |
| ART | Antiretroviral Therapy |
| AST | Aspartate amino transferase |
| CBC | Complete Blood Cell Count |
| CD | Cluster of differentiation |
| CFP-10 | Culture filtrate protein-10 |
| CFU | Colony forming unit |
| CTLs | Cytotoxic T Lymphocytes |
| D4T | Stavudine |
| DDI | Didanosine |
| DOTS | Directly Observed Treatment |
| DTH | Delayed type hypersensitivity |
| EC | ESAT-6 & CFP-10 |
| ECP | ESAT-6, CFP-10 & PPD |
| EFV | Efavirenz |
| EHNRI | Ethiopian Health and Nutrition Research Institute |
| ELISPOT | Enzyme Linked Immunospot |
| ESAT-6 | Early Secretory Antigenic Target-6 |
| FBS | Fetal Bovine Serum |

| | |
|---|---|
| FDA | Food and Drug Administration Authority |
| FRPC | Faculty Research Publications Committee |
| FTC | Emtricitabine |
| HAART | Highly active Antiretroviral Therapy |
| HAPCO | HIV/AIDS Prevention & Control Office |
| HIV | Human Immunodeficiency Virus |
| IFN-γ | Interferon Gamma |
| IL | Interleukin |
| iNOS | Inducible nitric oxide synthase |
| INSHI | International Network for the study of HIV-associated IRIS |
| IPT | Isoniazid Preventive Treatment |
| IQR | Inter quartile range |
| IRIS | Immune Reconstitution Inflammatory Syndrome |
| MOH | Ministry of Health |
| NO | Nitric Oxide |
| $NOS_2$ | Nitric Oxide Synthase |
| NRTI (s) | Nucleoside Reverse Transcriptase Inhibitor (s) |
| NtRTI (s) | Nucleotide Reverse Transcriptase Inhibitor (s) |
| NVP | Nevirapine |
| PBMC | Peripheral Blood Mononuclear Cell |
| PBS | Phosphate Buffer Saline |
| PBS-T | Phosphate Buffer Saline plus Tween 20 |
| PHA | Phytohemagultinin |
| PI (s) | Protease Inhibitor (s) |
| PLWHA | People living with HIV/AIDS |
| PMTCT | Prevention of Mother-to-Child Transmission |
| PPD | Purified protein derivative |
| P-Y | Person-Year |
| R10 | RPMI with 10% FBS |
| RPMI | Roswell Park Memorial Institute |

| | |
|---|---|
| RT | Room temperature |
| SFC | Spot forming cell |
| TB | Tuberculosis |
| TB/HIV | TB and HIV |
| TDF | Tenofovir |
| TGF-β | Transforming growth factor Beta |
| TLC | Total lymphocyte count |
| TNF-α | Tumor necrosis factor Alpha |
| TST | Tuberculin skin test |
| UNAIDS | The Joint United Nations programme on HIV/AIDS |
| VCT | Voluntary counseling and testing |
| WHO | World Health Organization |
| ZDV | Zidovud |

**ABSTRACT**

**BACKGROUND:** HAART greatly reduces the risk of developing tuberculosis in HIV-infected persons. However, individuals who initiate HAART may still be reported as having TB, either because they are developing active TB due to persistent immunodeficiency or because they have sub clinical TB that becomes apparent in the immune reconstitution inflammatory syndrome (IRIS). Despite the severity of the problem, little information is available on the extent to which HAART restores TB specific immune response or CD4 cell and characteristics of patients who develop TB while taking HAART and proportion of immune reconstitution syndrome associated with TB.

**OBJECTIVES:** To assess specific immune response to tuberculosis in HIV-1 infected patients before and during HAART and to assess the characteristics of patients who developed ART associated TB and to determine the incidence rate of TB IRIS.

**METHODS:** In a longitudinal prospective cohort study, 177 study participants naive to HAART were enrolled and followed for six months after starting HAART at ALERT Hospital, Addis Ababa Ethiopia. The study period was from June 2006 to September 2008. Blood samples were collected before initiation of HAART (at baseline), at 3$^{rd}$ and 6$^{th}$ month of HAART and at occurrence of suspected TB IRIS. T lymphocyte sub set enumerated and the immune response to tuberculosis was assessed *in vitro*, using ELISPOT assay in PBMC stimulated with TB antigens (PPD, ESAT-6, and CFP-10), and *in vivo* using tuberculin skin test (TST) at the specified time points.

**RESULTS**

**Recovery of TB specific immune response:** The proportion of TST positive responses increased significantly from baseline 17.5% to 26.8% at 3$^{rd}$ month and to 28.9% at the 6$^{th}$ month (p= 0.02). TST response increased significantly among groups with CD4 cell count <50 cells/mm$^3$ when the group is stratified according to baseline CD4 cell count and among group with active TB at baseline investigation when the group is stratified according to baseline TB status. Quantitative IFN-γ ELISPOT response to TB antigens (PPD, ESAT-6 and CFP-10) also increased after HAART in the whole cohort. Median SFC/million to PPD

and ESAT-6 significantly increased among group with baseline CD4<50 cells/$mm^3$ when the group is stratified according to baseline CD4 cell count. Although the difference was not statistically significant (except for quantitative ELSPOT response to PPD), the proportion of TST response and median SFC/million ELISPOT response to TB antigens after HAART were also higher in those with baseline CD4 ≥100 cells/$mm^3$ than < 100 cells/$mm^3$.

**ART associated TB, unmasking and paradoxical TB IRIS:** the incidence rate of ART associated TB in our cohort was 12.7 cases /100 PY during the first 6 months of HAART. The majority (62.5%) of TB cases occurred within the first month of HAART. Baseline TST response > 5 mm induration, baseline hemoglobin < 12 gm/dl and being male were the strongest predictors of occurrence of TB during HAART. Only 3/8 (37.5%) episodes of TB were presented as unmasking tuberculosis IRIS. The proportion of paradoxical IRIS was 9.6 % (3/31) and the incidence rate was 22.5 cases per 100 persons per year.

**CONCLUSIONS AND RECOMMENDATIONS:** The majority of the study participants attained CD4 cell counts above the zone of severe immunodeficiency within the first 3 months of treatment. However, the relative risk of attaining CD4 cell > 200 cells/$mm^3$ decreased significantly with decreasing baseline CD4 cell count. Thus, individuals who initiated HAART with CD4 < 50 cells/$mm^3$ are likely to have high risk of morbidity and mortality for longer period. This underscores the need of designing strategy to identify and treat patients before they get into a stage of profound CD4 lymphcytopenia. Our study indicates that TB specific T cell repertoire may not be totally lost in HIV infected individuals with CD4 < 50 cells/ $mm^3$ and their levels can still be improved by HAART. Our findings provide useful baseline information for further research to assess overall potential of HAART in the restoration of immunity to tuberculosis. Finally, in this study, we observed a high rate of tuberculosis in our cohort during the initial months of HAART indicating the need for careful screening for TB before initiation of HAART. More sensitive and specific diagnostic methods for TB may assist in early diagnosis. In addition, our study confirmed that a sub set of patients who developed TB during HAART manifested as IRIS.

Key words: *Tuberculosis, CD4 cell count, IRIS, HAART, immune response and Ethiopia.*

# CHAPTER 1. INTRODUCTION

## 1.1 General Introduction

Human immunodeficiency virus (HIV) is the most important factor fuelling the tuberculosis (TB) epidemic in populations with high HIV prevalence (WHO, 2004b). This is because of the impaired cell-mediated immune responses associated with HIV, which results in enhanced susceptibility to tuberculosis and rapid progression of latent tuberculosis to active disease. In addition, HIV infection may also have an indirect effect on the incidence of TB by increasing transmission rates of *Mycobacterium tuberculosis* (*M. tuberculosis*), with negative consequences for both HIV-negative and HIV positive persons (Odhiambo *et al.*, 1999; Sonnenberg *et al.*, 2004). Thus, in communities with high HIV rates, TB rates have been risen dramatically even where effective TB control strategies like Directly Observed Treatment short-course (DOTS) is available. This underscored the need to improve TB control wherever HIV-coinfection is common. In response to this, the World Health Organization (WHO) has developed a strategic framework with the goal of integrating TB and HIV/AIDS control programmes. One of the elements of this strategic framework is the widespread use of HAART as effective tool to TB control at the community level in countries with either an overlapping TB or HIV epidemic (WHO, 2003a).

Currently, the widespread use of HAART in developing countries has been shown to reduce the risk of treated HIV-infected persons developing TB by 70%–90%, compared with untreated individuals (Badri *et al.*, 2002; Girardi *et al.*, 2000; Jones *et al.*, 2000; Santoro-Lopes *et al.*, 2002). However, despite major reductions in TB incidence among individuals receiving HAART, TB risk remains elevated among those receiving treatment. Recent studies conducted in developing countries have shown that among individuals who initiated HAART, 4.8–17.6 cases per 100 person-years reported having TB, either because they are developing active TB or sub clinical TB may become apparent as the immune reconstitution inflammatory syndrome (Bonnet *et al.*, 2006). This ongoing burden of tuberculosis during HAART is emerging as a substantial problem in Sub Saharan countries where there is an overlapping TB/HIV co-epidemic. Therefore, assessing immune response to tuberculosis in HIV infected individual during HAART and identification of the characteristics of HIV-

infected persons who still develop tuberculosis in countries where there is large availability of HAART may provide further insight into the potential effects of the widespread use of antiretroviral therapy on the tuberculosis/HIV epidemics, and help to design control interventions. In light of this, this prospective cohort study was conducted to assess immune response to tuberculosis before and after initiation of HAART in HIV infected patients using different immune parameters such as *in vivo* PPD based tuberculin skin test and *in vitro* IFN-γ ELISPOT assay in PBMC stimulated with ESAT-6/CFP-10 and PPD antigen.

### *1.2* Mycobacterium tuberculosis

*Mycobacterium tuberculosis* belongs to the family Mycobacteriaceae, order Actinomycetales and genus Mycobacterium. There are three species under this genus and these include *M. tuberculosis complex*, the non-tuberculosis mycobacteria and *M. leprae* (Shinnick and Good, 1994). *M. tuberculosis complex*, mycobacteria that causes tuberculosis in human includes *M. tuberculosis, M. bovis, M. africanum, M. microtti* and *M. canetti*. These are genetically closely related sub-species where repetitive DNA elements such as insertion sequence IS 6110 and direct repeat have been found to be restricted to the *M. tuberculosis complex*. Of the pathogenic species belonging to the *M. tuberculosis complex*, the most frequent and important agent of human disease is *M. tuberculosis* (van Soolingen et al., 1997).

*M. tuberculosis* is rod-shaped, non-motile and non-sporulated. It has high lipid content in the wall, probably the highest in proportion among all bacteria. The complete genome of the mycobacterial strain H37Rv has been sequenced and is known to contain 4,411,529 base pairs, about 4000 genes with a G+C content of 65.6% (Cole *et al.*, 1998). Mycobacteria, including *M. tuberculosis*, are often neutral on Gram's staining. However, once stained, the bacilli cannot be decolorized by acid alcohol, a characteristic justifying their classification as acid-fast bacilli. Acid fastness is due mainly to the organisms' high content of mycolic acids, long-chain cross-linked fatty acids, and other cell-wall lipids. In the mycobacterial cell wall, lipids (e.g., mycolic acids) are linked to underlying arabinogalactan and peptidoglycan. This structure confers very low permeability of the cell wall, thus reducing effectiveness of most antibiotics. Another molecule in the mycobacterial cell wall,

lipoarabinomannan, is involved in the pathogen-host interaction and facilitates the survival of *M. tuberculosis* within macrophages (Mario, 1995).

The important protein characteristic of *M. tuberculosis* include those in purified protein derivative (PPD) tuberculin, a precipitate of non-species-specific molecules obtained from filtrates of heat-sterilized, concentrated broth cultures (Manchester, 1984; Mario, 1995). The other important proteins are early secretory antigen target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10). They are encoded by the RD1 genomic segment of *M.tuberculosis*, which is absent from all BCG strains and the vast majority of environmental mycobacteria. Thus, ESAT-6 and CFP-10 can be used in enzyme-linked immunospot (ELISPOT) assays to differentiate between*M. tuberculosis* infection and immune sensitization by BCG vaccination or exposure to environmental mycobacteria (Ritacco *et al.*, 2007).

### 1.3 Pathogenesis of *M. tuberculosis*

*M tuberculosis* infections occur by airborne transmission of droplet nuclei containing a few viable, virulent organisms produced by a sputum-positive individual. The bacilli are deposited in the alveolar spaces of the lungs, where they are phagocytosed by alveolar macrophages (McMurray, 2000). On entry into a host macrophage, *M. tuberculosis* and other intracellular pathogens initially reside in a phagosome. Pathogenic mycobacteria escape hostile environment by inhibition of phagosome-lysosome fusion and prevent acidification of the phagolysosome, perhaps by modulating the activity of a membrane proton pump (Armstrong and Hart, 1975). Pathogenic mycobacteria also prevent phagosomal maturation and stay in early endosome. Although it is not clear how the blocking of endosomal maturation is essential for *M. tuberculosis s*urvival in macrophages, it has been postulated that a selective advantage to *M. tuberculosis* staying in an early endosome is that, there would be less host immuno-surveillance by CD4 T cell (Smith, 2003).

Later, infected macrophages in the lung, through their production of chemokines, attract monocytes, lymphocytes, and neutrophils (van Crevel *et al.*, 2002), none of which kill the bacteria very efficiently (Fenton and Vermeulen, 1996). Then, granulomatous focal lesions composed of macrophage derived giant cells and lymphocytes begin to form. This process is

generally an effective means of containing the spread of the bacteria. As the cellular immunity develops, macrophages loaded with bacilli are killed and this results in the formation of the caseous center of the granuloma surrounded by a cellular zone of fibroblasts, lymphocytes, and blood-derived monocytes. Although *M. tuberculosis* bacilli are postulated to be unable to multiply within this caseous tissue due to its acidic pH, the low availability of oxygen, and the presence of toxic fatty acids, some organisms may remain dormant but alive for decades. This enclosed infection is referred to as latent or persistent TB and can persist throughout a person's life in an asymptomatic and non transmissible state. The strength of the host cellular immune response determines whether an infection is arrested here or progresses to the next stages (Smith, 2003).

In persons with efficient cell-mediated immunity, the infection may be arrested permanently in granuloma. The granulomas subsequently heal, leaving small fibrous and calcified lesions. However, if an infected person cannot control the initial infection in the lung or if a latently infected person's immune system becomes weakened by immunosuppressive drugs, HIV infection, malnutrition, aging, or other factors, the granuloma center can become liquefied by an unknown process and then serves as a rich medium in which the previously contained bacteria can revive and replicate in an uncontrolled manner. At this point, viable *M. tuberculosis* can escape from the granuloma and spread within the lungs (active pulmonary TB) and even to other tissues via the lymphatic system and the blood (miliary or extrapulmonary TB). When this happens, the person becomes infectious and requires Anti-TB therapy to survive (Dannenberg, 1982)

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### 1.4 Epidemiology of Tuberculosis

#### 1.4.1 Global Situation

Tuberculosis remains a major public health problem worldwide. Almost one third of the world's population is infected with *M. tuberculosis*. It is the foremost cause of death from a single infectious agent in adults**,** which kills a person in the world at every 15 seconds

(Aliyu and Salihu, 2003; Dye *et al.*, 2005). Figure 1.1 shows estimated worldwide TB incidence rates from data collected in 2006, there were an estimated 9.2 million new cases of TB, of which 709 000 (8%) were HIV-positive. This is an increase from 9.1 million cases in 2005, reflecting population growth in Asia, Africa and Europe. The countries that rank first to fifth in terms of absolute numbers of TB cases are India, China, Indonesia, South Africa and Nigeria, while Africa has the highest incidence rate per capita and accounts for 12 of the 15 countries with the highest TB incidence rates. There were an estimated 1.7 million deaths due to TB in 2006, of which 0.2 million were among HIV-positive people (WHO, 2008).

### 1.4.2 Ethiopian Situation

Ethiopia stands on the 7$^{th}$ rank of the world's top 22 tuberculosis (TB) high-burden countries with an estimated annual incidence of 379 cases and prevalence of 643 cases per 100,000 populations (WHO, 2008) and the prevalence of HIV among TB patients is up to 41% (Demissie *et al.*, 2000a; Yassin *et al.*, 2004). The high burden of TB in Ethiopia might in part be attributed to the rapid increase of HIV infection, because available data indicate that HIV/ AIDS accounted for an estimated 32% or 141,000 total TB cases in 2005 (MOH, 2007).

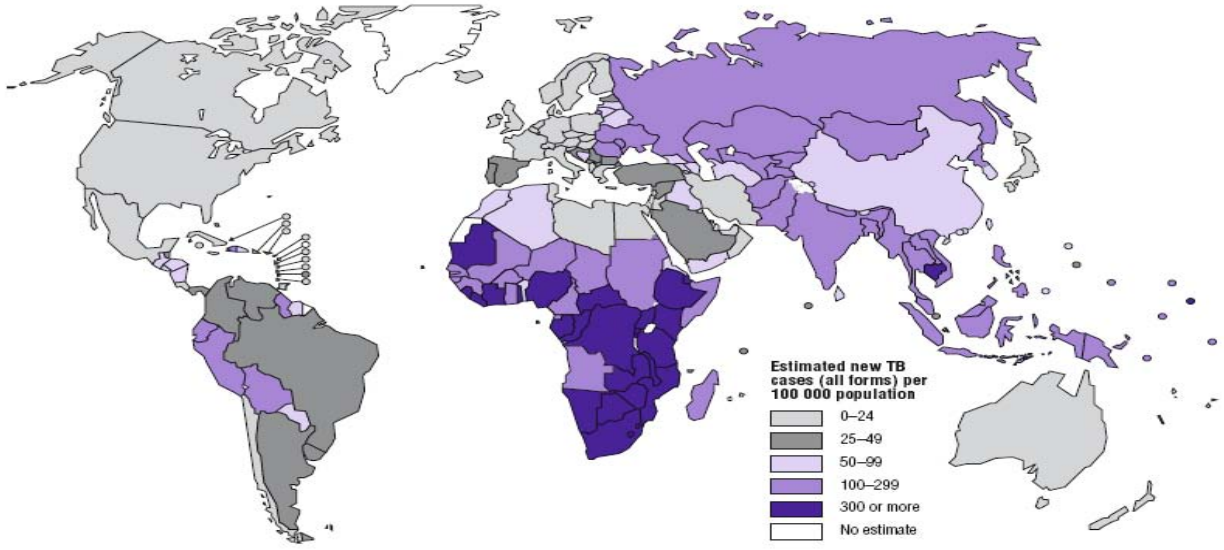



**Figure 1.1 Estimated worldwide TB incidence rates from data collected in 2006 (Adapted from WHO, 2008)**

## 1.5 Immunity to Tuberculosis

The immune responses against tuberculosis are complex involving both innate and adaptive arms of the immune system. It plays a fundamental role in the outcome of *M. tuberculosis* infection. It is clear that the immune system reacts efficiently in the vast majority of infections. This is particularly evident in the case of TB, where most people infected by the tubercle bacillus (~ 90 %) never develop the disease throughout their lifetime. Nevertheless, the risk of developing the disease increases considerably when TB infection co-exists with an alteration in the immune system, such as co-infection with HIV (Hernández-Pando, 2007).

### 1.5.1 Innate immunity

The innate immune response to tuberculosis comprises several different cells types and molecules, has its own receptor system to recognize the presence of pathogens and is a key to the initiation of an adaptive immune response in the host. The phagocytosis and the subsequent secretion of IL-12 are processes initiated in the absence of prior exposure to the antigen and hence form a component of innate immunity. The other components of innate immunity are natural resistance associated macrophage, neutrophils, natural killer cell (NK) etc (Raja, 2004).

### 1.5.2 **Adaptive immunity**

As for other intracellular infections, the primary protective immune response is cells mediated rather than antibody mediated (Mackaness, 1964). This has been demonstrated by remarkably increased risk of TB in patients with reduced cell mediated immunity, such as in those infected with HIV or individuals undergoing immunosuppressive therapy, compared to patients with impaired humoral immunity, such as those with multiple myeloma, who show no increased predisposition to tuberculosis (Barnes *et al.*, 1993).

a) **$CD4^+$ T cells**

*M. tuberculosis* resides primarily in a vacuole within the macrophage, and thus, major histocompatibility complex (MHC) class II presentation of mycobacterial antigens to CD4 T cells is an obvious outcome of infection. These cells are most important in the protective response against *M. tuberculosis*. Murine studies with antibody depletion of CD4 T cells or

the use of gene-disrupted mice have shown that the CD4 T cells is required for control of infection (Muller *et al.,* 1987). In humans, the remarkable susceptibility of patients with AIDS to TB has demonstrated the critical role of CD4 lymphocytes in protective immunity. The dually infected individual (PPD skin test–positive, HIV-1–infected) in most cases is infected with *M. tuberculosis* before HIV-1 and has a 170-fold increased risk (5%–10%/year) of reactivation of TB. This indicates that in the absence of immuno surveillance by CD4 cells, 5%–10% of persons with latent foci of TB have potential to break down and reactivate each year (Ellner, 1997).

The main function of CD4+ T cell is the production of cytokines including IFN-γ, which activates macrophages and promotes bacterial destruction. Recently, another function has been ascribed to these cells, i.e., helping to develop the CD8+ T cells mediated response (Serbina *et al.,* 2001). In the same way, CD4+ T cells may participate in the induction of apoptosis of infected cells and the subsequent reduction of bacterial viability through the CD95 Fas ligand system (Oddo *et al.,* 1998). In addition, CD4+ T cells might prevent reactivation of latent tuberculosis. This role could be associated with their function in maintaining organized granulomatous structure. Depletion of CD4+ T cells from the chronically infected mice could result in the loss of structural integrity of the tuberculous granuloma thus leading to TB reactivation (Scanga *et al.,* 2000).

b) **$CD8^+$ T cells**

The participation of CD8+ T cells in the control of TB infection is well recognized. Mice deficient in molecules such as CD8α, transporter associated with antigen processing (TAP), and perforin, were shown to be more susceptible to *M. tuberculosis* infection than animals which produced these molecules (Behar *et al.,* 1999). The mechanisms used by these cells for the control of TB seem to be mainly cytokine production and bacterial lysis. In addition, $CD8^+$ T cells have the potential to affect antimycobacterial immunity in a number of ways. For instance $CD8^+$ T cells are a source of type 1 cytokines such as IFN-$\gamma$ and TNF-α that help to activate macrophages for killing of the intracellular bacilli (Hernández-Pando, 2007).

c) **Gamma/delta T cells** ($\gamma\ \delta$ T cells)

Gamma/delta T cells (γ δ T cells) also participate in the innate immune response to TB. Murine studies have indicated that the induction of $\gamma\ \delta$ T cells in the immune response against TB precedes that of conventional CD4 and CD8 cells and hence plays an important role in modulating the effector response against tuberculosis. Following infection, the early recruitment of cells to the lung is mediated by the chemokines, CXCL2 and CXCL10, and the cytokine, IL-12 released by macrophages and dendritic cells in the lungs (Ferrero *et al.*, 2003). Once activated, $\gamma\ \delta$ T cells secrete IFN$\gamma$ and TNF$\alpha$. The productions of these cytokines strengthen the bactericidal capacity of macrophages by induction of NOS2. Recently, it has been shown that $\gamma\ \delta$ T cells secrete IL 17 in response to IL 23 secreted by dendritic cells, thereby implicating them as a main player in the resistance against infection at the initial stage (Lockhart *et al.*, 2006). The role of γδ T cells in protective immunity is not limited to cytokine secretion (such as IL-17 and IFN-γ) and cytotoxic activity. These cells also behave as antigen- presenting cells. Like dendritic cells, they can process and efficiently present antigens and give the co-stimulating signals needed to induce proliferation of αβ T cells. As noted, γδ T cells act as a link between the innate immune response and the adaptive immune response (Brandes *et al.*, 2005).

### 1.5.3 **Immunopathology**

Several studies have demonstrated that excessive Th2 cytokine production and release of prostaglandin E and TGF-β is implicated in the pathogenesis of TB. There is substantial evidence that demonstrates an increased expression of Th2 cytokines, especially IL-4, in patients with advanced pulmonary TB. IL-4 messenger ribonucleic acid (mRNA) and T-cells containing IL-4 are increased in human pulmonary TB. This high IL-4 expression correlates significantly with serum IgE, serum soluble CD-30, and also with the extent of activation (Lienhardt *et al.*, 2002; Seah *et al.*, 2000). It has been demonstrated that CD8+ cells are another source of IL-4, and this correlates with cavitation (van Crevel *et al.*, 2000). The presence of IL-4 at late stages of the disease has a direct pathogenic role because it downregulates the protective Th1 responses (Biedermann *et al.*, 2001)

An increased in expression of Th2 cytokines has been demonstrated to influence the effect of some cytokines. For instance, TNF-α plays an essential role in protection but may also be

a significant factor in its pathology. When TNF-α released into a relatively pure Th1-mediated inflammatory site it may act merely as a supplementary macrophage activating cytokine. However, when released into a mixed Th1/Th2 site with high IL-4 concentration, it causes damage. This has been demonstrated in a study that used a Balb/c mice model of progressive pulmonary TB. In the early stage of infection (21 days after infection), while the Th1 cytokine response predominated and controlled the growth of bacilli, the delayed-type hypersensitivity (DTH) response was the highest and DTH sites were not vulnerable to necrosis by TNF-α. In contrast, during the progressive phase of the disease (50 days after infection) extensive tissue damage and high IL-4 production are manifested, the DTH response was very low, and TNF-α administration in the DTH sites provoked extensive inflammation with necrosis (Hernandez-Pando and Rook, 1994; Hernandez-Pando *et al.*, 2004).

Anther cytokine associated with pathogenesis of human and murine TB is TGF-β. It is a potent cell-mediated immune response suppressant and anti-inflammatory cytokine, which has also been implicated in the pathogenesis of TB. Blood mononuclear cells from TB patients were shown to release increased levels of TGF-β (Toossi *et al.*, 1995) and Balb/c mice infected by the intratracheal route showed very high expression of TGF-β during the progressive phase of the infection. Treatment with recombinant β-glycan (type III TGF-β receptor) a potent inhibitor of TGF-β, caused increased expression of IFN-γ and IL-2, with strong downregulation of IL-4, and a significant reduction in lung bacterial counts to an extent similar to that achieved by conventional antimicrobial treatment (Hernandez-Pando *et al.*, 2006).

Another factor that deregulates the protective immune response against TB is prostaglandin production. Prostaglandins, in particular PGE-2, are potent mediators of intercellular communication. Indeed, at high concentrations, PGE-2 is immunosuppressive for T-cell-mediated immunity (Phipps *et al.*, 1991). When prostaglandin production was suppressed in animals suffering from advanced disease, a significant reduction of pneumonia and bacillary load, with a striking increment in the size of the granuloma was seen, and the expression of IFN-γ, TNF-α and iNOS was also improved. Therefore, PGE-2 is a significant factor participating in the pathogenesis of pulmonary TB and has contrasting functions depending

on its concentrations. During the early phase of the infection, the low PGE-2 concentrations contribute to iNOS expression, permitting the temporal control of bacterial growth; while the high PGE-2 concentrations during the late phase contributes to the downregulation of cell-mediated immunity, allowing disease progression (Rangel *et al.,* 2002).

Adrenal steroids may also contribute to the dysfunction of Th1 responses in TB. Reactivation or progression of infection is sensitive to activation of the hypothalamic-pituitary adrenal axis. The exposure of humans to the stress of war or poverty (Spence *et al.*, 1993), or cattle to the stress of transportation, is efficient in causing reactivation of latent infection. In mice, it has been demonstrated that this is due to glucocorticoid release (corticosterone in mice) (Brown *et al.*, 1995) which reduces macrophage activation and Th1-cells activity (Daynes *et al.*, 1991), while synergizing with some Th2 functions (Rook *et al.*, 1994).

Above all, an increase in antigen load is clearly a precipitating factor in deregulation of immune response, as shown by the striking linkage of the Th1/Th2 balance to the dose after immunization with particulate antigens such as mycobacteria. Thus, low antigen loads, such as the low dose of *M. vaccae* ($10^7$ CFU) used to presensitize mice, or the relatively low bacterial lung burden during early infection in the Balb/c model of progressive pulmonary TB, prime the Th1 response. In contrast, high antigen loads, for example the $10^9$ CFU of *M. vaccae* or the high bacillary loads produced in the lungs during the progressive phase of the Balb/c model, efficiently induce the Th2 response (Hernandez-Pando and Rook, 1994).

### 1.6 HIV Trend and Interaction with tuberculosis

#### 1.6.1 Global Trend

In 2007, globally an estimated 33.2 million (30.6–36.1 million) persons living with HIV, a total of 2.5 million (1.8–4.1 million) persons newly infected and about 2.1 million (1.9–2.4 million) adult and child deaths occurred due to AIDS. Although percentage prevalence has stabilized, continuing new infections (even at a reduced rate) contribute to the estimated number of persons living with HIV, being greater than ever before as shown in the Figure 1.2, HIV prevalence tends to reduce slowly over time as new infections decline and through the death of HIV-infected persons; it can increase through continuing HIV incidence and

through reduced mortality of HIV-infected persons on antiretroviral treatment (UNAIDS/WHO, 2007).

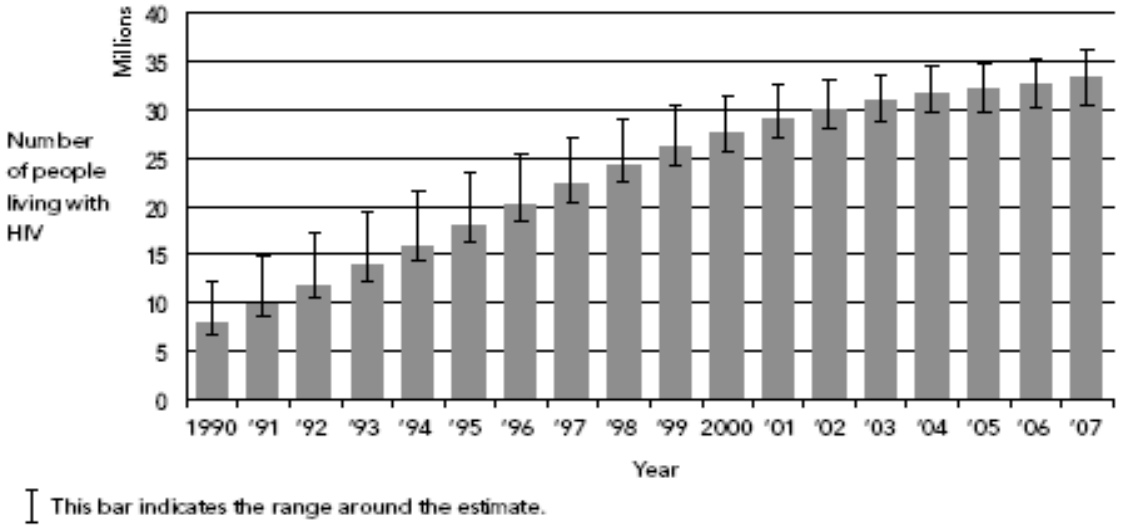



**Figure 1.2 Estimated number of people living with HIV globally, 1990–2007 (Adapted from UNAIDS/WHO, 2007)**

Sub-Saharan Africa remains the most affected region in the global AIDS epidemic. More than two thirds (68%) of all HIV-positive people live in this region where more than three quarters (76%) of all AIDS deaths in 2007 occurred. It is estimated that 1.7 million (1.4 million–2.4 million] people were newly infected with HIV in 2007, bringing to 22.5 million (20.9 million–24.3 million) the total number of people living with the virus. About 1.6 million (1.5 million–2.0 million) adult and child deaths occurred and there are an estimated 11.4 million (10.5 million–14.6 million) orphans due to AIDS in this region. Unlike other regions, the majority of people living with HIV in sub-Saharan Africa (61%) are women (UNAIDS/WHO, 2007)

### 1.6.2 National Trend

In Ethiopia, the 2005 Demographic and Health Survey, national adult HIV prevalence was estimated to be 1.4%, with infection levels highest in the Gambela (6%) and Addis Ababa (4.7%) regions (Central Statistical Agency & ORC Macro, 2006). However, the prevalence is a little higher according to the estimates made based on reports taken from voluntary counseling and testing (VCT) centers, blood banks, and ART programs: the national HIV

prevalence in 2005 was 3.5%; 3 % among males and 4% among females. The estimated prevalence in urban areas was 10.5% (9.1% and 11.9% among males and females respectively) and 1.9% in rural areas (1.7% among males and 2.2% among females). Ethiopia's epidemic stabilized in urban areas in 1996–2000, after which HIV infection levels declined slowly, notably in parts of the capital, Addis Ababa. In rural Ethiopia, where the majority of the population resides, the epidemic has remained relatively stable since HIV prevalence peaked in 1999–2001 (FOMOH/HAPCO, 2006).

Although the HIV/AIDS epidemic has stabilized, it continues to pose a threat to the development of Ethiopia where 1.3 million people are living with the virus, 744,100 are orphaned due to AIDS, and AIDS accounted for an estimated 34% of all young adult deaths (15-49 yrs) in Ethiopia and 66.3% of all young adult deaths (15-49 yrs) in urban Ethiopia. Moreover its poses serious threats to TB control. According to FMOH/HAPCO report HIV/AIDS accounted for about 32% of the estimated 141,000 total TB cases in 2005. The effect of HIV on TB is expected to continue from 2005 through 2010. As shown in the Figure 1.3, bars indicate the estimated number of TB cases and the line denotes the proportion of TB cases due to AIDS per 100 TB cases.

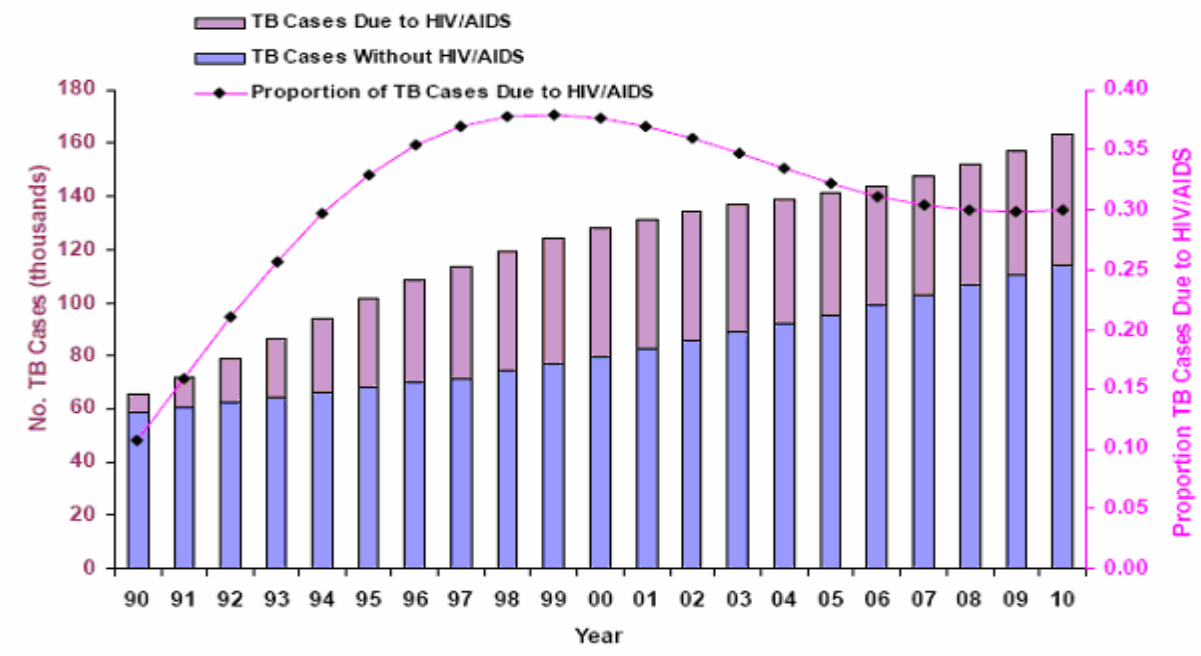



**Figure 1.3 Estimated and Projected TB Cases due to HIV/AIDS, 1990-2010 (source FOMOH/HAPCO, 2006)**

### 1.6.3 Interaction of TB and HIV

HIV and TB form a lethal combination, each speeding the other's progress. HIV is the most important factor fuelling the tuberculosis (TB) epidemic in populations with high HIV prevalence (WHO, 2004b). Approximately 0.7 million cases of TB attributable to HIV infection occur worldwide each year, and tuberculosis is the leading cause of death among people living with HIV. Among 1.7 million deaths caused by TB in 2006, 0.2 million occurred among people living with HIV/AIDS. The prevalence of HIV in incident TB patients in Africa has been reported to be about 22% (WHO, 2008) and the incidence of TB is more than 8 times higher in HIV-positive than in HIV negative people (Corbett *et al.*, 2006). In Ethiopia, TB/HIV co-infection is high, about 19 % - 45% of sputum-smear-positive pulmonary TB patients were reported to be HIV positive in same areas (Demissie, M. *et al.*, 2000b; Yassin *et al.*, 2004) .

**a) Effect of HIV on TB**

HIV infection impairs cell-mediated immunity. The impaired immunity leads to increased number of cases of primary TB and reactivation of TB in HIV-infected people (Hoffmann, Christian, 2007a). Patients with HIV and latent tuberculosis infection have an extremely high rate of development of active tuberculosis, perhaps as much as 10%/year, as opposed to a 10% lifetime risk in otherwise healthy persons with latent tuberculosis infection (Schluger and Rom, 1998). This high rate of development of active tuberculosis may be directly related to the loss of interferon gamma (IFN-$\gamma$)–producing CD4+ T cells, because this cytokine has been demonstrated to be of major importance in the human host response against tuberculosis (Huebner *et al.*, 1993). In addition, several impaired function of host response has been documented in HIV patients. For example, impaired receptor mediated phagocytosis and oxidative burst in macrophage and impaired interaction of T cells with antigen presenting cells (APCs) in the context of MHC class II has been shown in HIV patients (Lawn *et al.*, 2005b). In addition to increasing individual susceptibility to TB following *M. tuberculosis* infection, a high burden of HIV-associated TB cases also expands *M. tuberculosis* transmission rates at the community level, threatening the health and survival of HIV-negative individuals as well. In several countries, HIV has been associated

with epidemic outbreaks of TB. Many of the reported outbreaks involved multidrug-resistant (MDR) strains, which respond poorly to standard therapy (Aaron *et al.*, 2004; Corbett *et al.*, 2003; WHO, 2006a, b).

**b) Effect of TB on HIV**

*M. tuberculosis* enhances HIV replication, accelerating the natural evolution of HIV infection (Rosas-Taraco *et al.*, 2006). *M. tuberculosis* is potent activator of the immune system, which in turn leads to profound changes in the cytokine network, providing favorable environment for viral replication (Toossi *et al.*, 1999). Several studies have demonstrated increased expression of CCR5 and/or CXCR4, major chemokine HIV co-receptors, as well as their chemokine ligands, in human monocyte derived macrophages, alveolar macrophages, and CD4+ T cells in the course of *in vivo* and *in vitro M. tuberculosis* infection (Fraziano *et al.*, 1999; Wolday *et al.*, 2005). The expression of these co-receptors seems to depend on the activation state of these cells which then further amplifies viral replication by enhancing entry into uninfected cells. In addition, proinflammatory cytokine production by tuberculous granulomas (in particular TNFα) has been associated with increased HIV viraemia, which might accelerate the course towards severe immunosuppression (Garrait *et al.*, 1997). Tuberculosis also increases mortality. Study shows, the risk of death in HIV-infected patients with TB is twice that in HIV-infected patients without TB with matched CD4 cell counts, with most deaths caused by progressive HIV infection, rather than TB (Whalen *et al.*, 1995).

## 1.7 Highly Active Antiretroviral Therapy and Immunological Recovery

### 1.7.1 Highly Active Antiretroviral Therapy (HAART)

Highly active antiretroviral therapy (HAART) has changed the scenery of HIV infection since 1996, with the introduction of combined antiretroviral treatment. HIV infected individuals are living longer and AIDS has become a chronic and manageable disease (Steven *et al.*, 2003). Currently, 25 single or combination preparations from four classes of drugs are licensed four HIV treatment: nucleoside (NRTIs; Abacavir (ABC), Didanosine (ddl), Lamivudine (3TC), Stavudine (d4T), Zidovudine (ZDV), Emtricitabine (FTC)) and nucleotide analogs (NtRTIs, Tenofovir (TDF)); protease inhibitors (PIs: Lopinavir (LPV),

ritonavir (RTV, r), Nelfinavir (NFV) ) and fusion inhibitors (Enfuvirtide (T-20), Maraviroc (MVC) (Hoffmann and Mulcahy, 2007b).

World health organization (WHO) recommends a combination of 2 NRTIs+1NNRTI as first-line regimen for resource poor countries. The use of five-formulary approach (d4T or ZDV) +3TC+ (NVP or EFV) gives four possible combinations and provides options for substitution of toxicity. Each of the possible combinations are equally potent. Protease inhibitors (PI) are suggested in case resistance to NNRTIs is known to exist. HIV-2 and HIV-1 group O are often resistant to NNRTIs-based regimens (WHO, 2003b). In Ethiopian ART guideline as shown in Table 1.1, ABC and TDF are included in the first line regimen to expand the choice and tailor to specific patient needs. ABC is used in triple NRTI regimen in rare patients with TB and pregnancy, or other situations when the standard first-line regimens may not be given. Both ABC and TDF are reserved for the second-line regimen. However, in situations where other NRTI cannot be used or causes adverse effects or intolerance, they can still be considered in the first-line regimen (FMOH/HAPCO, 2007).

**Table 1.1 First Line ARV Regimens in Adolescents and Adults (adopted from FMOH/HAPCO, 2007)**

| **Recommended ARV Regimens for Adults and Adolescents: One of the following should be used unless there are contraindications:** |
|---|
| **ZDV/3TC/NVP or EFV** |
| **D4T/3TC/NVP or EFV** |
| **TDF/3TC/NVP or EFV selective setting** |
| **ABC/3TC/NVP or EFV selective setting** |

In treatment failure, the entire first line drugs should be changed to a second line regimen. The second-line drugs should include at least three new drugs, one or more of them from a new class. This increases the likelihood of treatment success and minimizes the chance of cross-resistance. The second-line regimen requires a switch from (d4T or ZDV) +3TC+ (NVP or EFV) to (TDF or ABC) +ddI+ (LPV/r or SQV/r) (WHO, 2003b) (Table 1.2)

**Table 1.2 Second-Line ARV Regimens in Adolescents and Adults (adopted from FMOH/HAPCO, 2007)**

| First - line Regimen | Second line Regimen (during treatmetn failure) |
|---|---|
| D4T or ZDV<br>+<br>3TC<br>+<br>NVP or EFV | DdI[a] or TDF<br>+<br>ABC<br>+<br>LVP/r b OR SQV/r[b] or NFV or IND/r |
| a *Didanosine alone must be taken on an empty stomach, at least one hour before or at least 2hr after (< 50% absobed after) a meal. Tableets should be dissolved in at least 30ml of water, on other liquids may be used to dissolve the tablets. The enteric coated version will not need to be dissolved.*<br>b *LPV/r and SQV/r require secure cold chain. Therefore, NFV can be considered as an alternative in resource-limited settings with out cold chain.* | |

### 1.7.2 Immunological recovery

a) **Recovery of CD4 cell**

After initiation of HAART, plasma viral load is reduced by more than 90% within the first 1–2 weeks, which is accompanied by CD4 lymphocyte count increases in two principal phases (Pakker *et al.*, 1997). The first phase of rapid increase in the number of circulating CD4 lymphocytes can usually be detected within 1–2 weeks of starting treatment and extends over the first 2–3 months of treatment. This increase largely represents a redistribution of activated CD45Ro memory cells previously sequestered in lymphoid tissue and a reduction in apoptotic cell death (Carcelain *et al.*, 2001; Lederman, M. M., 2001). A second phase of gradual increase follows, approaching stable CD4 cell counts at 4–6 years. This second phase represents expansion of naive CD45Ra cell as thymic function is restored and is largely responsible for the long-term sustained rise in CD4 lymphocyte count (Pakker *et al.*, 1998).

The factors that determine CD4 cell recovery are only partly known and depend on both the host and the virus. Several studies demonstrated that CD4 cell recovery in patients who maintain a good control of viral load with antiretroviral therapy are associated with age and baseline CD4+ T-cell counts (Kaufmann *et al.*, 2002; 2005). On the other hand some studies have shown that the increase of CD4+ T-cell count is independent from baseline CD4+ T-cell count at which HAART started (Gulick *et al.*, 2000; Tarwater *et al.*, 2001). Nevertheless, achieving a CD4 cell count over specific thresholds (eg, 200 cells/$mm^3$)

depends on baseline CD4 cell count and may take substantially longer in patients who initiate antiretroviral therapy at lower values (Kaufmann *et al.*, 2000a; 2000b).

b) **Recovery of pathogen specific immune response**

A number of studies have described improvement in immune function during HAART. In general, *in vitro* T-cell proliferative responses to recall antigens and mitogens improve during treatment (Autran *et al.*, 1997; Li *et al.*, 1998) and cytokine responses shift from a predominantly type 2 to a type 1 pattern, with increases in IFN-γ and IL-2 production (Hardy *et al.*, 2003; Imami *et al.*, 1999; Martinon *et al.*, 1999). Later increases in circulating CD45RA naive lymphocytes are associated with reversal of HIV-associated defects in the T-cell receptor (TCR) repertoire (Connors *et al.*, 1997; Gorochov *et al.*, 1998) and restoration of immune responses to neoantigens (Valdez *et al.*, 2000). Delayed type hypersensitivity (DTH) responses to antigens assessed by skin testing also improve and correlate with the magnitude of viral load reduction (Wendland *et al.*, 1999).

Qualitative and quantitative recovery of pathogen-specific cellular and humoral immune responses are observed among a number of organisms including mycobacteria, cytomegalovirus, Epstein-Barr virus, hepatitis B and C virus, and *Candida albicans,* but not for HIV-1 itself (Rinaldo *et al.*, 1999). In observational and randomized prospective clinical studies, it has been proven safe to discontinue primary and secondary prophylaxis against a variety of pathogens—eg, *Pneumocystis carinii, Mycobacterium avium* complex (MAC) infections, and *Cryptococcus neoformans*—when CD4 cell count has reached a minimum of at least 200 cells per μL for 3–6 months. Secondary prophylaxis against cytomegalovirus was even successfully discontinued at 75 CD4 cell per μL (Battegay *et al.*, 2006).

c) **Recovery of tuberculosis specific immune response**

It has been demonstrated that suppression of HIV-1 replication by antiretroviral therapy results in restoration of tuberculosis specific immune response. The mechanism how suppression of HIV-1 replication with HAART improves immune function is unclear. Three possibilities have been proposed to underline this mechanism (Hengel *et al.*, 2002):

1. HIV-1 induces *M. tuberculosis* -specific CD4 T cell or antigen-presenting cells anergy (Hazenberg *et al.*, 2000; Lempicki *et al.*, 2000).When HAART reverses this anergy, CD4 T cells are then free to expand and / or differentiate into cytokine-producing cells (Hengel, 2002).

2. HIV-1 infection, by increasing immune activation, causes trapping of *M. tuberculosis*-specific CD4 T cells in secondary lymphoid tissue. When HAART reverses this trapping, CD4 T cells are redistributed from lymphoid tissues and are then free to expand and / or differentiate into cytokine-producing cells (Autran *et al*., 1997; Bucy *et al.*, 1999).

3. HIV-1 depletes *M. tuberculosis* -specific CD4 T cells as quickly as they arise, through HIV-1-related cell death. In this model HIV-1 "uncouples" pathogen-driven exponential cell expansions (Hengel *et al.*, 2001; Ho *et al.*, 1995). When HAART reverses this depletion, CD4 T cell are free to expand and/ or differentiate into cytokine-producing cells (Hengel *et al.*, 2002). Regardless of the actual mechanism, increased *M. tuberculosis* burden, brought on by HIV-1 disease, likely contributes to increased *M. tuberculosis* specific cells expansions after HAART (Narita *et al.*, 1998).

Although the mechanism by which HAART restores tuberculosis specific immune response is unclear, several studies have demonstrated restoration of tuberculosis specific immune response using different approach; one approach is using Peripheral Blood Mononuclar Cell (PBMC) proliferation assay. Studies conducted using this assay have shown a trend towards increased proliferation of PBMC stimulated *in vitro* with PPD during HAART; the proportion of positive responders increases steeply during the first 6 months (Autran *et al*., 1997; Wendland *et al.*, 1999) and is sustained at 12 months (Li *et al*., 1998). In a prospective cohort of 10 patients followed up at 2-monthly intervals over 1 year, also demonstrated that trends toward increased proliferation of PBMCs in response to *M. tuberculosis*-specific stimuli. However, responses at 1 year were markedly less than those among HIV-negative control groups with either positive or negative PPD skin test responses. Furthermore, production of IL-2 and IL-12 increased minimally (Schluger *et al.*, 2002).

A second assay of antigen-specific CD4 T cells immunity measures the elaboration of intracellular cytokine expression measured at the level of a single CD4 T cell, using flow

cytometry (Waldrop *et al.*, 1997). With this assay 13 patients prospectively studied during HAART for 1 year, using expression of the lymphocyte activation marker CD69 following *in vitro* stimulation of PBMC with PPD as a correlate of *M. tuberculosis* -specific immune responses. In a majority of patients, the percentage of CD4 and CD8 cells responding to PPD increased during the first year of HAART. However, although substantial increases in CD4 cell count were observed in all patients, a small group of patients failed to restore these responses after 1 year of HAART. These patients were more likely to have baseline CD4 cell counts of $<50 \times 10^6$ cells/mm$^3$ compared to those who had good restoration of responses (Hsieh *et al.*, 2000). This suggests that delaying initiation of HAART in chronic HIV infection may lead to long-term impairment of functional immune responses to *M. tuberculosis* (Hsieh *et al.*, 2000).

The third is a novel approach of an in-vitro model that that was developed to measures the growth of reporter-gene tagged mycobacteria (bacillus Calmette-Gue´rin; BCG lux) in whole blood as a functional read-out of antimycobacterial immune responses (Kampmann *et al.*, 2000). Using this method, a study evaluated prospectively a cohort of 15 BCG-vaccinated HIV-infected children for in-vitro antimycobacterial immune responses before and during the first year of HAART. They found that after HAART blood from children showed rapid and sustained reconstitution of specific antimycobacterial immune responses, measured as the decreased growth of mycobacteria (Kampmanna *et al.*, 2006).

Although, restoration of *M. tuberculosis* specific immune responses have been demonstrated using different approaches, little information is available on the quantitative restoration of *M. tuberculosis* specific IFN-γ secreting cells during HAART. In a study conducted using intracellular cytokine expressions assay in single patient with disseminated *M. tuberculosis* and HIV infection who started anti –TB treatment and HAART concurrently. The proportion of peripheral blood CD4 T cell expressing *M. tuberculosis* -specific IFN-γ increased from 8.6% at 11 days to 11% at 33 days and 33% at 95 days after starting HAART (Hengel et *al*., 2002). Another study also conducted using IFN-γ ELISPOT assay in 19 HIV-TB co-infected patients who were receiving both anti –TB treatment and HAART. The frequency of IFN-γ spot forming cell (SFC) increased in 7 patients who experienced IRIS, from median 44 SFC/$10^6$ PBMC at baseline to 2970 SFC/$10^6$ PBMC at

occurrence IRIS (Bourgarit et *al*., 2006). Both studies did not address whether the observed expansion of *M. tuberculosis* specific T cells represent an effect of HAART or an effect of Anti-TB treatment. Further studies are needed to determine the extent to which *M. tuberculosis* specific T cell clones are restored during HAART.

### 1.8 Immune Reconstitution Inflammatory Syndrome (IRIS)

Immune reconstitution inflammatory syndrome (IRIS) in HIV-infected patients is an adverse consequence of the restoration of pathogen-specific immune responses during the initial months of HAART. Previously subclinical infections are "unmasked" or pre-existing opportunistic infections clinically deteriorate as host immunopathological inflammatory responses are "switched on'' (Lawn *et al.*, 2005c). IRIS has been associated with a range of opportunistic infections, including cytomegalovirus, *Pneumocystis carenii*, *Cryptococcus neoformans*, herpesviruses, varicella zoster virus (VZV), hepatitis B virus (HBV) and hepatitis C virus (HCV), progressive multifocal leucoencephalopathy (caused by JC virus), leishmaniasis, and cerebral toxoplasmosis (French *et al*., 2004; Shelburne, S. A., 3rd *et al.*, 2002). Non infectious, including Sarcoid IRIS and autoimmune IRIS (systemic lupus erythematosus, polymyositis, rheumatoid arthritis, Guillain– Barre syndrome, Graves' disease) are the least frequently observed IRIS (French *et al.*, 2004) .

#### 1.8.1 Tuberculosis associated immune reconstitution inflammatory syndrome

IRIS associated with tuberculosis may manifest as either an inflammatory 'unmasking' of a previously untreated infection, or as the paradoxical clinical deterioration of an infective process for which the patient is on appropriate antimicrobial therapy. Patients who have tuberculosis that is unrecognized prior to ART initiation may present with accelerated and inflammatory features of tuberculosis soon after ART initiation (the unmasking form) (Goldsack *et al.*, 2003; John *et al.*, 2005). Patients receiving tuberculosis treatment may manifest with recurrence or worsening of their symptoms after starting ART (termed 'paradoxical deterioration') (Lawn *et al.*, 2005c).

a) **Paradoxical tuberculosis IRIS**

Paradoxical tuberculosis IRIS manifests in patients who are diagnosed with tuberculosis prior to starting ART and are typically improving on tuberculosis treatment. After starting

ART, they develop recurrent, worsening or new clinical or radiological manifestations of tuberculosis (Lawn *et al.*, 2005c; 2007a). Paradoxical reactions are thought to be due to intensification of cell-mediated immune responses and may be associated with conversion of pretreatment cutaneous anergy to PPD to a positive response following antituberculosis treatment (Markman and Eagleton, 1981). In those with active tuberculosis, proinflammatory and immunosuppressive immune mechanisms are present concomitantly, the balance of which might be altered during the early stages of antituberculosis treatment (Vanham *et al.*, 1997).

It has been reported that after initial clinical improvement, paradoxical worsening of disease develops in up to 13- 36% of patients treated with concurrent anti-tuberculosis treatment and HAART (Manosuthi *et al.*, 2006; Narita et al., 1998). The most commonly reported manifestations of *M. tuberculosis* -associated IRIS were fever, lymphadenopathy, and worsening respiratory symptoms. Lymphadenopathy was the most frequent manifestation, occurring in 61 (71%) of the patients. Lymphadenopathy with or without overt lymphadenitis was most frequently peripheral (cervical, supraclavicular, axillary, or inguinal) (Lawn *et al.*, 2005c). Worsening radiographic pulmonary infiltrates occurs in up to 45% of patients. Central nervous system (CNS) involvement with tuberculoma or tuberculous meningitis carries the worst prognosis (Murdoch *et al.*, 2007).

The risk of paradoxical reactions is strongly associated with starting HAART within the first 2 months of tuberculosis treatment (Breen *et al.*, 2004; Navas *et al.*, 2002). Other factors associated with paradoxical IRIS include extrapulmonary and disseminated tuberculosis, a lower CD4 lymphocyte count and higher viral load before starting HAART, and a greater reduction in viral load and greater CD4 increment during HAART(Breton *et al.*, 2004; Michailidis *et al.*, 2005).

b) **Unmasking tuberculosis IRIS**

Unmasking tuberculosis IRIS is less well defined. This manifests in patients who have unrecognized tuberculosis on starting ART, which then manifests with a particularly accelerated or in- flammatory presentation or course during immune reconstitution (Breen *et al.*, 2005; Goldsack *et al.*, 2003; John *et al.*, 2005). This term does not refer to all patients who develop tuberculosis while receiving ART. In high tuberculosis incidence settings, in

particular, reactivation and re-infection of tuberculosis that is not related to ART-mediated immune reconstitution commonly occurs in patients on ART. Indeed, many cases may occur because of persistent immunodeficiency (French, 2007).

The mechanisms underlying development of TB during HAART is complex. Three possibilities have been proposed to underline this mechanism. (1) Shortening of the time for sub clinical TB to become symptomatic (a phenomenon often referred to as ‘‘unmasking’’), (2) increased rapidity of initial onset of TB symptoms, and (3) heightened intensity of clinical manifestations. It has been suggested that the term ‘‘ART-associated TB’’ to be used to refer collectively to all cases of TB presenting during ART and ‘‘immune reconstitution disease’’ or ‘’ Immune reconstitution inflammatory syndrome’’ to be used to refer to the subset of ART-associated TB cases in which the effect on disease severity results in exaggerated and overtly inflammatory disease (Lawn *et al.*, 2008).

Although the mechanism underlying development of TB is heterogeneous, high rates of tuberculosis have been reported in individual receiving HAART, especially in the initial months of treatment in ART programmes in resource-limited settings (Lawn *et al.*, 2006a). Little information is available on the relationship between risk of TB after starting HAART and immunological response to HAART. Studies are needed to determine the incidence risk factor and immunological characteristic of HAART associated TB and Paradoxical IRIS.

**1.9 Significance of the Study**

In Ethiopia, tuberculosis remains one of the major causes of morbidity and mortality. According to the MOH hospital statistics data, tuberculosis is the leading cause of morbidity, the third cause of hospital admission, and the first cause of hospital death in Ethiopia. The ongoing HIV-epidemic is an important contributing factor in the growing caseload and presents a massive challenge to the control of tuberculosis (MOH, 2008).

To combat the growing burden of tuberculosis in Ethiopia, the new TB/ HIV collaborative activities implementation guideline was developed in 2005. During 2005 and 2006, the TB/HIV collaborative activities were scaled-up in many sites of the country, reaching the actual number of over 338 implementing sites (FMOH, 2007). One of the elements of this strategy is Isoniazid preventive treatment (IPT) with the aim to reduce the risk of developing

active TB among HIV Infected individuals. The other element of this strategy is the hope that the widespread use of HAART will prove an effective tool in TB control at the community level (WHO, 2003a, 2004b)

Widespread use of HAART in developing countries has been demonstrated to reduce the incidence of TB among HIV infected individuals receiving HAART (Badri *et al.*, 2002; Girardi *et al.*, 2000; Jones *et al.*, 2000; Santoro-Lopes *et al.*, 2002). Nonetheless, HIV-associated tuberculosis continues to occur in countries where HAART is widely used. Recent studies conducted in developing countries have shown that up to 17.6 cases per 100 person-years reported having TB among individuals who initiated HAART (Bonnet *et al*., 2006). The high burden of tuberculosis during HAART may be attributed to baseline immune status at which HAART is initiated. Existing data indicate that the ongoing risk of TB during HAART is much greater among those with low nadir CD4 cell counts and advanced clinical stage of disease (Seyler *et al.*, 2005).

Understanding the extent of reconstitution of tuberculosis specific immune response after HAART in relation to baseline immune status and assessing the occurrence of tuberculosis after HAART in developing countries like Ethiopia may be critical to maximize the potential benefit of HAART to TB control measures. In light of this, the present study was undertaken to assess immune response to tuberculosis before and after initiation of HAART in HIV infected patients using different immune parameters such as *in vivo* PPD based tuberculin skin test and *in vitro* IFN-γ ELISPOT assay in PBMC stimulated with ESAT-6/CFP-10 and PPD antigens. The information obtained from the study may help in the management of patients with HIV and active / latent tuberculosis. Moreover, it may provide useful information that can optimize the potential benefit of HAART to tuberculosis control. In addition, this study in Ethiopia is the first and preliminary one to assess immune response to tuberculosis after HAART in HIV infected patients and may thus provide baseline information for future research in this area.

## 1.10 HYPOTHESIS

The extent of tuberculosis specific immune restoration in HIV patients depends on the baseline immune status at which HAART is started.

## 1.11 OBJECTIVES OF THE STUDY

### General objective

To assess specific immune response to tuberculosis in HIV-1 infected patients following initiation of HAART.

### Specific objectives

- To compare the different immune parameters in HIV infected patients before and during HAART:
  - Proportion of Tuberculin Skin test reactivity
  - Frequency of IFN-γ spot forming cell in PBMC stimulated with PPD, ESAT-6 and CFP-10 (IFN-γ ELISpot assay)
  - CD4 T lymphocyte cell count
- To assess the incidence, risk factor & and immune response characteristics of tuberculosis IRIS (ART associated TB).

## CHAPTER II. MATERIALS AND METHODS

### 2.1 Study Design

A longitudinal prospective cohort study was conducted on informed and consented study participants naive to HAART recruited from ALERT Hospital, enrolled and followed up for six months after starting HAART. The study period was from June- 2006 to September 2008.

### 2.2 Study Area

The study was conducted at ALERT hospital (All Africa TB and Leprosy Eradication, Rehabilitation and Training Center) in Addis Ababa, Ethiopia. The hospital has been providing free ART since 2004. The hospital is one of the 329 currently operational ART sites in Ethiopia. By May 10, 2008; 5621 HIV infected individuals had started ART at ALERT hospital (MOH-HAPCO, 2008).

### 2.3 Study Population

The study participants included consecutive HIV-1 infected patients who were enrolled at ALERT Hospital ART program and recruited by the following inclusion and exclusion criteria:

**Inclusion criteria**

1. At least 18 years of age and not older than 55 years
2. Naive to HAART
3. Willing to give informed consent
4. Resides in Addis Ababa and is available for follow up at ALERT ART center for at least six months after enrolment

**Exclusion criteria**

1. Starting HAART before the commencement of the study
2. Receiving immunosuppressive treatment such as corticosteroids.
3. Known or suspected history of diabetes mellitus or lymphoma,or leukemia, hemophilia or malignant neoplasms
4. Being pregnant or having a plan to become pregnant during the study period.

The sample size of the study population was calculated based on the expected proportion of individuals who develop TB (ART associated TB) in the study population after initiation of HAART. Assuming a maximum of 10% ART associated TB in a population of infinite size, a precision of ±5%, and a 5% significance level, 138 study participants are needed for the study using the following formula;

$$N = \frac{(Z\alpha/2)^2 \times P(1-P)}{d^2}$$

$$= (1.96/0.05)^2\ 0.1(1-0.1)$$

$$= 138$$

Where N – number of samples Z – confidence interval (95%)

P – Proportion (10%) d – the level of confidence is 5%

Assuming 20% enrolled study participants were expected to be excluded in the analysis of ART associated TB because of prevalent tuberculosis at baseline investigation and assuming a loss-to-follow-up rate of 10%, 30% of study participants were added and a total of 179 subjects were recruited and 177 enrolled in the study (as shown in Figure 3.2)

**2.4 Data Collection**

Consecutive study participants, who were eligible to HAART at ALERT hospital, were recruited by attending physician and the purpose of the study was explained to them and their consent sought. Patients who fulfilled the inclusion criteria and signed written informed consents were enrolled in the study and all relevant data were collected at enrolment (baseline) and during follow up (see annex I, II and III).

a) **Baseline Data**

Baseline data were obtained at the time of enrolment before initiation of HAART as part of the routine work of the ART clinic, data on socio-demographic variables were collected and study participants underwent standard clinical examination. The attending physician recorded relevant history and physical findings in standardized form and then classified the

disease according to the WHO clinical staging (WHO, 2005) (see Annex IV). Following clinical staging the following laboratory investigations were done: CD4 and CD8 cells count, complete blood cell count, Clinical chemistry (renal function tests and liver function tests). All participants were screened for tuberculosis using sputum smear for acid fast bacilli (AFB), X-ray and also, if needed histopathology examination. In addition to routine ART care investigations blood sample was also collected to isolate peripheral blood mononuclear cell (PBMC) for subsequent ELISPOT assay to assess *in vitro* immune response to tuberculosis. Tuberculin skin test was also performed to assess *in-vivo* immune response to TB.

Following baseline assessment, HAART was initiated in accordance with guidelines for use of antiretroviral drugs in Ethiopia**,** which define ART initiation criteria as: WHO stage 4 disease (clinical AIDS), regardless of CD4 count, WHO stage 3 disease with consideration of CD4 count <350/$mm^3$ and WHO stages 1 or 2 disease with CD4 count<200/$mm^3$ (FMOH/HAPCO/DACA, 2003)( see Annex V).

b) **Follow-up Data**

After initiation of HAART, as part of the National ART Program, patients received scheduled clinical assessments at regular intervals; at weeks 2, 4, 8 and 12, and 24. They were also encouraged to visit the clinic any time when they felt sick. During every visit, relevant laboratory investigations were done as per clinical indication. In addition, the following laboratory investigations were performed regularly: ELISPOT assay for *In vitro* immune response to TB, tuberculin skin test, CD4 and CD8 cell count, complete blood cell count, renal function test and liver function tests were done at week 12 ($3^{rd}$ month), week 24 ($6^{th}$ month) and at the time when tuberculosis associated IRIS was clinically suspected.

### 2.5 Sample Collection and Handling

1. **Blood sample collection**:

Blood sample was collected in four different pre-labeled evacuated tubes (BD, USA) for each study participant. Two 10 ml heparin coated evacuated tubes labeled for PBMC isolation, one 5ml evacuated tube containing 7.5mg EDTA pre-labeled for CBC**,** CD4 cell count and a 5ml evacuated tube without anticoagulant labeled for clinical chemistry.

2**. Sputum**

Three consecutive sputum samples (spot, early morning, spot) were collected from each study participant at enrolment before initation of HAART and from individuals wth suspected TB during HAART.

3**. Samples for pathology investigation of TB**

Fine needle aspirate (FNA) of lymph nodes, pus or body fluids (peritoneal or plelural) was collected at ALERT/ AHRI pathology sample collection site by experienced pathologist. The sample was collected using a 21- 22gauge needle with an attached 10-ml syringe and smeared on clean slides on the spot of specimen collection. The slides were air dried and transported to AHRI laboratory using a slide box for subsequent staining by wright stain.

## 2.6 Laboratory Investigations

### 2.6.1 Isolation of PBMC

PBMC was isolated within 6 hours of blood sample collection. Meantime the blood sample was maintained at room temperature (RT) ($20^0$C - $25^0$C). Twenty ml of heparinized whole blood was resuspended with complete medium (RPMI with L-glutamine (Sigma-Aldrich), plus 100U of penicillin per ml, and 100mg of streptomycin (Sigma-Aldrich) per ml); in 1:1 proportion and mixed gently. The mixed blood was carefully layered into Ficoll-Hypaque and spinned at 1800 rpm (600g) for 30 minutes. Then the lymphocyte layer was aspirated aseptically and washed 3X in complete medium at 1500 rpm (405g) at $4^0$C for 10 minutes. Cell viability was determined by trypan blue and PBMC were frozen using freezing medium [10% Dimethyl sulphoxide (DMSO) in Fetal Bovine Serum (FBS)], and stored at -$80^0$ C.

### 2.6.2 IFN-γ ELISPOT Assay

The *in vitro* ELISPOT assays for IFN-γ producing cells were performed using standard protocol as described elsewhere (Lalvani *et al.*, 1997). The assay template, which indicates the position of individual antigens, mitogen and negative control in a 96 - well ELISPOT plate was prepared before the commencement of the assay (see Annex vi). Similar template was used throughout the assay.

On day one, Millipore Multiscreen 96-well ELISPOT Filtration plate (MAIPS-4510) (Millipore Corp, France), was labeled with the plate number and coated with capture anti-IFN-γ. monoclonal antibody (Mab1D1K, MABTECH) 50 µl per well in carbonate bicarbonate buffer solution (Sigma-Aldrich) at the final concentration of 10µg/ml. Then the plate was incubated overnight at $4^0$C.

On day two (after 20 hour incubation), the coating solution was flicked off and the plate was washed 6X with sterile PBS to remove unbound antibodies and blocked with 100µl/well of R10 (RPMI with 10% FBS) and incubated in the hood at room temperature for 2 hours to avoid non specific binding. Meanwhile frozen PBMC samples were thawed at $37^0$C in water bath and washed two times with R10. Then cells were counted and adjusted to the desired concentration with R10. After 2 hour incubation, the blocking solution was flicked off and 50µl/well of the antigens ESAT-6 and CFP-10 at a final concentration of 10µg/ml and PPD at a final concentration of 5 µg/ml was added in duplicates. PHA at a final concentration of 5µg/ml as a positive control and medium (R10) as a negative control were also added in duplicate wells. Then $2 \times 10^5$ PBMC suspended in 50 µl R10 per well were seeded and the plate was incubated for 20 hours at $37^0$C in 5% $CO_2$ cell culture incubator.

On day three, the plate was washed 6X with PBST (PBS with 0.05%Tween 20). Following washing, biotinylated detector monoclonal antibody (Mab - 7-B6-1-biotin) was added at the concentration of 1:1000 in PBS and incubated for 2 hours at RT. To remove unbound detector antibody the plate was washed 6X with PBST and 50µl of 1:1000 PBS diluted streptavidin-ALP was added and incubated for 1h at RT in the dark. Then the plate was washed 6X with PBST and 50µl/well of chromogenic alkaline phosphatase conjugate substrate (prepared as per manufacturer's specifications, for one plate using 200µl of 25x development buffer, 4.8ml of 0.22µl filtered deionised water, 50µl of each AP Colour Reagent A and B) added and incubated at RT in dark until the negative control wells start to give faint background. Then colour development was stopped by washing the plate in running tap water for 2-4 minutes and then soaked in water for about 10 minutes. Finally the plate was dried well in air and spots were visualized and counted using an ELISPOT reader (AID, GmBH, Strasburg Germany).

**Reading and interpretation of ELISPOT assay**

The intensity and size of a spot was set before counting and the same setting was used throughout. The mean spot count from the duplicate wells was transferred and analyzed using SPSS statistical software version 13. Failed ELISPOT response (*in vitro* anergy) was defined as less than 20 spots/2 x $10^5$ PBMCs or 100 spots/$10^6$ PBMCs in the PHA positive control wells. Assay responses were evaluated as qualitative results (positive or negative responses) and quantitative results (SFC / $10^6$ PBMC). The qualitative result was pre defined as positive if test wells contained a mean of at least 5 spot-forming cells more than the mean of the negative control wells and were at least twice the mean of the negative control wells in numbers. This predefined cutoff point is based on threshold used by several published studies elsewhere (Chapman *et al.*, 2002; Dosanjh *et al.*, 2008; Lalvani *et al.*, 2001a; Lalvani *et al.*, 2001b; Liebeschuetz *et al.*, 2004; Richeldi *et al.*, 2004). Quantitative counts were represented as SFCs/$10^6$ PBMC (spot-forming cell per million PBMC) above the negative control wells by multiplying with 5 (5x SFC/200,000 PBMC = SFC/$10^6$ PBMC). EC ELISPOT response was defined as positive if qualitative ELISPOT response was positive for ESAT-6 and /or CFP-10. ECP ELISPOT response was defined as positive if qualitative ELISPOT response was positive for ESAT-6 and /or CFP-10 and/ or PPD.

2.6.3 **CD4 and CD8 cell count**

CD4 and CD8 cell count was performed on FACSCount system (Becton Dickinson Biosciences) Fifty µl of blood sample was added into each twin-tube reagent tubes containing monoclonal anti-human CD3 antibody conjugated to the tandem dye phycoerythrin +Cy5 (PECy5) and a monoclonal anti-human CD4 antibody conjugated to phycoerythrin (PE) or a monoclonal anti-human CD8 antibody conjugated to PE. After a fixative solution had added, the sample was run on the instrument. The calculation of absolute CD3+, CD4+ and CD8+ T-cell was determined automatically by using the built-in attractors software programme and automatically printed out the result.

### 2.6.4 **Direct microscopy**

Direct microscopic examination for acid fast bacilli (AFB) was done on three consecutive sputum samples (spot, morning, spot) after staining with the hot Ziehl Neelsen technique according to the national guidelines (MoH, TB and Leprosy Control manual) (FMOH, 2002).

### 2.6.5 **HIV Test**

Testing for HIV was done at ALERT laboratory according to the national guidelines for HIV testing (Rapid HIV test Algorithm) that includes a combination of three tests namely, Determine® HIV -1/2, CapillusTM HIV-1/HIV-2 and Uni-GoldTM. (Annex 3).

### 2.6.6 **Tuberculin Skin Test (TS**T)

According to the guidelines specified in the WHO standard tuberculin test technical guide (Sokal, 1975), (Statens Serum Institute, Denmark) of PPD tuberculin was injected intracutaneously on the volar surface of the forearm, and the transverse diameter of induration was measured 48 hour later. For accurate measurement of induration, the edge of induration was marked by moving a ball point pen on the skin from the outer edge of the erythema or swelling inwards until resistance was felt. This spot was marked as the edge on one side. The same procedures were used to mark all four margins and the transverse diameter of the indurations was measured using caliper. Then, the result was recorded in millimeters. A positive TST result was defined as an induration of 5 mm or larger.

### 2.6.7 **Cytomorphological Staining and Examination**

At AHRI laboratory, previously prepared slides were flooded with freshly filtered Wright's stain and buffered with clean tap water. The buffered slides were continuously stained with Wright's stain for 10 minutes, washed with water and air dried. Finally, the slides were examined by the pathologist to evaluate whether the morphology was suggestive for tuberculosis or not. Cytological examination of FNA smears was considered diagnostic of TBLN when they contained a thick, yellowish material showing either necrotic back ground associated with the presence of lymphohistiocytic and the presence of a significant

polymorph nuclear cell population or the presence of a granulomatous inflammatory reaction consisting of giant cells, and/or epithelioid cell clusters and lymphohistiocytic cell population.

## 2.7 Case Definitions

### 2.7.1 **Tuberculosis case definitions and classification**

A case of tuberculosis were defined and classified according to Ethiopian National TB and Leprosy Control Programme Guideline (FMOH, 2002). Accordingly, smear positive pulmonary tuberculosis (PTB+) was diagnosed if two or more initial sputum examinations were positive for AFB, or one sputum positive for AFB plus radiographic abnormalities consistent with active TB as determined by a physician. Smear negative pulmonary tuberculosis (PTB-) was diagnosed if at least three sputum specimens negative for AFB, and radiologic abnormalities consistent with TB, and no response to a course of broad-spectrum antibiotics, and decision by a physician to treat with a full course of antituberculosis chemotherapy. Extra pulmonary TB (EPTB) was defined as TB of organ other than the lung eg. pleura, lymph nodes, abdomen , genitourinary tract, skin, joint, bones, or meninges. Diagnosis was made based on fine-needle aspiration of lymphadenopathy for cytology or abdominal ultrasonography and or strong clinical evidence consistent with active extra pulmonary TB followed by a decision by clinician to treat with full course of anti- TB treatment (FMOH, 2002; WHO, 2004a).

### 2.7.2 **Past history of TB**

Patients were considered to have a past history of active TB at HAART initiation if they reported a history of active TB diagnosis and had received or completed full course of anti-TB therapy before enrolment into this study.

### 2.7.3 **Prevalent TB**

Prevalent TB was defined as TB diagnosis made prior to enrolment for which Anti-TB treatment was still being received at enrollment or new TB diagnosis established in the interval between enrollment and initiation of ART.

### 2.7.4 **Incident TB (ART associated TB)**

Incident TB (ART associated TB) was defined as the occurrence of TB in a patient who, prior to ART, exhibited no signs or symptoms of disease and in whom adequate TB screening and clinical assessment had been performed (Meintjes *et al.*, 2008; Ratnam I, 2005).

### 2.7.5 **Unmasking TB IRIS and paradoxical IRIS**

Since there are no established standard diagnostic criteria for the diagnosis of IRIS, for the purpose of this study, we used the published proposed diagnostic criteria (Breton *et al.*, 2004; French *et al.*, 2004; Shelburne, S. A. *et al.*, 2005). Thus, for our purposes (and as in the literature), Paradoxical TB IRIS was defined as the clinical or radiological deterioration of preexisting tuberculous lesions or the development of new lesions in patients initially responding to both Anti-TB and HAART (Lawn *et al.*, 2005c) . Unmasking TB IRIS was defined as incident TB cases which occurred within the first three months of HAART and presented with unusual clinical manifestation or exaggerated inflammatory reaction.

a) **Paradoxical TB IRIS**

Diagnosis of ‘paradoxical’ TB IRIS was accepted if a patient had the following two criteria and fulfilled the subsequent three major criteria:

1. Diagnosis of active TB at baseline investigation or a recent history of TB prior to starting of HAART (patients who were initiated on HAART within the 6 months after the initiation of antituberculosis therapy) (Breton *et al.*, 2004) or diagnosis of TB while taking HAART and at least followed up for three months while taking both ART and Anti-TB therapy (Breen *et al.*, 2005).
2. Initial clinical response to TB therapy; cessation of fever, relief of pulmonary symptoms, decrease in lymphoid size, termination of meningeal signs, depending on the original presentation (Shelburnea et al., 2005)

   The three major criteria (Colebunders *et al.*, 2006):

1. Radiological examinations showing worsening or emergence of intrathoracic lymphadenopathy, pulmonary infiltrates, pleural effusions, abdominal lymph nodes, hepatosplenomegaly
2. A good virological response and/or increase in CD4+ lymphocyte count, and/or conversion of tuberculin skin test from negative to positive, and/or adequate adherence to ART and tuberculosis treatment
3. A clear exclusion of other conditions that could explain the clinical manifestations of the patient, such as tuberculosis treatment failure or other concomitant infections, tumours, or allergic reactions.

b) **Unmasking Tuberculosis IRIS**

Diagnosis of 'Unmasking' TB IRIS was accepted if a case of incident TB/ART associated fulfils the following criteria:

1. No evidence of active TB at enrolment (at baseline investigations)
    - No clinical evidence consistent with active TB and/ or
    - AFB negative and/or no evidence of TB by pathology investigation
2. Diagnosis af active TB within 3 months of starting ART and one of the following two criteria must be met:
    i. Unusual clinical manifestations / exaggerated inflammatory reaction at presentation active TB
        - Radiological evidence showing intra thoracic lymphadenopathy, and/or pulmonary infiltrate, and/ or cavitating TB, and/or pleural effusion, abdominal lymph nodes and/or hepatosplenomegaly

    ii. Once established on tuberculosis treatment, a clinical course that is complicated by a paradoxical reaction

3. A good virological response and/or increase in CD4+ lymphocyte count, and/or conversion of tuberculin skin test from negative to positive, and/or adequate adherence to ART.

2.7.7 **Immunological Super responder**

Defined as if individual had achieved an absolute CD4 cell count of 500 cells /$mm^3$ at $6^{th}$ month of treatment (Lawn *et al.*, 2006b)

2.7.8 **Immunological failure**

Defined as fall of CD4 count to pre-therapy baseline (or below) and or 50% fall from the on-treatment peak value (if known) and or persistent CD4 levels below 100 cells/$mm^3$ (FMOH/HAPCO, 2007). For the purpose of this study, we adopted the guideline definition of Immunological failure as follows: If baseline CD4 count $\geq$ $6^{th}$ month and or if $3^{rd}$ month CD4 count $\geq$ twice the value of CD4 cell count at $6^{th}$ month and or if both $3^{rd}$ and $6^{th}$ month CD4 cell count < 100 cells/ $mm^3$.

**2.8 Data Entry and Management**

2.8.1 **Data entry**

All Information obtained from the clinical and laboratory data were double entered by two different data entry clerks into microsoft access spread sheet, cleaned, verified and prepared for analysis

2.8.2 **Statistical analysis**

Statistical analyses were performed in three steps. The first step of analysis was done on data collected at baseline, which describes baseline characteristics of study participants and assesses the effect of level of immunodeficiency on baseline immune parameters such as TST and ELISPOT assay. The second step of analysis comprises data collected at baseline, $3^{rd}$ and $6^{th}$ month of HAART. The data included in the analyses were from study participants who had at least one follow up ($3^{rd}$ or $6^{th}$ month) laboratory investigation. Finally, the third step of analysis was conducted on patients who developed TB during HAART and IRIS. In all steps of analysis nonparametric statistics were used as appropriate because the data were

either non-parametrically distributed or the sample size in each category was too small to be analyzed through parametric statistics. SPSS for Windows version 13.0 (SPSS Inc, Chicago, USA.) and GraphPad Prism 5 statistical software were used for analyses. All reported P values are 2 sided and P value <0.05 was considered as significant.

a) **Step one: baseline analysis**

Baseline characteristics of study participants were described using median and inter quartile range for quantitative variables and proportion for qualitative variables. To assess effect of level of immunodeficiency on baseline immune parameters, study participants were classified in 4 groups according to baseline CD4 cell: <50 cells/ mm3, 50 - 99 cells/ $mm^3$, 100–199 cells/$mm^3$, and $\geq$ 200 cells/$mm^3$. Baseline TST results were dichotomized into TST negative <5 mm indurations and TST positive $\geq$ 5mm indurations. Difference on proportion of TST positive result across CD4 strata was compared using chi square test for trend. The differences in proportion of baseline positive ELISPOT response across CD4 strata were also analyzed using chi square test. Quantitative baseline ELISPOT assay results for each antigen were described as the median frequency of spot forming cell per million PBMC (SFC/$10^6$ PBMC). Difference in frequency of spot forming cell across CD4 cell strata were compared using Kruskal-Wallis test.

b) **Step two: follow-up analysis**

To assess the effect of HAART on the reconstitution of CD4 cell, we compared the difference in median CD4 T cell at $3^{rd}$ and $6^{th}$ months with the baseline CD4 T cell count using Wilcoxon signed rank test. Effect of baseline CD4 count on $3^{rd}$ and/or $6^{th}$ month recovery of CD4 cell count grater than the critical threshold of 200 cells/$mm^3$ (zone of severe immunodeficiency) or 350 cells/$mm^3$ were assessed through dichotomized CD4 cell count at $3^{rd}$ and $6^{th}$ month ( <200 and $\geq$200 ) and (< 350 and $\geq$ 350). The proportion of study participants having CD4 count >200/>350 at $3^{rd}$ and $6^{th}$ month in respect to baseline CD4 count strata were compared using chi square test for trend. The risks of $3^{rd}$ or $6^{th}$ month CD4 count being $\geq$200 cells/$mm^3$ relative to baseline CD4 count $\geq$200 cells/$mm^3$ were estimated for baseline CD4 count <50 cells/$mm^3$, 50–99 cells/$mm^3$, and 100- 199 cells/$mm^3$.

To assess effect of HAART on qualitative TB specific immune response, proportion of positive TST and ELISPOT response at baseline were compared with 3rd and 6th month using chi square test for trend. Effect of baseline CD4 count on 3rd and 6th month qualitative response was also assessed by stratifying baseline CD4 count and analyzing the difference in the proportion of positive response at baseline, 3rd and 6th month result in each category. To avoid the confounding effect of Baseline TB status the data were also categorized according to baseline TB history and analyzed.

To assess the effect of HAART on reconstitution of quantitative IFNγ ELISPOT response, median SFC/million at 3rd and 6th month were compared against baseline median SFC / million for each TB antigens ( PPD, ESAT-6 and CFP-10) using Wilcoxon signed rank test. The effect of baseline level immunodeficiency on reconstitution quantitative TB specific immune response was also assessed through baseline CD4 cell count stratified analysis.

c) **Step three: analysis on ART associated TB and TB IRIS**

To determine incidence rate of TB (ART associated TB) study participants were categorized based on baseline TB status (Figure 3.10). TB incidence rates (ART associated TB) were calculated among individuals without prevalent TB at start of HAART and who had at least one follow up investigation. Paradoxical TB IRIS incidence rate or proportion was calculated among individuals with prevalent TB cases at start of HAART or incident TB cases (who were followed at least three months after start of Anti-TB therapy). In both cases, to avoid bias due to variation in follow up duration at the result of lost to follow up and end points, incidence rate was calculated as cases per 100 person-year observation by taking the duration between enrolment and last time investigation for those lost to followed up or taking the duration between enrolment and end point. (The dates of TB diagnosis and dates of Paradoxical TB IRIS diagnosis while taking HAART was considered as end points for incidence rate ART associated TB and paradoxical TB IRIS respectively).

In univariate analysis, we compared the baseline demographic, laboratory, and clinical characteristics between patients with ART associated TB and those without (participant who had past history of TB at enrolment plus number of participants who do not have any history of TB), using chi square or Fisher's exact test for categorical variables and Mann- Whitney

U test for quantitative variables. Multivariate logistic regression analyses were used to identify independent risk factors for ART associated TB. Variables at baseline with $p<0.05$ in the univariate analysis were included in the multivariate model.

### 2.9 Ethical Considerations

Before the commencement of this research project, the proposal was approved by Department of Microbiology, Parasitology and Immunology, and ethically cleared by Faculty Research Publications Committee-II (FRPC-II), endorsed by AC, FM. Addis Ababa University. Ethical clearance was also obtained from AHRI/ALERT Ethics Review Committee, and the National Ethics Review Committee (NERC).

At enrolment, each and every study participant was informed about study objectives, benefits and risks of the study including procedures. They were also informed that they have the right to withhold information, refuse cooperation or to drop out of the study at any time regardless of need to explain the reason to anyone and that their withdrawal will not have any repercussions on their management.

Only participants who had consented were enrolled in the study and all information including their HIV status was kept confidential. The names of an individual were not written in any of the data collection or sample-processing forms. Only the treating physician had access to patients' identifiers. Schedule time table was prepared in cooporation with ALERT laboratory in order not to disturb patient follow up and the study participants were getting free care according to the national guideline.

## CHAPTER 3. RESULTS

### 3.1 Socio-Demographic Characteristics

Socio-demographic characteristics of study population are presented in Table 3.1. The majority (68.9%, 122/177) of enrolled participants were females. The highest proportion of the participants are in the age range 28-37 (49.7%, 88/177), followed by the age range 18-27 (25.5 %, 45/177), 38-47 (20.3%, 36/177), and 48-55 (4.5%, 8/177). Almost half of the participants 47.5% (84/177) were married. Thirty-six point seven percent (65/177) of the study participants' educational level was primary followed by 35% (62/177) secondary. The majority of 76.3% (135) participants were of the Orthodox Christian faith.

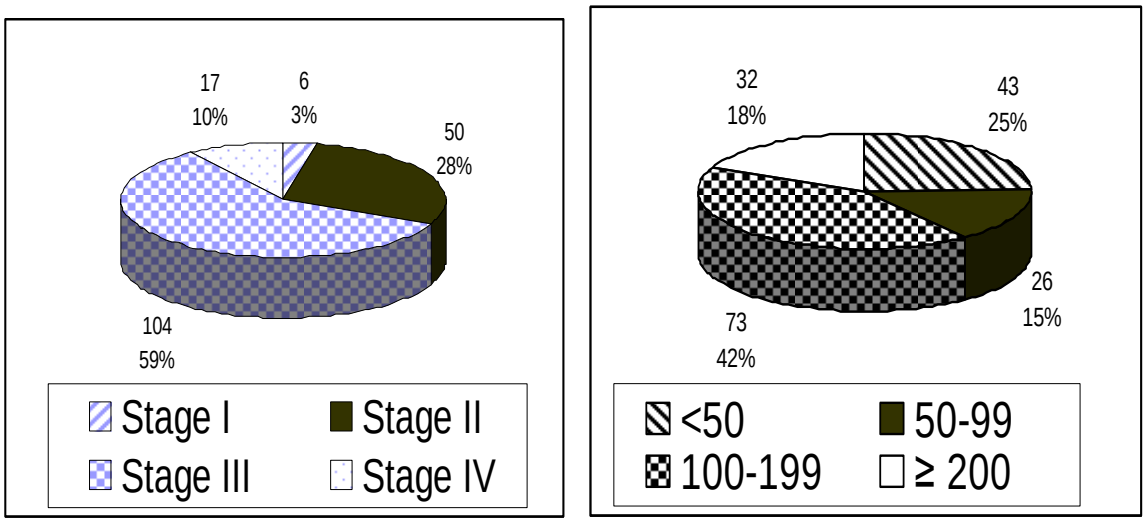
**Table 3.1 Socio-demographic characteristics of study participants (n =177)**

| Characteristics | No (%) |
|---|---|
| Sex | |
| Male | 55 (31.1) |
| Female | 122 (68.9) |
| Age ( in years) | |
| 18-27 | 45 (25.4) |
| 28-37 | 88 (49.7) |
| 38-47 | 36 (20.3) |
| 48- 55 | 8 (4.5) |
| Marital Status | |
| Single | 32 (18.1) |
| Married | 84 (47.5) |
| Separated | 4 (2.3) |
| Divorced | 20 (11.3) |
| Widowed | 37 (20.9) |
| Education | |
| Illiterate | 45 (25.4) |
| Able to read and write only | 2 (1.1) |
| Primary | 65 (36.7) |
| Secondary | 62(35) |
| Tertiary | 3(1.7) |
| Religion | |
| Muslim | 19 (10.7) |
| Orthodox | 135 (76.3) |
| Protestant | 23 (13) |

### 3.2 Baseline Clinical and Laboratory data

#### 3.2.1 WHO clinical staging of HIV and CD4 cell count

At enrollment, 104/177 (59%) of the participants had WHO stage III, followed by 50/177 (28%) WHO stage II, 17/177 (10%) WHO stage IV and 6/177 (3%) WHO stage I (Figure 3.1a). The highest proportion, 76/177 (43%) of the participants had baseline CD4 cells count within the range of 100-200 cells/mm$^3$, followed by 43 (24%) < 50 cells/mm$^3$ , 32 (18%) ≥ 200 cells/mm$^3$, and 26 (15%) within the range of 50-100 cells/mm$^3$ (Figure 3.1 b).




(a) (b)

**Figure 3.1 Distribution of study participants by WHO clinical staging (a) and CD4 cell strata (b) ([cell/mm$^3$])**

#### 3.2.2 Hemoglobin and Total Lymphocyte Count

The median total lymphocyte count (TLC) was 1310 (IQR, 0.9-1.9) cells/mm$^3$. Twenty two (12%) participants had baseline hemoglobin value less than 10 g/dl. The proportion of participants who had hemoglobin < 10 g/dl was 8/43 (19 %), 4/26 (15.4%), 8/76 (10.7%), 2/32 (5.9%) for those with CD4 cell count category < 50, 50-99, 100-199 and ≥ 200 cells/mm$^3$ respectivly (Table 3.2).

**Table 3.2 Characteristics of the study patients according to baseline CD4 cell count**

| | Baseline CD4 cell count (cells/mm3) groups | | | | |
|---|---|---|---|---|---|
| | **Total** | **< 50** | **50-99** | **100-199** | **≥ 200** |
| **Number of participant *** | 177 | 43 (23) | 26 (14.7) | 76 (42.9) | 32 (18.1) |
| **Age (y)**** | 30 (27 – 37) | 30 (27-35) | 32(28-40) | 31(27-36) | 30(26-37) |
| **Baseline CD4 cell (cells/mm$^3$)**** | 123 (63-182) | 31(14- 39 ) | 79 (68-86) | 152 (121-174) | 222 (210-245) |
| **TLC (10$^3$cells/mm$^3$)**** | 1.31(0.9-1.9) | 0.9 (0.6-0.14) | 1.12 (0.8-1.5) | 1.13 (0.8-1.5) | 1.67 (1.2-2) |
| **HGB≤ 12g/dl*** | 69 (38.8) | 22 (52) | 16 (61.5) | 23 (30.7) | 8 (23.5) |
| **≤ 10g/dl*** | 22 (12.4) | 8 (19) | 4 (15.4) | 8 (10.7) | 2 (5.9) |
| **History of TB *** | | | | | |
| **Yes** | 68 (38.4) | 22 (51.2) | 15(57.7) | 25 (32.9) | 6(18.8) |
| **No** | 109 (61.6%) | 21 (48.8) | 11 (42.3) | 51 (67.1) | 26 (81.3) |

***Number (%) ** median ( IQR)**

### 3.2.3 History of TB at enrolment

As described in Table 3.3, among 68 (38.4%) study participants who had a history of TB at enrolment, 40 (22.6%) had completed Anti-TB treatment prior to enrolment, and the remaining 28 (15.7%) prevalent TB cases were either newly diagnosed active TB [15 (8.4%)] at baseline investigation or were already started taking Anti-TB treatment before enrolment [13 (7.3%)]. When the proportion of all patients in different CD4 cell categories was compared for prevalent TB (n=28) at baseline, 8/43 (19%), 8/26 (31%), 11/76 (14.6%), and 1/32 (2.9%) patient who had TB had baseline CD4 cell < 50, 50 - 99, 100 - 199 and ≥ 200 CD4 cell respectively. Among patients with past history of TB, 25 (67.5%) were

pulmonary TB, 13 (32.5%) extra pulmonary and 2(5%) disseminated TB. There was statistically significant decreasing trend in the proportion of study participants with past history of TB from 43.9% (18/42) to 38.5% (10/26), 22.7% (17/75) and 17.6% (6/34), as CD4 cell increases from >50 to 50-99, 100-199, and ≥ 200 cells/mm$^3$ respectively (p= 0.0406).

**Table 3.3 History of TB at enrolment according to baseline CD4 cell count.**

| | Baseline CD4 cell count (cells/mm3) groups | | | | | |
|---|---|---|---|---|---|---|
| | **Total (n=177)** | **< 50 (n=43)** | **50-99 (n=26)** | **100-199 (n=76)** | **≥ 200 (n=32)** | p value |
| **Number of participant with history of TB** | **68 (38.4)** | **22 (51.2)** | **15(57.7)** | **25 (32.9)** | **6(18.8)** | |
| **Past History of TB** | **40 (22.6)** | **14 (32.6)** | **7 (26.9)** | **14 (18.4)** | **5 (15.6)** | 0.0406 |
| **PTB** | 25 (62.5) | 7 (50) | 5 (71.4) | 9 (64.3) | 4 (80) | |
| **EPTB** | 13 (32.5) | 5 (35.7) | 2 (28.6) | 4 (28.6) | 1 (10) | |
| **DTB** | 2 (5) | 2 (14.3) | – | 1 (7.1) | – | |
| **Prevalent TB*** | **28 (15.8)** | **8 (19)** | **8 (31)** | **11 (14.6)** | **1 (2.9)** | **>0.05** |
| **PTB** | 19 (67.9) | 7(87.5) | 5 (62.5) | 7 (63.6) | | |
| **EPTB** | 8 (28.6) | 1(12.5) | 2 (25) | 4 (36.4) | 1 (100) | |
| **Pt with untreated active TB** | **15 (8.5)** | **3 (6.9)** | **5 (19.2)** | **7 (9.3)** | – | |
| **Pt on anti-TB** | **13 (7.3)** | **5 (12)** | **3 (11.2)** | **4 (5.3)** | **1 (2.9)** | |

***Number (%) , ** median PTB = Pulmonary TB EPTB = Extra pulmonary TB DTB = Disseminated TB**

### 3.3 Baseline TB Specific Immune Response

#### 3.3.1 In vivo Immune Response to TB

a) **TST Response**

Among 177 study participants, 4 participants didn't undergo TST for unknown reason and other 4 participants did not return for test reading. Baseline TST results were available for 169 study participants (Figure 3.2). Among these, 80.5% (136/169) had cutaneous anergy (TST response = 0 mm), 17.2 % (29/169) had TST≥5 mm indurations and 2.4% (4/169) had TST result > 0 and < 5 mm.

b) **TST response in relation to CD4 count**

TST responses were significantly associated with CD4 cell count (p = 0.0002, test for trend); the proportion of baseline TST response ≥5 mm among participants increased as CD 4 cell count increased. Of study participants with CD4 cell count ≥ 200 cells/$mm^3$, 29% (9/31) had positive TST result compared to 23.9% (17/71) with CD4 cell count 100 to 199 cells/$mm^3$, 12 % (3/25) with CD4 cell count 50-99 cells/$mm^3$and no positive TST results with CD4 cell counts of less than 50 cells/$mm^3$ (Figure 3.3).

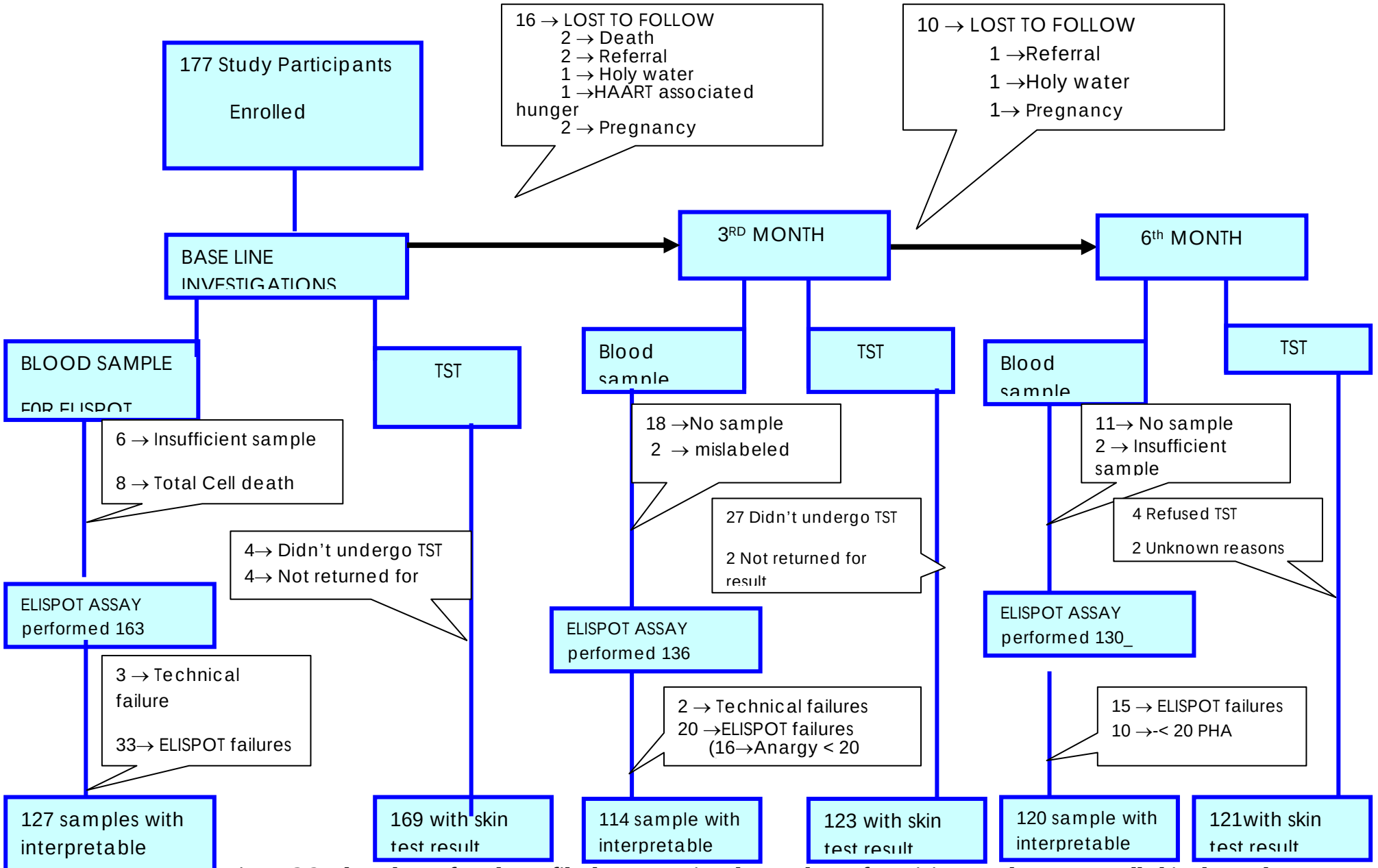



**Figure 3.2 Flow chart of study profile demonstrating the numbers of participants who were enrolled in the study and were on follow up and number of TST and ELISPOT tests performed and analyzed.**

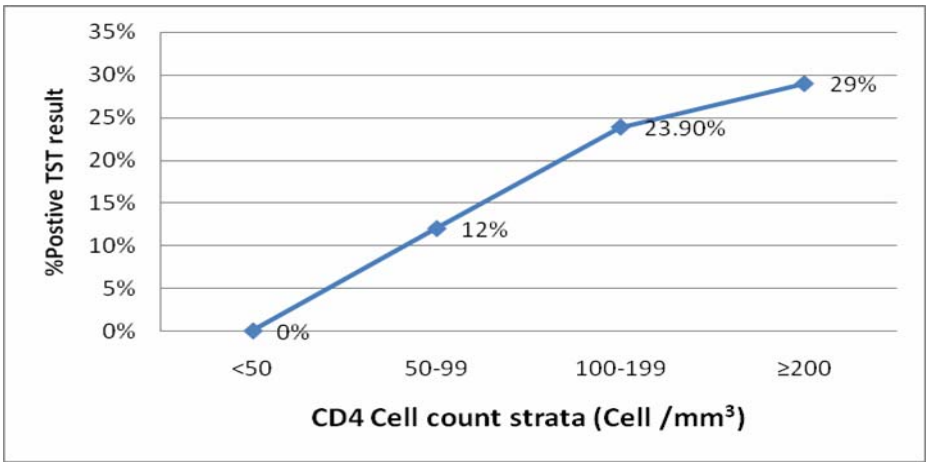



**Figure 3.3 Relationship between tuberculin skin test reactivity (TST > 5 mm) and CD4 cell count**

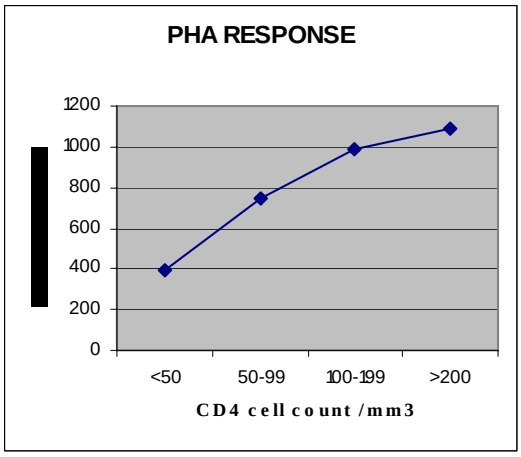
### 3.3.2 In-vitro Immune Response to TB

As described in Figure 3.2, 163 PBMC samples were challenged *in vitro* with TB antigens ESAT-6, CFP-10 and with PPD and mitogen (PHA) to measure IFN-γ response using ELISPOT assay. Out of 177 samples, 6 samples had insufficient volume for PBMC isolation and 8 frozen PBMC samples were not recovered after thawing. Of those performed assays, three samples were lost due to technical failure and 33 (20.5%) had *in vitro* anergy for mitogen (PHA response < 20 SFC/200 000 PBMC). Interpretable result was available for 127 (78%) at baseline.

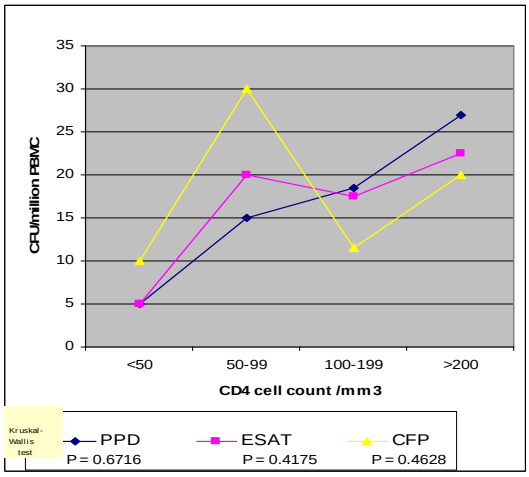
#### a) Quantitative ELISPOT Response

To assess the effect of CD4 cell count on frequency of spot forming cell, baseline quantitative ELISPOT responses was analyzed by stratifying CD4 cell count as: > 50, 50-99, 100 – 199, and $\geq$ 200. Quantitative response to mitogen was significantly affected by CD4 cell count (p= 0.0025). The median PHA response increased from 390 SFC/$10^6$ of those with CD4 cell strata < 50 to 750 SFC/$10^6$, 985 SFC/$10^6$ and 1193 SFC/$10^6$ with CD4 cell strata: 50-99, 100 – 199, and $\geq$ 200 respectively (Figure 3.4, a). Quantitative ELISPOT response to PPD increased with CD4 cell count (not significant; P = 0.16: median (IQR) responses to PPD were: 5 (0-20), 15 (0-150), 22.5 (0-60) and 27.5 (0-175) SFC/Million in CD4 cell strata; 50-99, 100 – 199, and $\geq$ 200 cells/$mm^3$ respectively). As shown in Figure 3.4, (b), variable patterns of quantitative responses to ESAT- 6 and CFP – 10 were observed as

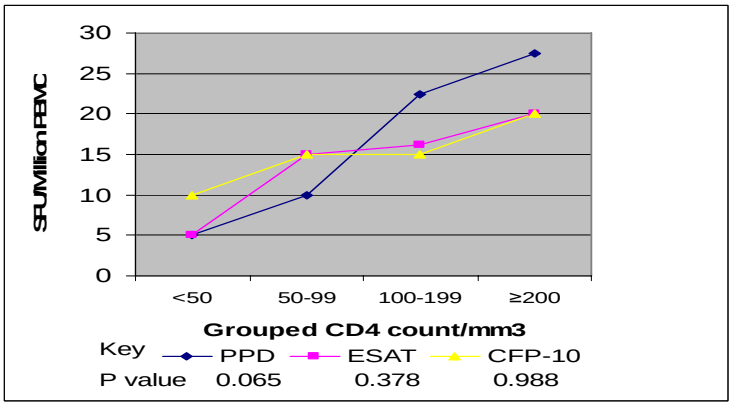
grouped CD4 cell count increases (median response to both ESAT- 6 and CFP – 10 increased in respective order from 5 (0-35) and 10 (0-40) in CD4 cell count <50 to 20 (0-55) and 25 (5-75) in CD4 cell group 50-100, then decreased to 18.75 (0-50) and 17 (0-70) in CD4 cell group 100-200 and finally increased to 22.5 (0-68.75) and 20 (0-42.5) in CD4 cell group >200 cells/mm$^3$. The result was farther analyzed by excluding individuals with prevalent TB. Although the difference ws not significant, we found similar pattern on increasing quantitative response to PPD, ESAT-6 and CFP-10 as grouped CD4 cell count increased (Figure 3.4, c).




(a) PHA




b) PPD, ESAT-6 and CFP-10 (All study participants)




(c) PPD, ESAT-6 and CFP-10 (study participant with prevalent TB excluded)

Figure 3.4 Median frequency of ELISPOT response (SFC/ million) to PHA (a) and TB antigens (PPD, ESAT-6, and CFP-10) (b) and (c) by CD4 cell strata.

b). **Qualitative ELISPOT Response**

**i. EC/ECP ELISPOT Response**

Proportion of positive response to EC ELISPOT (ESAT-6 and /or CFP Positive) and to ECP ELISPOT positive (ESAT-6 and /or CFP and or PPD positive) were analyzed according to pre defined criteria. Thus, of 127 valid ELISPOT results, 67(52%) had positive EC ELISPOT response and 74 (60%) had ECP ELISPOT response (Table 3.4).

**Table 3.4 Baseline qualitative response for individual antigen and ELISPOT result interpreted based on the combination EC (EAST-6 and CFP-10) or ECP (EAST-6, CFP-10 and PPD)**

| Possible result combination | INDIVIDUAL ANTIGEN RESULT | | | ELISPOT RESULT | |
|---|---|---|---|---|---|
| | ESAT-6 | CFP-19 | PPD | EC ELISPOT | ECP ELISPOT |
| 1 | + | + | + | 24 | 24 |
| 2 | + | + | – | 9 | 9 |
| 3 | + | – | + | 8 | 8 |
| 4 | + | – | – | 10 | 10 |
| 5 | – | + | + | 6 | 6 |
| 6 | – | + | – | 10 | 10 |
| **7** | – | – | + | 0 | 10 |
| 8 | **–** | **–** | **–** | 0 | 0 |
| **Total** | | | | 67(52%) | 77(60%) |

**ii. EC ELISPOT Response in relation with CD4 cell count**

To assess whether the observed effect of CD4 cell count on proportion of TST positive response would also affect qualitative results or not, proportion of ELISPOT positive response was analyzed by CD4 cell strata. We found both qualitative EC and ECP ELISPOT tests were not affected by level of CD4 cell count as there was no significant difference on proportion of positive response in both EC and ECP ELISPOT tests ($p = 0.6947$, and 0.7750 respectively) [Figure 3.5].

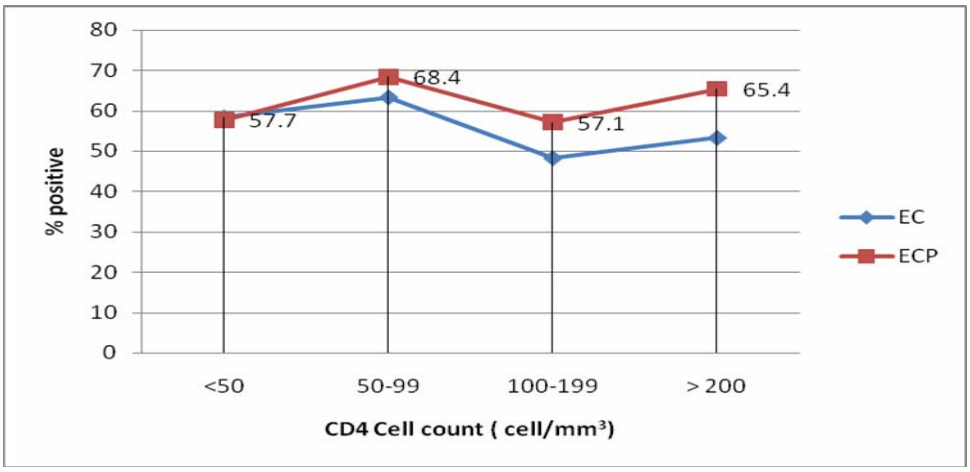



Figure 3.5 Baseline qualitative ELISPOT result interpreted based on the combination EC (EAST-6 and CFP-10) and ECP (EAST-6, CFP-10 and PPD)

### iii. EC ELISPOT response in relation with baseline TB status

To assess the performance of qualitative EC ELISPOT assay in different baseline TB groups, we stratified and analyzed the data according to baseline TB history as shown in Figure 3.6. Although the difference is not statistically significant (p = 0.275), we observed high proportion 8/11 (72.7%) of positive EC ELISPOT response among patients with active TB compared to 5/8 (62.5%) among patients receiving Anti-TB treatment and 11/28 (39.3%) among patients who had past history of TB treatment.

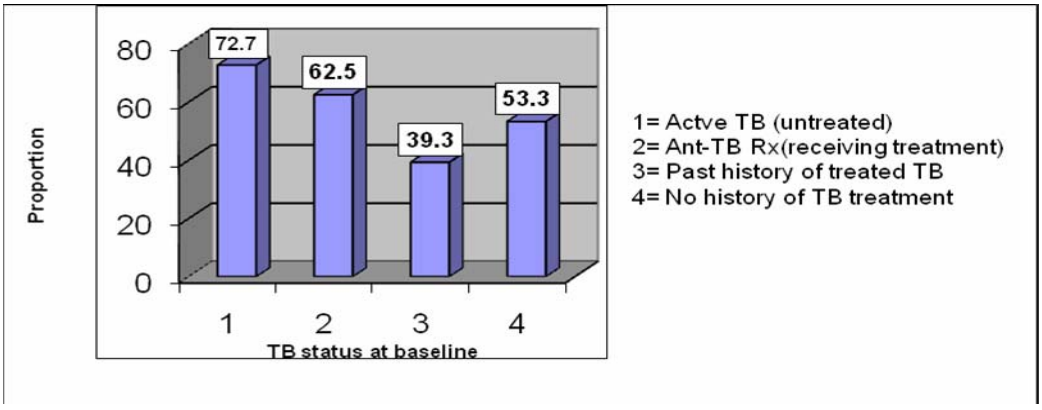



Figure 3.6 Proportions of positive EC ELISPOT assays according to TB history

### iv. EC-ELISPOT and TST response in identification of latent TB

To investigate the performance of both TST and EC ELISPOT assays in the identification of patients with latent TB, the data were analyzed by excluding those individuals who had prevalent TB at enrolment or individuals who subsequently developed TB while taking HAART within the first 3 months of HAART (since they may have had active sub-clinical TB at the time of testing). Thus, the analysis was made on dichotomized data; proportion of positive response stratified by past history of TB treatment (individuals with past history of TB and with no history of TB) and proportion of positive responses stratified by CD4 count ($< 100$ and $\geq 100$ cells/$mm^3$). As shown in Table 3.5 a, TST positive responses were significantly associated with CD4 cell count (P = 0.0139); the proportion of patients with a TST response >5 mm was much lower among those with CD4 cell counts <100 cells/$mm^3$ which is 4.2 % (2/48) compared to those with CD4 cells > 100 cells/$mm^3$of 18.1 % (17/87). Whereas, EC ELISPOT positive responses were not significantly associated with CD4 cell count [51.5% (17/33) vs 47.1% (32/68); P = 0.67 among groups with CD4 count $\geq$100 and < 100 cells/$mm^3$ respectively] (Table 3.5 b).

In stratified analysis according to TB history, although the difference was not statistically significant, lower proportion of both TST and EC ELISPOT positive response was observed among patients with past history of TB compared to responses among individuals with no history of TB. The proportions of positive TST response were (5.6% (2/36) vs 17.2% (17/99); P = 0.0861 among individuals with past history of TB and with no history of TB respectively. The proportions of positive EC ELISPOT positive response were 34.6% (9/26) vs 53.3% (40/75); P = 0.10 among individuals with past history of TB and with no history of TB respectively. Since past history of TB was found confounded by CD4 count in this study, both past history of TB and CD4 cell count were included in multivariate analysis to confirm the result. Nonetheless, only TST response was significantly associated with CD4 cell count (Table 3.5 a.).

**Table 3.5 Univariate analysis and multivariate analysis on proportion of positive TST response (a) and EC ELISPOT response (b)**

(a)

| | Proportion positive TST response | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|---|
| | | OR | P value | OR | P value |
| CD4 count | | | | | |
| ≥ 100 | 18.1 % (17/87) | 1 | | | |
| <100 | 4.2 % (2/48) | 0.18 (0.04–0.81) | 0.0139 | 0.21 (0.04-0.81) | O.026 |
| History of TB | | | | | |
| No | 17.2% (17/99) | 1 | | | |
| Yes | 5.6% (2/36) | 0.28 (0.06 –1.3) | 0.0861 | 0.39 (0.8–1.8) | 0.217 |
| OR = odds ratio | | | | | |

(b)

| | Proportion positive EC ELISPOT response | Univariate anllysis | | Multivariate analysis | |
|---|---|---|---|---|---|
| | | OR | P | OR | P |
| CD4 count | | | | | |
| > 100 | 51.5% (17/33) | 1 | | | |
| < 100 | 47.1% (32/68) | 1.1 (0.72 –1.7) | 0.67 | 1.4 (0.58–3.3) | 0.472 |
| History of TB | | | | | |
| No | 53.3% (40/75) | 1 | | | |
| Yes | 34.6% (9/26) | 0.65 (0.37 – 1.1) | 0.10 | 0.44 (0.17–1.1) | 0.085 |
| OR = odds ratio | | | | | |

### 3.4 Treatment and Follow-up

#### 3.4.1 Highly Active Antiretroviral Therapy

After baseline assessment, 177 study participants started HAART at ALERT hospital. Among them 76 (43 %) received combination of stavudine, lamivudine and nevirapine (d4t - 3TC-NVP), while 48 (27.2%) received the combination of stavudine, lamivudine and efavirenz (d4t-3TC-EFV), 37(20.9%) received the combination of Zidovudine, lamivudine and nevirapine (AZT- 3TC-NVP), and 16 (9%) received the combination of Zidovudine, lamivudine and efavirenz (AZT - 3TC-EFV).

During follow up substitutions of ART were made for 14 study participants. Nevirapine substituted with efavirenz in 7 study participants (5 patients who developed ART associated TB and 2 patients who developed hepatotoxicity). Zidovudine substituted with efavirenz in 3 patients who developed severe anemia, stavudine substituted with Zidovudine in 1 patient who developed peripheral neuropathy , efavirenz substituted with tenofovir (TDF) in 1 patient who developed severe skin rush. stavudine and nevirapine substituted with Zidovudine and efavirenz in 1 patient who developed hepatotxicicty and severe skin rush and Zidovudine and nevirapine substituted with stavudine and efavirenz in 1 patient.

### 3.4.2 Follow-up

Among 177 study participants enrolled in the study, 161 (91%) study participants (151 who had completed 6 months of follow up duration and 10 who had at least one follow up investigation) were included in the follow up analysis. 16 study participants with no follow up investigation were included in the baseline analysis but excluded from follow-up analysis. The reasons of 26 study participants who didn't complete the study period include, pregnancy 3 (11.5%), death 2 (7.7%), preferring the remedy of holy water ''tesbel" 2 (7.7%), being referred to other health institutions 3 (11.5%), and 1(3.8%) dropped out from the study complaining inability to feed her self as the result of ''HAART induced hunger'' and the remaining 15 (57.7%) were lost to follow up for unknown reasons (Figure 3.2).

## 3.5 Follow-up Laboratory Data

### 3.5.1 Effect of HAART on Reconstitution of CD4 T cell count

a) **Recovery of median CD4 cell count over time**

To assess the effect of HAART on the reconstitution of CD4 T cell, we compared CD4 T cell levels at the $3^{rd}$ and $6^{th}$ months after treatment with levels at baseline using Wilcoxon signed rank test. The result shows that CD4 cell count was significantly increased ($p<0.0001$) from a median of 130 (IQR, 65-184) at baseline to 238 (IQR, 140 - 342) at month three and 228 (IQR, 158- 356) at month six of HAART (Figure 3.7). Although the median CD4 cell decreased slightly from 238 (IQR, 140 - 342) at third month to 228 (IQR, 158- 356) at $6^{th}$ month, the difference is not statistically significant ($p=0.2513$).

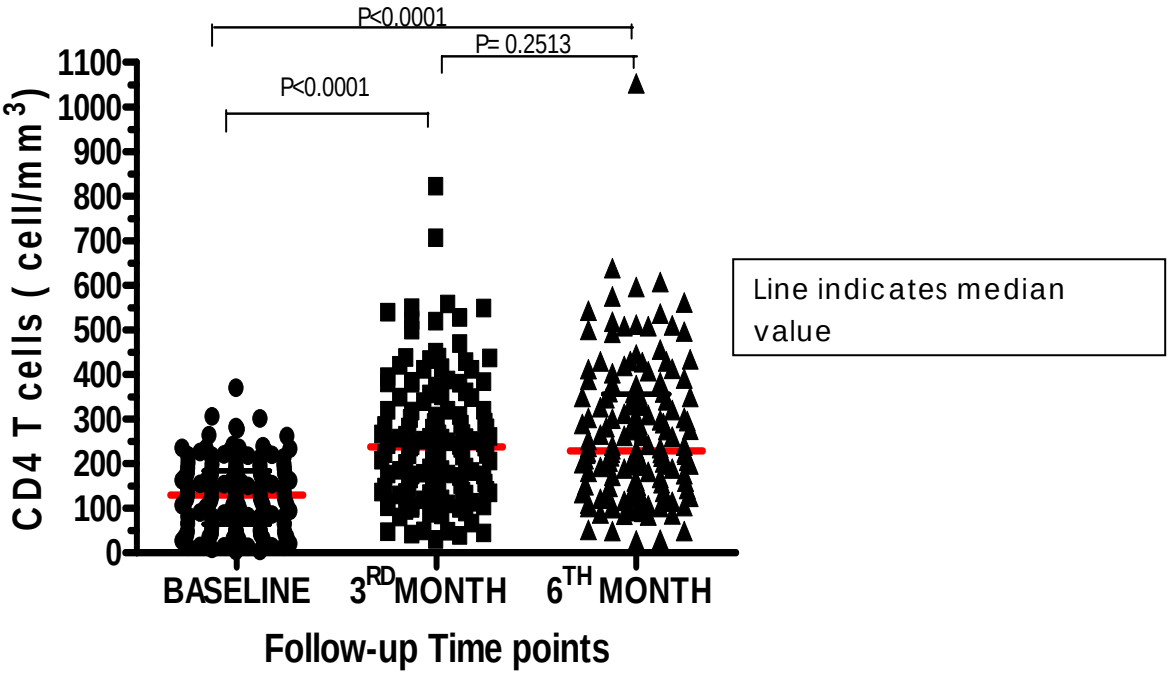



**Figure 3.7 Median CD4 cell count at baseline, 3rd and 6th month**

b) **Attaining CD4 cell count ≥ 200 cells/mm$^3$ over time**

We also assessed effects of HAART on recovery of CD4 count by the proportion of patients who had achieved CD4 cell count above the zone of severe immunodeficiency at 3rd and 6th months compared to baseline (Figure 3.8). The proportion of patients with CD4 counts > 200 cells/mm$^3$ significantly increased from baseline 19.9% (32/161) to 60.6% (83/137) and 64.4% (94/146) at 3rd and 6th months, respectively (p< 0.0001).

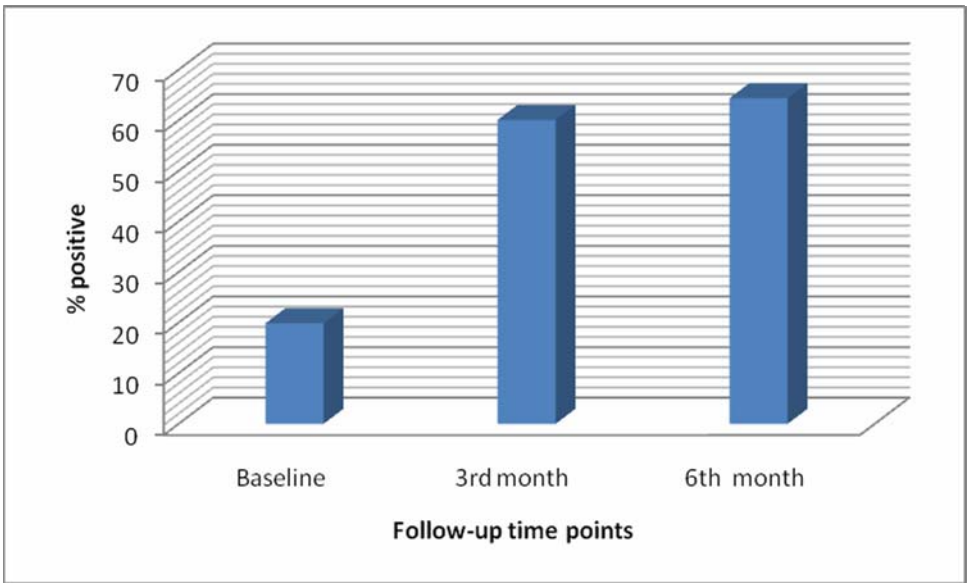



**Figure 3.8 Proportion of patients with CD4 cell count > 200 cells/mm3 at baseline and at the 3rd and 6th months of HAART**

c) **Attaining CD4 cell count ≥ 350 cells/mm$^3$ overtime**

Effect of baseline CD4 cell count on recovery of CD4 cell count at 3rd and 6th month of treatment was assessed by grouping baseline CD4 cell is shown in Table 3.6. There was no significant difference on the change in magnitude of median CD4 cell count between groups at 3rd month (comparison among 4 groups, p= 0.567) or at 6th month (p= 0.458). Among study participants, 23.3% (32) and 26% (38) had achieved CD4 cell count ≥ 350 cells/mm$^3$ at 3rd and 6th month respectively. The proportion of participant who have achieved CD4 cell count ≥ 350 cells/mm$^3$ at 3rd and 6th month increased significantly as baseline CD4 cell group increased (comparison among the 4 groups at 3rd month P<0.0001; at 6th month p= 0.01).

**Table 3.6 Proportion of study participants having 3rd and 6th month CD4 T-cell counts ≥200 or ≥350 cells/mm3 classified by baseline CD4 T strata**

| | Baseline CD4 cell count (cells/mm$^3$) groups | | | | **All the Participant** | P value |
|---|---|---|---|---|---|---|
| | <50 | 50-99 | 100- 199 | ≥ 200 | | |
| **No participants** | 36 | 22 | 71 | 32 | 161 | |
| Age >40* | 11.1% (4/36) | 27.3%(6/22) | 15.5%(11/71) | 12.5%(4/32) | 15.5%(25/161) | 0.381 |
| Change of CD4 cont** | | | | | | |
| 3rd month – baseline | 89(48-177) | 120(59-217) | 91(43-156) | 133(15-224) | 91(48-173) | 0.567 |
| 6th month – baseline | 94(70-180) | 124(59-244) | 110(49-229) | 75(14-217) | 104(50-210) | 0.458 |
| CD4 cell count* ≥200 cells/mm$^3$ at | | | | | | |
| 3rd month | 26.7(8/30) | 50(10/20) | 65.6(40/61) | 96.2(25/26) | 60.6(83/137) | 0.000*** |
| 6th month | 31.3(10/32) | 52.6(10/19) | 70.8(46/65) | 93.3(28/30) | 64.4(94/146) | 0.000*** |
| CD4 cell count* ≥350 cells/mm$^3$ at | | | | | | |
| 3rd month | 6.7(2/28) | 20(4/20) | 18(11/61) | 57.7(15/26) | 23.3(32/137) | 0.000*** |
| 6th month | 9.4(3/32) | 21.1(4/19) | 27.7(18/47) | 46.7(14/30) | 26.(38/146) | 0.01*** |

*% (Number of patients). ** median CD4 cell count cells/mm$^3$

d) **Relative risk of having CD4 count > 200 or >350 cell count over time in relation to baseline CD4 cell level**

Tables 3.7 and 3.8 describe the relative risk of having CD4 count ≥ 200 or ≥350 cell count at 3rd and 6th month determination. Compared with the group with baseline CD4 cell ≥200 cells/mm$^3$, the relative risk of having a 3rd and 6th month determination of CD4 cell counts >200 cells/mm$^3$ was significantly lower in the patients with a baseline CD4 cell count < 50, 50-99 and 100-199 cells/mm$^3$. The chance of achieving CD4 count ≥ 200 cells/mm$^2$ at 3rd

and 6th month in respective order is 28% and 33% for those with baseline CD4 count < 50 cells / $mm^3$ (p<0.0001), 52% and 56% for baseline CD4 group 50-99, 68% and 66% for baseline CD4 group 100-199, compared with the group with a baseline CD4 cell count ≥200 cells/$mm^3$ (Table 3.7).

**Table 3.7 Relative risk of having 3rd or 6th month values of CD4 cell counts >200 cells/$mm^3$**

| *Baseline CD4 cell count* | At 3rd month | | | At 6th month | | |
|---|---|---|---|---|---|---|
| | Relative risk | (95% CI) | P value | Relative risk | (95% CI) | P value |
| <50 | 0.28 | 0.15 – 0.50 | 0.0000 | 0.33 | 0.19-.56 | 0.0000 |
| 50-99 | 0.52 | 0.33 - 0.81 | 0.0004 | 0.56 | 0.36-0.87 | 0.0015 |
| 100- 199 | 0.68 | 0.56 - 0.83 | 0.0024 | 0.66 | 0.54-0.81 | 0.0080 |
| ≥ 200 | 1 | 0.89 - 1.12 | 1 | 1 | 0.87-1.15 | 1 |

Compared with the group with a baseline CD4 cell ≥200 cells/mm3, the relative risk of having a 3rd determination of CD4 cell counts ≥350 cells/mm3 was significantly lower among patients with a baseline CD4 cell count < 50, 50-99 and 100-199 cells/$mm^3$. However, the relative risk of achiving CD4 count at 6 months is only significantly different for those with baseline CD4 cell count group <50 cells / $mm^3$ (RR .33 (CI, 0.06-0.63), p= 0.0014] (Table 3.8).

**Table 3.8 Relative risk of having 3rd or 6th month values of CD4 cell counts >350 cells/$mm^3$**

| *Baseline CD4 cell count (cells/$mm^3$)* | At 3rd month | | | At 6th month | | |
|---|---|---|---|---|---|---|
| | Relative risk | (95% CI)= | P value | Relative risk | (95% CI) | P value |
| <50 | 0.12 | 0.03-0.49 | 0.0000 | 0.33 | 0.06-0.63 | 0.0014 |
| 50-99 | 0.35 | 0.14-0.88 | 0.0156 | 0.45 | 0.17-1.17 | 0.1272 |
| 100- 199 | 0.31 | 0.17-0.59 | 0.006 | 0.82 | 0.48-1.39 | 0.4872 |
| ≥ 200 | 1 | 0.63-0.16 | 1 | 1 | 0.58-1.74 | 1 |

e) **Effect of age on recovery of CD4 cell**

We have also assessed effect of age on recovery of CD4 cell after treatment by stratifying age into two groups < 40 years and ≥40 years (Table 3.9). We found no significant difference

between age groups (<40 and ≥40 years) in median CD4 cell count before treatment at baseline (p= 0.644), and after treatment at 3rd month (p = 0.300) or 6th month (p= 0.442).

**Table 3.9 Median CD4 cell count at baseline, 3rd and 6th month stratified by age**

| | CD4 cell count cells/mm$^3$ (Median ( IQR) | | |
|---|---|---|---|
| | Baseline | 3rd month | 6th month |
| Age | | | |
| < 40 | 131( 63-189) | 244 (158-346) | 232(188-353) |
| ≥ 40 | 108 (78-166) | 154 (132-350) | 216 (117-412) |
| P vale | 0.644 | 0.300 | 0.442 |

f) **Effect of baseline CD4 count on proportion of immunological failure and immunological super responder**

We also assessed effect of baseline CD4 count on the proportion individuals achieving CD4 cell count ≥ 500 cells/mm$^3$ ('immunological super responder') or on the proportion of individuals who fail to respond to HAART (''immunological failure") at 6th moth. We found that only 9.6% (14/146) of study participants had achieved CD4 cell count ≥ 500 cells/mm$^3$ at 6th month. The proportion of immunological responders was significantly associated with baseline CD4 cell count; none of the individuals with baseline CD4 < 100/mm$^3$ had achieved CD4 count ≥ 500/ mm$^3$ compared to 12.3% (8/65) and 20% (6/30) of individuals with baseline CD4 cell strata of 100-199 and ≥ 200 cells/mm$^3$ respectively (p =. 0.021). Immunological failure was observed in 9.9% (16/146) of the study participants. There was no significant difference in the proportion of immunological failure among CD4 strata (p=0.631) (Table 3.10).

**Table 3.10 Proportion of immunological super responder and immunological failure according to baseline CD4**

| | **Baseline CD4 cell count (cells/mm3) groups** | | | | | |
|---|---|---|---|---|---|---|
| | **Total** | **< 50** | **50-99** | **100-199** | **≥ 200** | **p value** |
| Immunological Super responder* | 9.6(14/146) | 0 (0/28) | 0 (0/22) | 12.3(8/65) | 20(6/30) | 0.021 |
| Immunological failure** | 11(16/146) | 12.5(4/28) | 0(0/22) | 9.2(6/65) | 20(6/30) | 0.163 |

Immunological Super responder* = Defined as if individual had achieved an absolute CD4 cell count of 500 cells /mm$^3$ at 6th month of treatment

Immunological failure** = According to Ethiopian ART guideline, defined fall of CD4 count to pre-therapy baseline (or below) and or 50% fall from the on-treatment peak value (if known) and or persistent CD4 levels below 100 cells/mm$^3$. (FMOH/HAPCO, 2007)

For the purpose of this study, we adopted the guideline definition of immunological failure as follows If baseline CD4 count ≥ 6th month and or if 3rd month CD4 count ≥ twice the value of CD4 cell count at 6th month and or if both 3rd and 6th month CD4 cell count < 100 cells/ mm$^3$

### 3.5.2 Effect of HAART on restoration of TB specific immune response

### I) TST response after HAART

#### a) TST response in relation to baseline CD4 count

To assess the effect of HAART on restoration of skin test response, we have analyzed the proportion of skin test positive response at baseline, 3rd and 6th month of treatment. The proportion of positive response increased significantly from base line 17.5% to 3rd month 26.8% and to 6th month 28.9% (p= 0.02). Significant increment in proportion of TST response was observed on participants having baseline CD4 cell count less than 50 cells /$mm^3$ when the result was categorized by baseline CD4 strata (Table 3.11).

**Table 3.11 Proportion of study participants having TST >5mm indurations at baseline, 3rd and 6th month classified by baseline CD4 cell strata**

| *Baseline CD4 cell count (cells/$mm^3$)* | *BASLINE n = 154* | *$3^{RD}$ MONTH n= 123* | *$6^{TH}$ MONTH n=121* | p value |
|---|---|---|---|---|
| <50 | 0/36 (0%) | 2/25 (8%) | 4/23 (17.4%) | 0.0122* |
| 50-99 | 3/22 (13.6%) | 7/18 (38.9%) | 5/19 (26.3%) | 0.3208 |
| 100- 199 | 15/66 (22.7%) | 13/54 (24.1%) | 15/55 (27.3%) | 0.5671 |
| ≥ 200 | 9/ 31(28.1%) | 11/26 (42.3%) | 11/24 (45.8%) | 0.1918 |
| Total | 27/154 (17.5%) | 33/123 (26.8%) | 35/121(28.9%) | 0.02* |

#### b) TST response in relation to enrolement TB status

We also compared the proportion skin test reactivity before and after treatment with stratified analysis according to baseline TB status as shown in Table 3.12, we found a significant increment in proportion of TST positive response from baseline 21% to 66% at 3rd month (P= 0.015) and 53.4 % at 6th month (P=0.05) among group with active TB at baseline. However, no significant increment was observed in the remaining groups.

**Table 3.12 Proportion of study participants having TST >5mm indurations at baseline, 3rd and 6th month classified by baseline TB status**

| ***Baseline TB status*** | **Proportion of TST positive response** | | | | | **Pt trained** |
|---|---|---|---|---|---|---|
| | BASE | $3^{RD}$month | P3 | $6^{TH}$ MONTH | P6 | |
| Active TB | 3/14 (21.4%) | 8/12 (66.7%) | **0.015** | **7/13 (53.8%)** | **0.05** | **0.0554*** |
| On Anti-TB Rx | 2/9 (22.2%) | 1/6 (16.7%) | **> 0.05** | **2/7 (28.6%)** | **> 0.05** | **0.8768** |
| Past History of TB | 3/33 (9.1%) | 5/24 (20.8%) | **> 0.05** | **5/23 (21.7%)** | **> 0.05** | **0.3460** |
| No History of TB | 19/98 (19.4%) | 19/81 (23.5%) | **> 0.05** | **21/78 (26.9%)** | **> 0.05** | **0.4939** |

P3 = p value for difference between baseline and 3rd month
P6= p value for difference between baseline and 6th
Pt= p value for trained (baseline, 3rd & 6th month)

Since we observed the confounding effect of active TB on the proportion of TST after HAART, we excluded individuals with prevalent TB at baseline and individuals who subsequently develop TB while taking ART and analyzed the data, to assess change on the proportion of TST reactivity after HAART among group stratified according to baseline CD4 strata. We found a significant increment in proportion of TST reactivity among groups with baseline CD4 < 50 cells/mm$^3$ after HAART (P = 0.0386) (Table 3.13).

**Table 3.13 Proportion of TST reactivity over time among individuals with out prevalent TB according to baseline CD4 cell strata**

| Baseline CD4 cell count | *BASLINE N = 123* | *3$^{RD}$ MONTH N = 101* | *6$^{TH}$ MONTH N=94* | p value |
|---|---|---|---|---|
| <50 | 0/26 (0%) | 0/20 (0%) | 2/16 (12.5%) | 0.0386 |
| 50-99 | 2/14 (14.3%) | 4/11 (36.7%) | 2/12 (16.7%) | 0.3626 |
| 100- 199 | 8/54 (14.8%) | 9/45 (20%) | 7/44 (15.9%) | 0.7760 |
| ≥ 200 | 7/29 (24.1%) | 10/25 (40%) | 9/22 (40.9%) | 0.3468 |
| Total | 17/123 (13.8%) | 23/101 (22.8%) | 20/94(21.3%) | 0.1819 |

P < 0.05 (Chi-square test for trend)
Patients with active TB at baseline or who were taking anti TB treatment at enrolment were excluded from analysis

**c) Effect of baseline CD4 cell level on proportion of TST reactivity over time**

Effect of baseline CD4 cell level on proportion of TST reactivity over time was farther assessed among individuals without prevalent TB at enrolment. The difference on the proportion of TST reactivity was compared at each time points (baseline, 3$^{rd}$ and 6$^{th}$ month) by stratifying the result according to baseline CD4 cell count < 100 and ≥ 100 cells/mm$^3$. We found difference on the proportion TST reactivity only at baseline (5% Vs 18.1%, P=0.0491 groups with CD4 < 100 Vs ≥ 100 cells/mm$^3$ respectively). Although, the proportion of TST reactivity after HAART was higher among groups with baseline CD4 cell count ≥ 100 cells/mm$^3$ compared with group with baseline CD4 cell < 100 cells/mm$^3$, the difference was not statistically significant (Table 3.14).

**Table 3.14 Comparison TST reactivity between individuals with baseline CD4 < 100 and ≥ 100 cells/mm3 before and after HAART**

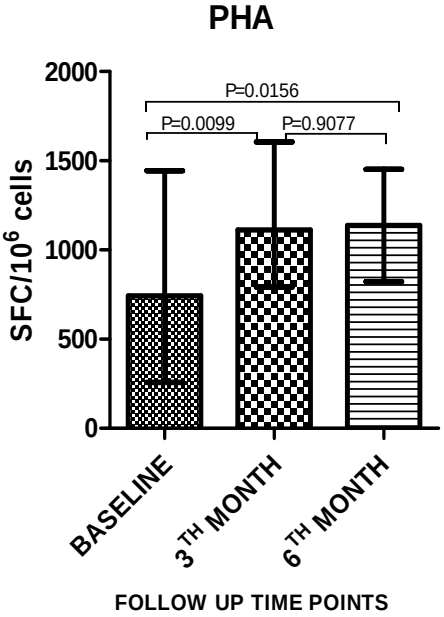
| Baseline CD4 cell strata ( cells/mm$^3$ ) | Proportion of TST positive response | | |
|---|---|---|---|
| | **Baseline** | **3$^{rd}$ Month** | **6$^{th}$ Month** |
| < 100 | 2/40 (5%) | 4/31 (12.9%) | 4/28 (14.3) |
| ≥100 | 15/83 (18.1%) | 19/70 (27.1%) | 16/66 (24.2) |
| P value | 0.0491 | 0.1155 | 0.2807 |

II. **Effect of HAART on ELISPOT response**

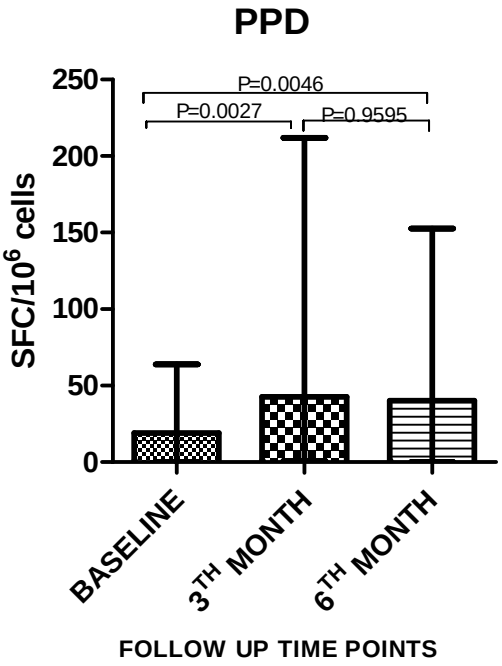
a) **Quntitative ELISPOT response for mitogen and TB antigens**

Figure 3.9 shows quantitative IFN γ ELISPOT response over time in PBMC stimulated with mitogen (PHA) and TB antigens including; PPD, ESAT-6 and CFP10. The result indicated that the median SFC/million was significantly increased for mitogen and for all individual TB antigens after HAART at 3rd and 6th month. Median response SFC/$10^6$ PBMC increased for PHA from baseline 743 (IQR, 243, 1444) to 1113 (IQR, 793, 1605, p=0.0099 ) at 3rd month and to 1138 (IQR, 823, 1453, p =0.0156 ) at 6th month, for PPD increased from baseline 19 (IQR, 0, 64) to 43 (IQR, 1, 212, p=0.0027 ) at 3rd month and to 40 (IQR, 0, 153, p = 0.0046) at 6th month, for ESAT-6 from baseline 18 (IQR, 0,45) to 35 (IQR, 117, p=0.0123 ) at 3rd month and to 40 (IQR; 0, 148, p = 0.004) at 6th month, and for CFP-10 from baseline 15 (IQR, 0,48) to 30 (IQR, 0, 101, p=0.0026 ) at 3rd month and to 31(IQR, 0, 131, p = 0.0032) at 6th month. However, there is no difference in median SFC/$10^6$ PBMC response between 3rd and 6th month response for mitogen and individual TB antigens (p>0.05).

(A)




(B)



(C) (D)

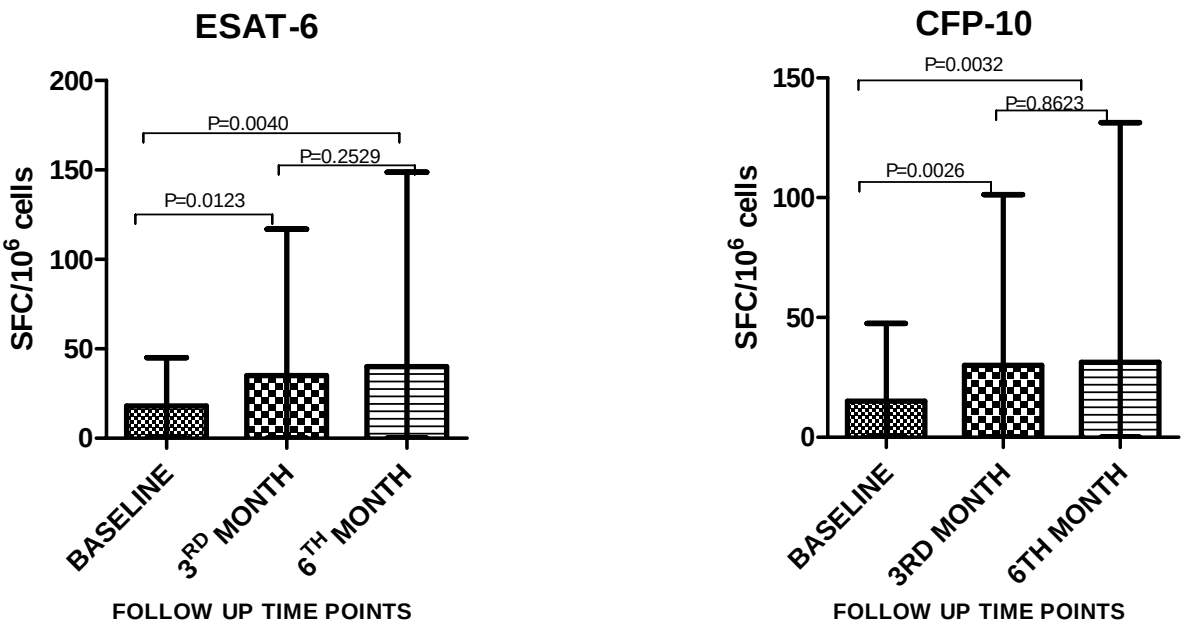



**Figure 3.9 Effect of HAART over time on median frequency of spot forming cell after stimulation of PBMC with PHA (A), PPD (B), ESAT- 6 (C), and CFP-10(D)**

b) **Quntitative ELISPOT response for mitogen and TB antigens in relation with basline CD4 cell count**

To assess effect of baseline level of immunodeficiency on reconstitution of response to mitogen and TB specific immune response, the median frequency of SFC/$10^6$ PBMC at 3rd and 6th month was compared by stratifying the result with baseline CD4 cell count. As shown in the Table 3.15, baseline CD4 cell count strata, which is associated with an increase in median frequency of SFC/$10^6$ PBMC, was CD4 cell <50 cells/ $mm^3$ for PHA response at 3rd and 6th month ( p= 0.009 and p=0.02 respectively, Table 3.15, a), for PPD response at 3rd month (p=0.0017, Table 3.15b, and for ESAT-6 response at 3rd and 6th month (p=0.05 and p=0.024 Table 3.15, c). Baseline CD4 cell strata 50 -99 cells/$mm^3$ was only associated with an increase PHA response at 3rd (p=0.05, 3.15, a). Baseline CD4 strata $\geq$200 cells/$mm^3$ was associated with an increase in median frequency of SFC/$10^6$ PBMC CFP-10 response at 6th month (p= 0.006, Table 3. 15, d).

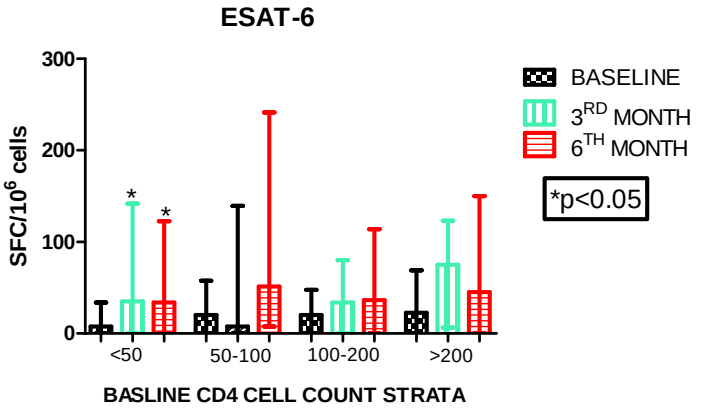
### c) Effect of baseline CD4 level on restoration of quantitative ELISPOT response to mitogen and TB antigens

Since the confounding effect of current Anti-TB treatment on quantitative ELISPOT response reported in pervious studies elsewhere (Ulrichs *et al.*, 2000), we excluded study participants with prevalent TB at baseline and assessed effect of baseline CD4 cell level on the extent of restoration quantitative ELISPOT response. As shown in Table 3.16 A, the median SFC/$mm^3$ to PHA was significantly higher among individual with enrolment CD4 cells ≥ 100 at baseline compared to individuals with CD4 < 100 cells/$mm^3$ before HAART. However, no effect of baseline CD4 cell on reconstitution of PHA response observed after treatment. Although the difference was not statistically significant, before treatment higher quantitative ELISPOT responses to PPD, ESAT-6 and CFP-10 were observed among individuals with CD4 > 100 cells/ $mm^3$. After treatment similar pattern in magnitude of increased quantitative ELISPOT responses to these antigens were observed among both CD4 groups. However, except ESAT-6 response at $6^{th}$ month, the median SFC/million responses were still higher among the group with CD4 ≥100 cells/$mm^3$ but the difference was not significant except for quantitative PPD response at $6^{th}$ month (Table 3.16, B).

**Table 3.15 Effect HAART over time on median frequency of spot forming cell after stimulation of PBMC with PHA (a), PPD (b), ESAT- 6 (c), and CFP-10(d) stratified by baseline CD4 count**

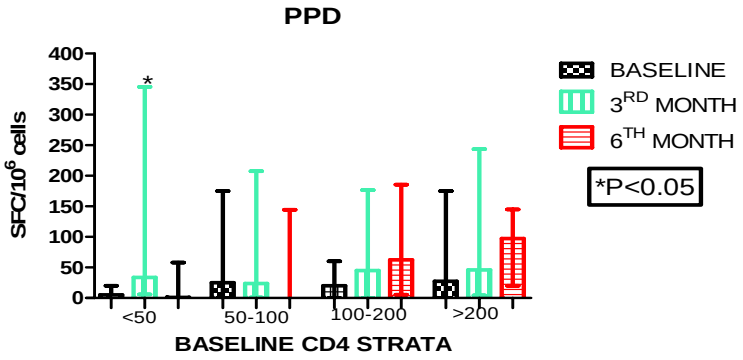
## a)PHA

| CD4 STRATA | BASELINE | 3RD MONTH | 6TH MONTH |
|---|---|---|---|
| <50 | 310 (100, 810) | 890 (429, 468)* | 1053(678, 1477)* |
| 50-99 | 880 (268, 1170) | 1118(821, 1703)* | 1125(711, 1605)* |
| 100- 199 | 970(413, 1743) | 1135(839, 1620) | 1146(819, 1551) |
| ≥ 200 | 1090(390, 2016) | 1069(418, 1531) | 1140(1100, 1205) |
| * p<0.05 | | | |



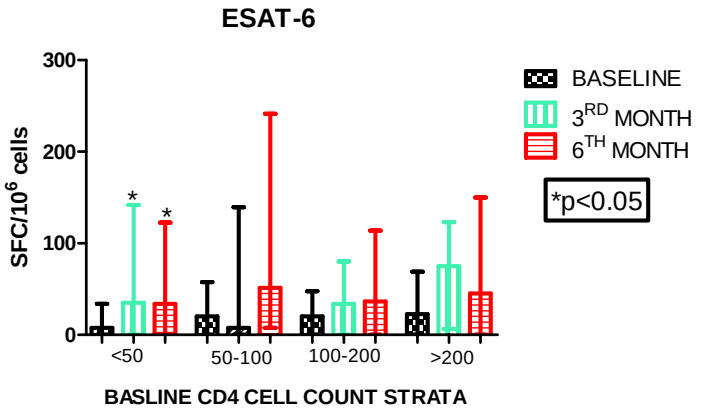

## b) PPD

| CD4 STRATA | BASELINE | 3RD MONTH | 6TH MONTH |
|---|---|---|---|
| <50 | 5(0, 20) | 34(6, 346)* | 1(0, 58) |
| 50-99 | 25(0, 175) | 24(1, 208) | 0(0, 144) |
| 100- 199 | 20(0, 60) | 45(0, 177) | 63(6, 186) |
| ≥ 200 | 28(0, 175) | 46(4, 244) | 98(20, 145) |
| * p<0.05 | | | |



## c) ESAT-6

| CD4 STRATA | BASELINE | 3RD MONTH | 6TH MONTH |
|---|---|---|---|
| <50 | 8(0, 34) | 35(0, 142)* | 34(0, 123)* |
| 50-99 | 20(0, 58) | 8(0, 139) | 51(8, 241) |
| 100- 199 | 20(0, 48) | 34(0, 80) | 36(0, 114) |
| ≥ 200 | 23(0, 69) | 75(6, 123) | 45(0, 150) |
| * p<0.05 | | | |




## d) CFP-10

| CD4 STRATA | BASELINE | 3RD MONTH | 6TH MONTH |
|---|---|---|---|
| <50 | 8(0, 39) | 30(9, 101) | 24(0, 56) |
| 50-99 | 25(3, 90) | 10(0, 195) | 15(0, 239) |
| 100- 199 | 15(0, 60) | 35(0, 85) | 25(0, 120) |
| ≥ 200 | 20(0, 43) | 45(1, 230) | 108(25, 185)* |
| * p<0.05 | | | |

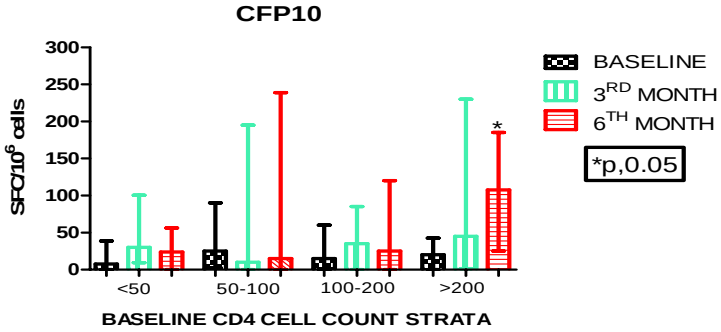


**Table 3.16 Effect of baseline CD4 level on restoration of quantitative ELISPOT response to PHA (A) and to TB antigens (B)**

(A)

| Baseline CD4 cell strata ( cell/$mm^3$ ) | PHA | | |
|---|---|---|---|
| | **Baseline** | **3rd Month** | **6th Month** |
| < 100 | 438 (136-989) | 1098 (498-1739) | 1075 (608-1285) |
| ≥ 100 | 1000 (385-1895) | 1120 (713-1644) | 1138 (918-1364) |
| P value | 0.0020 | 0.8392 | 0.1846 |
| Median SFC/million PBMC ( Inter Quartile Range ) | | | |

(B)

| Baseline CD4 cell strata Cells/$mm^3$ | **PPD** | | | **ESAT-6** | | | **CFP-10** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Baseline** | **3rd Month** | **6th Month** | **Baseline** | **3rd Month** | **6th Month** | **Baseline** | **3rd Month** | **6th Month** |
| < 100 | 5 (0, 20) | 23 (4, 256) | 10 (0, 110) | 10 (0,34) | 8 (0,88) | 40 (3, 180) | 10 (0, 48) | 25 (2, 96) | 28 (0, 105) |
| ≥ 100 | 25 (0, 63) | 43 (0, 124) | 78 (8, 186) | 20 (0,45) | 35 (0,111) | 40 (0,149) | 15 (0, 35) | 35 ( 0,108) | 41 (0, 170) |
| P value | 0.096 | 0.616 | 0.042 | 0.212 | 0.376 | 0.364 | 0.907 | 0.757 | 0.746 |
| Median SFC/million PBMC ( Inter Quartile range ) | | | | | | | | | |

### d) Qualitative ELESPOT response over time stratified by baseline CD4 cell count

Qualitative ELISPOT positive responses were analyzed based on the combination of antigen used in the assay. There was no significant difference on the proportions of EC ELISPOT positive responses between baseline, 3rd and 6th month [63 (52.9%), 60 (53.1%) and 62 (51.2%) p>0.9506 respectively] (Table 3.17 A) or ECP ELISPOT positive response between baseline, 3rd and 6th month (73 (61%), 69 (61.1%) and 75 (62%) p = 0.9878 respectively) (Table 3.17 B). Stratified analyses according to CD4 cell count also indicated baseline level of immunodeficiency have no effect on proportion of both EC and ECP ELISPOT positive response after HAART.

**Table 3.17 Proportion of positive ELISPOT responses (A. based on EC result and B on ECP result at baseline 3rd and 6th month of treatment by baseline CD4 cell count**

**A) EC (ESAT-6 and CFP-10) ELISPOT positive response**

| ***Baseline CD4 T cell Strata*** | **Proportion of ELISPOT positive response** | | | **p value** |
|---|---|---|---|---|
| | Baseline | 3rd Month | 6th Month | |
| < 50 | **13(56.5%)** | **13(52%)** | **14(51.9%)** | **0.9344** |
| 50- 99 | **10(58.8%)** | **8(53.3%)** | **10(55.6%)** | **0.9514** |
| 100 -199 | **26(49.1%)** | **26(50%)** | **26(49.1%)** | **0.9938** |
| ≥ 200 | **14(53.8%)** | **13(61.9%)** | **12(52.2%)** | **0.7867** |
| Total | **63(52.9%)** | **60(53.1%)** | **62(51.2%)** | **0.9506** |

**B) ECP (ESAT-6, CFP-10 and PPD) ELISPOT positive response**

| ***Baseline CD4 T cell Strata*** | **Proportion of ELISPOT positive response** | | | **p value** |
|---|---|---|---|---|
| | Baseline | 3rd Month | 6th Month | |
| < 50 | **14(60.9%)** | **16(64%)** | **15(55.6%)** | **0.8203** |
| 50- 99 | **11(64.7%)** | **9(60%)** | **11(61.1%)** | **0.9587** |
| 100 -199 | **31(58.5%)** | **29(55.8%)** | **35(66%)** | **0.5370** |
| ≥ 200 | **17(65.4%)** | **15(71.4%)** | **14(60.9%)** | **0.7614** |
| Total | **73(61%)** | **69(61.1%)** | **75(62%)** | **0.9878** |

**e) Qualitative ELISPOT response over time stratified by baseline TB status**

Qualitative responses were also analyzed by stratifying the result with baseline TB history. As shown in the table below (Table 3.18 A). There is no association between baseline TB status and the proportion of both EC ELISPOT and ECP ELISPOT positive response. However, there is a non significant trend in decreasing the proportion of EC ELISPOT positive response on patients who were on Anti-TB treatment at enrolment.

**Table 3.18 Proportion of positive ELISPOT response (a) based on EC result and (b) on ECP result at baseline 3rd and 6th month of treatment stratified by baseline TB history.**

**A) EC (ESAT-6 and CFP-10) ELISPOT positive response**

| ***Baseline TB status*** | ***Proportion of ELISPOT positive response*** | | | **p value** |
|---|---|---|---|---|
| | Baseline | 3rd Month | 6tth Month | |
| Active TB | **8(72.7%)** | **9(75%)** | **7(53.8%)** | **0.4681** |
| On Anti-TB Rx | **5(62.5%)** | **2(40%)** | **3(37.5%)** | **NA** |
| Past History of TB | **10(40%)** | **11(44%)** | **11(42.3%)** | **0.9595** |
| No History of TB | **40(53.3%)** | **38(53.5%)** | **41(55.4%)** | **0.9617** |
| **NA = analysis is not applicable** | | | | |

**B) ECP (ESAT-6, CFP-10 and PPD) ELISPOT positive response**

| ***Baseline TB status*** | ***Proportion of ELISPOT positive response*** | | | **p value** |
|---|---|---|---|---|
| | Base | 3rd Month | 6th Month | |
| Active TB | **9(81.8%)** | **10(83.3%)** | **9(69.2%)** | **0.6480** |
| On Anti-TB Rx | **6(75%)** | **3(60%)** | **5(62.5%)** | **NA** |
| Past History of TB | **13(52%)** | **14(56%)** | **13(50%)** | **0.9094** |
| No History of TB | **45(60%)** | **42(59.2%)** | **48(64.9%)** | **0.7455** |
| **NA = analysis is not applicable** | | | | |

### 3.6 ART associated TB, Unmasking and Paradoxical TB IRIS

To determine the incidence and characteristic of patients who develop ART associated tuberculosis and paradoxical TB IRIS, study participants enrolled in our study were categorized based on baseline TB status (current and past history of TB) (Figure 3.10). A total of 149 study participants (114 study participants who had no history of TB and 35 participants who had past history of treated TB) were eligible at enrolment for ART associated tuberculosis study cohort. A total of 31 study participants, 25 among 28 at enrolment (15 patients who had active TB at baseline investigation and 13 patients who had been diagnosed with active TB and already started anti TB therapy prior enrolment in this study) and 6 patients who developed TB while taking HAART were eligible for paradoxical TB IRIS study.

#### a) ART associated TB

Among 149 study participants without prevalent TB at enrolment, 127 (85.2%) had complete $6^{th}$ month follow up durations, 9 (6%) dropped out from the study after $3^{rd}$ month investigation (the time interval between HAART initiation and last visit date was taken for person-year calculation and included in the analysis), 13 (8.7%) lost to follow up soon after HAART initiated and excluded in the analysis. One hundred thirty-six (91.2 %) study participants who were included in the analysis contributed a total 62.79 person- year observations. Eight individuals developed active TB within the six month treatment, which represents the incidence of ART associated TB 12.7 cases per 100 person-year observation (Table 3.10).

Among eight ART associated tuberculosis, three cases were pulmonary TB (two cases of smear positive pulmonary tuberculosis and one case of smear negative pulmonary TB and four cases were extra pulmonary TB (two cases with peritoneal TB, one case with scrotal TB and one case with lymph node TB) and the remaining one case was with disseminated TB (Table 3.19).

The characteristics of patients with ART associated tuberculosis are described in Table 3.19. Among 8 individuals who developed TB 6 were male. At initiation of HAART, the median age of patients was 37 years (IQR, 31- 44 years), and the median CD4 cell count was 110 (IQR, 43-151). The median time to onset of the TB episode was 28 days (IQR, 18–100 days), and 75% (6/8) of events occurred within 3 months after initiation of HAART.

**b) Characteristics related with ART associated tuberculosis**

Comparison on the demographic, clinical, and laboratory characteristics at baseline and at 3rd and 6th months after initiation of HAART of 8 patients with ART associated TB and 128 control patients who did not develop TB are shown in Table 3.20. At baseline patients with ART associated TB, compared with patients without TB event, were older (median age, 37(IQR, 31- 44) years vs 31(IQR, 28-37) years p = 0.05), a significant proportion of them were male 6/8 (75 %) vs 37/128 (26.9 %); p=0.012), higher proportion of them had hemoglobin less than 12gm/dl 6/8 (75%) vs 42/127 (33.1%); p=0.024) and higher proportion of them had skin test positive response 'TST>5mm' 5/8 (62.5 %) vs 13.8 % (17/123; p=0.003). However, there is no difference between patients with ART associated TB and without in terms of baseline CD4 count, CD8 count, CD4/CD8 ratio, liver function test, qualitative ELISPOT positive response and quantitative ELISPOT response.

After initiation of HAART patient with ART associated TB compared to without TB, had lower median CD4 cells count at 3rd and 6th month (3rd month 124.5 (IQR, 94-158) vs 245 (IQR, 153 – 347, p = 0.0066) and 6th month 101 (IQR, 82-191) vs 235 (IQR, 171 – 360; p= 0.0034), higher proportion of them had 3rd month hemoglobin value less than 12gm/dl 4/5 (80%) vs 29/114 (25.4%); p=0.021) and had higher proportion of 6th month TST response >5mm 6/7 (85.7%) vs 21.3% (20/94); p=0.001).and had higher 3rd month quantitative ELISPOT response to TB antigens (median PPD response SFC/million, 228 (IQR, 45-362) vs 25 (IQR, 0-122, p = 0.032 ), median ESAT-6 response; 355 (IQR, 35-415) Vs 30 (IQR, 0-90; p= 0.019), and CFP-10 response; 330 (IQR, 30-395) vs 25 (IQR, 0-85, p= 0.029).

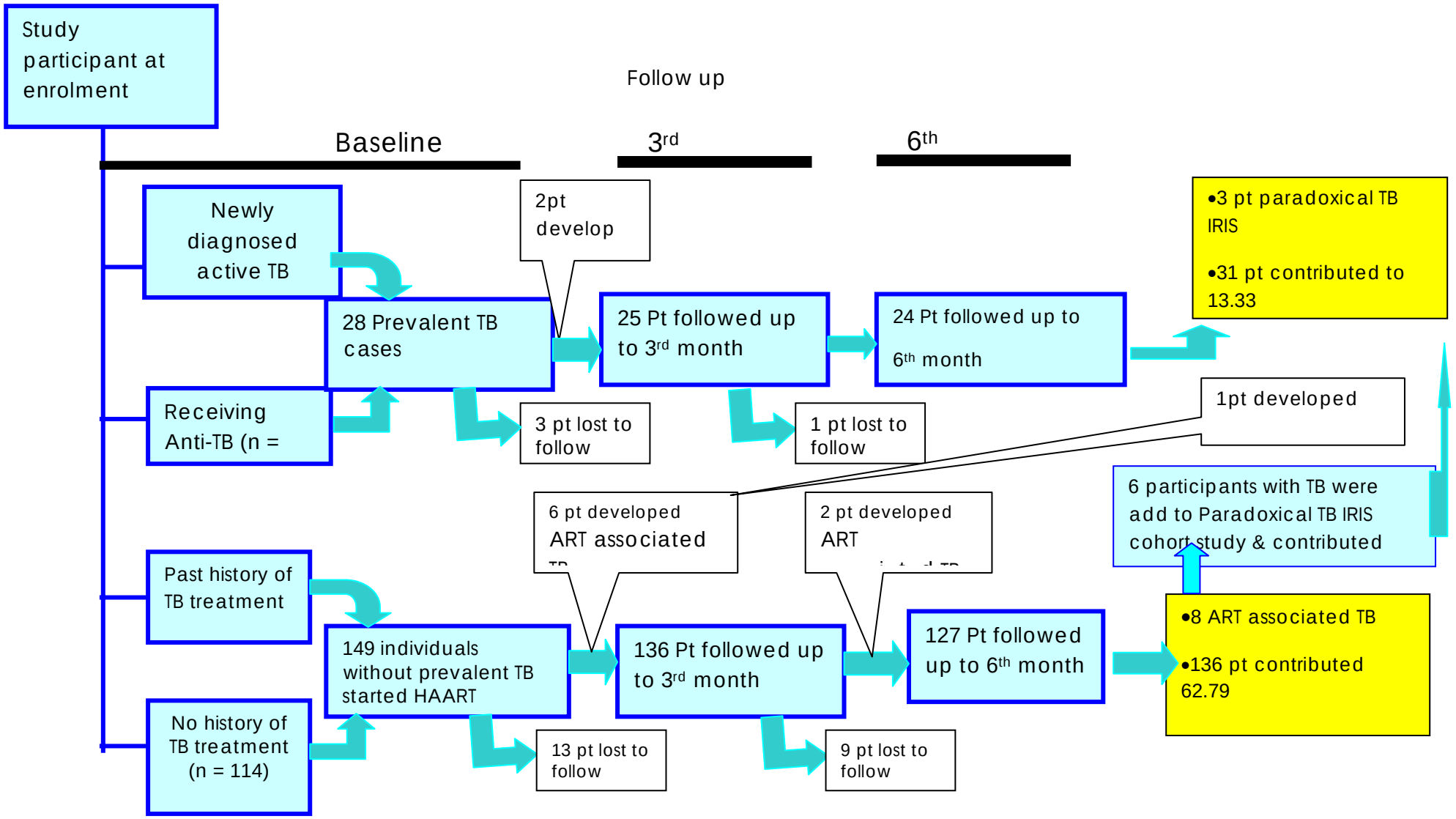



**Figure 3.10 Cohort profiles of study participants categorized according to baseline TB status to determine incidence of ART associated TB and IRIS**

**Table 3.19 Characteristics patients with ART associated tuberculosis**

| S N0 | Sex & Age | Time of on set (Day) | Site TB | Diagnosis | Characteristic at baseline | | | Characteristic at IRIS | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | CD4 cells/mm$^3$ | TST mm | ELISPOT (EC) | CD4 cells/mm$^3$ | TST mm | ELISPOT (EC) |
| 1 | M/38 | 28 | PTB | AFB + Ve | 54 | 0 | + Ve | 98$^{3rdm}$ | 13.5$^{3rdm}$ | + Ve |
| 2 | F/30 | 106 | PTB | AFB + ve | 39 | 0 | - Ve | 78 | 0 | + Ve |
| 3 | M/54 | 24 | PTB | AFB –ve [3]X-RAY | 92 | 10 | + Ve | 85 | 10 | + Ve$^{3rdm}$ |
| 4 | F/35 | 28 | EPTB TB Lymphadenitis Axillary | Pathology[1] | 275 | 28.5 | + Ve | 107$^{6TM}$ | 28$^{6thm}$ | No sample |
| 5 | M/28 | 79 | EPTB Peritoneal TB | Pathology[2] | 153 | 21 | + VE | 117$^{3d}$/182$^{6h}$ | 4$^{irs}$/20$^{6m}$ | pos |
| 6 | M/44 | 13 | EPTB Peritoneal TB | X-ray[5] & Pathology[6] | 11 | 0 | Anergy | 9$^{IRIS}$ / 48$^{6tm}$ | 9.5 | -Ve |
| 7 | M/32 | 180 | EPTB Scrotal TB | Pathology[4] | 128 | 18 | -VE | 176$^{3d}$ /95$^{6th}$ | 10 | +Ve$^{3rdm}$ |
| 8 | M/43 | 10 | Disseminated TB | X-ray, ultrasound and pathology[7] | 142 | 18 | + VE | 132 | 6.5 | +Ve |

[1]Pathology , Caseous necrosis, sheet of degenerating cells consistent with TB lymphadenitis
[2]Pathology: peritoneal fluid analysis, proteinaceous background containing sheet of lymphocytes consistent with TB peritonitis.
[3]CX-ray Bilateral infiltrate pul TB
[4]Pathology; 4x3cm firm, mobile mass between the testes, Asp-pus; Caseous necrosis, sheet of degenerating cells consistent with tuberculosis cold abscess
[5] CX-ray has basal infiltration and effusion obliterating the costophrenic angles
6Pathology; peritoneal fluid analysis, sheet of lymphocytes in a proteinaceous back ground, few mesothelial cells consistent with TB pleurisy
[7] Pathology pleura effusion analysis, X-ray, Ultrasound revealed mass 2 lap and plural effusion

**Table 3.20 Univariate analysis on characteristics of patients with and patients without tuberculosis events within the 6 months after initiation of HAART**

| Characteristics | ART associated TB | | p value |
|---|---|---|---|
| | Yes ( N=8 ) | No ( N= 128 ) | |
| Age | | | |
| Median | 37(31- 44) | 31(28-37) | 0. 05* |
| Sex | | | |
| Male | 75%(6/8) | 26.9%(37/128) | 0.012* |
| Weight | | | |
| Basline | 49(43-59) | 59(44-46) | 0.7091 |
| 3rd Month | 51(40-48) | 52(47-58) | 0.2020 |
| 6th Month | 50(42-65) | 53(49-61) | 0.3127 |
| Past history of TB | 25%(2/8) | 25.8%(33/128) | 1 |
| CD4 cell count | | | |
| <100 cells/mm$^3$ | 50%(4/8) | 31.3%(44/128) | 0.2714 |
| WHO Clinical Stage | | | |
| Stage3 or Stage4 | 62.5%(5/8) | 62.5%(40/128) | 1 |
| CD4 cell count, median | | | |
| Baseline | 110 (43-151) | 146 (75-196) | 0.3068 |
| 3rd Month | 124.5 (94-158) | 245 (153 – 347) | 0.0066* |
| 6th Month | 101 (82-191) | 235 (171 – 360) | 0.0034* |
| CD8 cell count, median | | | |
| Baseline | 1294(776-1838) | 1024(745-1404) | 0.3888 |
| 3rd Month | 682(561-1076) | 1050(698-1457) | 0.1128 |
| 6th Month | 1050(924-1513) | 963(653-1477) | 0.3841 |
| CD4/CD8ratio, median | | | |
| Baseline | 0.13(0.07-0.2) | 0.14(0.09-0.22) | 0.6984 |
| 3rd Month | 0.24(0.09-.52) | 0.27(0.15-0.36) | 0.7627 |
| 6th Month | 0.095(0.06-0.19) | 0.27(0.18-0.41) | 0.0028* |
| Hemoglobin (< 12 gm/dl) | | | |
| Baseline | 75%(6/8) | 33.1%(42/127) | 0.024* |
| 3rd Month | 80%(4/5) | 25.4%(29/114) | 0.021* |
| 6th Month | 33.3%(2/6) | 18.3%(20/109) | 0.323 |
| Hemoglobin (< 10 gm/dl) | | | |
| Baseline | 0%(0/8) | 7.9(10/127) | 0.531 |
| 3rd Month | 0%(0/5) | 7%(8/114) | 0.702 |
| 6th Month | 0%(0/6) | 3.7%(4/109) | 0.805 |
| Liver function test | | | |
| Baseline | | | |
| SGOT | 38(28-44) | 31(25-46) | 0.7001 |
| SGPT | 25(17-27) | 23(19-34) | 0.9514 |
| AP | 191(80-308) | 165(109-223) | 0.5361 |

| Characteristics | ART associated TB | | p value |
|---|---|---|---|
| | Yes ( N=8 ) | No ( N= 128 ) | |
| 3rd Month | | | |
| SGOT | 60(28-70) | 31(24-39) | 0.212 |
| SGPT | 18(16-63) | 27(19-36) | 0.6510 |
| AP | 459(404-514) | 159(89-271) | 0.0703 |
| 6th Month | | | |
| SGOT | 34(20-46) | 26(22-35) | 0.7845 |
| SGPT | 20(19-32) | 24(17-38) | 0.4958 |
| AP | 472(454-486) | 176(127-249) | 0.0613 |
| Skin test >5mm, proportion | | | |
| Baseline | 62.5%(5/8) | 13.8%(17/123) | 0.003* |
| 3rd Month | | | |
| 6th Month | 85.7%(6/7) | 21.3%(20/94) | 0.001* |
| Qualitative ELISPOT response | | | |
| Baseline | 71.4%(5/7) | 48.4% (45/93) | 0.436 |
| 3rd Month | 85.7%(6/7) | 46.3%(43/89) | 0.112 |
| 6th Month | 50%(3/6) | 50%(49/94) | 1 |
| Quantitative ELISPOT response | | | |
| Baseline | | | |
| PPD | 10(0-200) | 18(0-51) | 0.967 |
| ESAT-6 | 25(5-40) | 15(0-40) | 0.656 |
| CFP-10 | 15(0-50) | 15(0-35) | 0.973 |
| 3rd Month | | | |
| PPD | 228(45-362) | 25(0-122) | 0.032* |
| ESAT-6 | 355(35-415) | 30(0-90) | 0.019* |
| CFP-10 | 330(30-395) | 25(0-85) | 0.029* |
| 6rd Month | | | |
| PPD | 91(0-404) | 49(0-149) | 0.633 |
| ESAT-6 | 111(0-202) | 40(0-151) | 0.623 |
| CFP-10 | 32(0-294) | 40(0-169) | 0.953 |
| * Significant (p value ≤ 0.05) | | | |

In a multivariate model, baseline predictors that were independently associated with ART associated TB were male sex (0R, 8.719, 95% CI, 1.35 - 56.3), baseline skin test positive response (OR, 10.6; 95% CI, 1.78 - 62.5) and baseline hemoglobin less than 12 gm/dl (OR, 11.6; 95% CI, 1.69 -79.6) (Table 3.21).

**Table 3.21 Multivariate logistic regression analyses showing independent risk factors for ART associated tuberculosis**

| Baseline risk factors | Odds ratio(95% CI) | **P value** |
|---|---|---|
| **Male Sex** | **8.72 (1.35 - 56.3)** | **0.023** |
| **Baseline TST > 5mm** | **10.6 (1.78 - 62.5)** | **0.010** |
| **Hemoglobin <12gm/dl** | **11.6(1.69 -79.6)** | **0.013** |

**c) Unmasking TB**

Among eight ART associated tuberculosis, three cases were considered as unmasking TB IRIS on the bases of their clinical and or laboratory parameters. As shown in Table 3.22, at basline CD4 cell count of patients 1, 2 and 3 were 54, 91 and 11 cells/$mm^3$ respectively. Patient 1 and 3 had TST anargy (0 mm indurations) whereas patent 2 had positive TST response (10 mm indurations) at enrloment. Three of them developed TB with in the first month of ART. At the onest of TB IRIS CD4 cell count decresed and at 3$^{rd}$ month increased among three of them.

Among three unmasking TB IRIS cases, two ceses were pulmonary TB (one of which smear positive and the other one smear negative) and one case was extra pulmonary TB (peritoneal TB). Tuberculin skin test response converted from 0 mm induration of pretreatment value to 13.5 mm and 9.5 mm induration at on set IRIS in patient one and three respectively. After intiation of ant-TB treatment, initial clinical response to the treatment was observed on in three of them. However, the clinical condition of patient two then subsequently deterorated and developed paradoxical IRIS at 77 day following initation of HAART and 49 day after initation of ant-TB treatment.

**Table 3.22 Characteristics of patients with unmasking TB IRIS**

| S N0 | Sex & Age | Time of on set (Day) | Site TB | Diagnosis | Characteristic at baseline | | | Characteristic at IRIS | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | CD4 cells/$mm^3$ | TST mm | ELISPOT (EC) | CD4 cells/$mm^3$ | TST mm | ELISPOT (EC) |
| 1 | M/38 | 28 | PTB | AFB + Ve X -ray | 54 | 0 | + Ve | $26^{IRIS}$ /$98^{3rdm}$ | $13.5^{3rdm}$ | + Ve |
| 2 | M/54 | 24 | PTB | AFB –ve X-RAY | 92 | 10 | + Ve | $85^{IRIS}$ /$151^{3rd\ m}$ | 10 | + $Ve^{3rdm}$ |
| 3 | M/44 | 13 | EPTB Peritoneal TB | X-ray & Pathology | 11 | 0 | Anergy | $9^{IRIS}$ / $48^{6tm}$ | 9.5 | -Ve |

1. **Twenty eighth day on ART, Cough and fever with night sweet of 2 wks duration** CX-ray **has right basal infiltrate and cavity,** AFB; **2 sputum smear positive**
2. **Twenty forth day on ART, Worsening of fever, Cough with night sweating and has generalized weakness** CX-ray **reveled bilateral infiltrate of pulmonary TB.**
3. **Thirteenth day on ART, developed abdominal fullness since initiation of ART. It is associated with fever, sweating, and fatigue. Stoll is mucoid but not bloody. No pedal swelling, SOB or chest pain.** Pathology examination**; Peritoneal fluid analysis, sheet of lymphocytes in a proteinaceous back ground, few mesothelial cells consistent with TB pleurisy.** X-ray **has basal infiltration and effusion obliterating the costa phrenic angles**

### d) Paradoxical TB IRIS

Thirty one TB cases who were receiving both Anti-TB and HAART were followed and contributed to a total of 13.33 person year observation for paradoxical TB IRIS study (25 of them were from prevalent cases at enrolment and 6 of them were incident cases during HAART). As shown in Figure 3.10, 9.6 % (3/31) individuals developed paradoxical TB IRIS. The incidence rate of paradoxical TB IRIS was 22.5 cases per 100 persons per year. The clinical manifestation of the IRIS were exacerbation of existing disease manifestations 'radiological deterioration of pulmonary TB for two cases and development of new disease manifestations at another anatomic site '' abdominal lymphadenopathy' for one cases.

At the onset of IRIS, one of the patients was unconscious, thus we were unable to collect blood sample or perform TST because of ethical reasons. Thus, in this patient the diagnosis was made based on clinical manifestation. The laboratory results of the two patients have shown, an increase in CD4 cell counts by 167 and 59 cells/$mm^3$ in patient 2 and 3 respectively. Frequency PPD specific IFN-γ producing cell increases 8 fold (from 91 to 752 SFC/million PBMC ) in patient 2 and 6 fold in patient 3 (from 200 to 1120 SFC/million PBMC ). However, TST responses were Anergic (0 mm indurations) in patient 2 at baseline and at occurrence of paradoxical IRIS. TST decreased from baseline 10 mm indurations to 4 mm indurations at IRIS in patient 3 (Table 3.23).

**Table 3.23 Characteristics of patients with paradoxical TB IRIS**

| S No | Sex & Age | Time of on set IRIS(Day) | Diagnosis of TB before IRIS | Clinical manifestations paradoxical TB IRIS | Characteristic at baseline | | | Characteristic at IRIS | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | CD4 cells/$mm^3$ | TST mm | ELISPOT (EC) | CD4 cells/$mm^3$ | TST mm | ELISPOT (EC) |
| 1 | M/29 | 36 | Smear +ve PTB | Deteriorations of pulmonary TB | 96 | 0 | cell not recovered | --- | —— | No sample |
| 2 | F/28 | 56 | Smear –ve PTB | Deteriorations of pulmonary TB | 17 | 0 | + Ve | 185 | 0 | EC –ve ECP + ve |
| 3 | M/54 | 77 | X-ray AFB | Development of new abdominal lymphadenopathy | 92 | 10 | +Ve | 151 | 4 | EC –Ve ECP +ve |

1. Thirty-six days after initiation of HAART and six months after Anti-TB treatment, developed loss of conscious, complain of left ocular pain (post therapeutic), X-ray has basal infiltration and cavity.
2. Fifty-six days after initiation HAART and three months after Anti-TB treatment, started to cough with mucoid sputum one month durations. Has fever, sweating. Chest X-ray has right basal infiltrate and cavity.
3. Seventy seven days after initiation HAART and forty-nine days after Anti-TB treatment, developed abdominal swelling. Pathology, careous necrosis, clusters of epitelhoid cells consistent with TB lymphadenitis.

# CHAPTER 3. DISCUSSION

## 4.1 Baseline Immune Response to Tuberculosis

We have tried to assess prospectively immune response to tuberculosis in HIV infected individuals using different immune parameters such as *in- vivo* tuberculin skin test and *in-vitro* IFN-γ ELISPOT assay in PBMC stimulated with ESAT-6, CFP-10 and PPD antigens. The responses were assessed before initiation of HAART (at baseline) and at the third and sixth months of treatment and at the time when tuberculosis associated IRIS was clinically suspected. To provide further insight into the determinants of such responses, baseline data were analyzed separately to investigate the effect of the level immunodeficiency on ELISPOT and TST responses.

In this study, the overall proportion of positive baseline TST responses was 17.2 % (29/169) and qualitative EC ELISPOT positive responses were 52.8% (67/123) [Figure 3.3 & 3.5 respectively]. The proportion of positive TST responses was significantly affected by level of immunodeficiency in stratified CD4 cell count analysis. In contrast, the proportions of EC ELISPOT positive responses were not affected by level immunodeficiency. This is consistent with many previous studies indicating that TST responses were significantly affected by CD4 cell count (Lawn *et al.*, 2007b; Tegbaru *et al.*, 2006). Whereas, ELISPOT positive responses were not affected by CD4 cell count (Dheda *et al.*, 2005; Lawn *et al.*, 2007b).

Although qualitative ELISPOT responses were not affected by the level of immunodeficiency as measured by CD4 cell count, there was a non significant trend in decreasing quantitative ELISPOT responses to PPD, ESAT-6 and CFP-10 as CD4 cell decreased (Figure 3.4). Moreover, quantitative ELISPOT responses to mitogen (PHA) were also significantly affected by the level of CD4 count. In consistent with this finding, effect of CD4 cell level on quantitative ELISPOT response to PHA has been reported in previous studies (Dheda *et al.*, 2005; Lawn *et al.*, 2007b). In the study conducted to assess determinants of ELISPOT responses among patients with advanced HIV infection, the median SFC/million to PHA was significantly lower in patients with low CD4 count (< 100 cells/$mm^3$) compared to patients with CD4 $\geq$ 100 cells /$mm^3$ (Lawn *et al.*, 2007b). In another study conducted to compare the performance of T-cell-based tests for diagnosis of TB in

HIV-infected individuals, median quantitative ELISPOT response to PHA was significantly lower in patients with low CD4 count (< 200 cells/$mm^3$) compared to patients with CD4 > 200 cells /$mm^3$ (Dheda *et al.*, 2005).

Despite the observed effect of level of immunodeficiency on quantitative ELISPOT responses, no impact of CD4 cell count was observed on the proportion of positive ELISPOT responses. The possible reasons may include: 1) although the magnitude of the PHA response decreases with CD4 cell counts, responses were still well above the threshold (100 SFC/million) for the positive control as observed in this study the lowest median PHA response was 390 (IQR, 100 - 890) SFC/106 among patients with CD4 cell cont <50 cells /$mm^3$ (Table 3.15, A). 2) in ELSPOT assay, equal number of PBMC (2 x $10^5$cells/$mm^3$) is added in every test well of the culture plate regardless of whole blood CD4 count and this may normalize the number of CD4 cell and help to retain performance of the assay among patients with low CD4 cell counts. 3) the contribution of CD8 cell as IFN- γ secreting cell may also helps to retain the performance of ELISPOT assay (Shams *et al.*, 2001).

Our finding on dichotomized (CD4 cell count $\geq$100 and < 100 cells/ $mm^5$) multivariate analysis also supports that qualitative ELISPOT assay is probably not affected by level of CD4 cell deficiency (Table 3.5, b). Moreover, Although the difference was not significant, the observed variation in the proportion of positive responses between groups stratified according to history of TB (untreated active TB, receiving Anti-TB treatment, past history of TB treatment, and with no history of TB), indirectly indicates that qualitative EC ELISPOT result may provide useful information on HIV infected patients (Figure 3.6).

Effect of past history of TB treatment on TST and EC ELISPOT response were also assessed among study participants without prevalent TB at enrolment. We found no significant difference on proportion of positive response on both TST and EC ELISPOT among patient with history of TB compared to patient without TB history (Table 3.5, a and b). In contrast, Lawn and his colleagues (2007b) have shown that the proportion of ELISPOT positive response was associated with history of TB treatment. The possible reason for the disparity might be explained by variation on characteristics of study participants, because participant with a history of TB treatment were heterogeneous with regard to the time since completion of TB treatment (16 month of median duration since

completion of TB treatment in our study compared to the 4 month median duration in their study). Thus, in our study, participants with past history of TB treatment were likely to be at high risk of re-infection with TB. Nonetheless, our result indicates that qualitative ELISPOT assay results were not affected by CD4 cell count and that more work is needed to understand the reasons why.

However, despite the observed good performance of ELISPOT assay in HIV infected patient regardless of level of the immunodeficiency, 20% (33/163) of the assay failed in our study due to *in-vitro* anergy (PHA response < 20 SFC/2 x $10^5$ PBMC) [Figure 3.2]. This may raise a serious issue on the performance of the assays on HIV infected patients, because ELISPOT assay failure was also reported in previous studies conducted on HIV infected patients. In a study conducted in Senegal to assess the validity of ELISPOT assay for detection of latent TB among HIV infected patients, 13.3% ELISPOT assay failure was reported (Karam *et al.*, 2008). Twenty five percent ELISPOT assay failure was also reported in anther study conducted to evaluated on the performance of the ELISPOT assay for diagnosis of active tuberculosis among HIV infected individuals (Carrara *et al.*, 2004).

The characteristics of patients who failed to respond to PHA were compared with patients who responded to PHA. We found no significant difference on proportion of failed response between groups stratified with CD4 cell strata. However, when the CD4 cell count dichotomized in to <50 and $\geq$ 50 cells/$mm^3$ the proportion failed responses was higher 30.8% (12/39) among patients with CD4 < 50 cells/ $mm^3$ compared to 17.2% (21/122) among patients with CD4 >50 cells/ $mm^3$ (P = 0.068). Although the difference is not statistically significant, the observed numerical difference in proportion of failed PHA response could be due to higher proportion of apoptotic-prone cells among PBMC samples collected from patients with CD4 < 50 cells /$mm^3$. Because available studies indicate, apoptotic cell death is inversely associated with CD4 count and significantly higher at advanced HIV disease (Gougeon *et al.*, 1996; Patki *et al.*, 1997). These apoptotic-prone cells may likely escape trypan blue staining and are counted as viable cells and included in the final concentration of the PBMC on the ELISPOT plate.

In conclusion, our results on baseline immune response to tuberculosis have shown that the proportion of positive TST response were significantly affected by level of

immunodeficiency, whereas, the proportion of EC ELISPOT positive responses were not affected by the level of CD4 cell count. Thus, EC ELISPOT assay can perform well in HIV infected individuals regardless of CD4 count as indicated by proportion of positive response. However, both the observed statistically significant effect of CD4 cell count on quantitative PHA response and numerical effect (statistically not significant P = 0.068) of CD4 count on the proportion of *in-vitro* anergy (ELISPOT failure) indicates the need of further research which tests the utility of the ELISPOT assay through recently recommended method that proposes to allow the PBMC to "rest" overnight before plating in the ELISPOT assay. This will probably eliminate the effect by allowing fragile apoptotic-prone cell to die, so that a true count of the number of viable cell can be assessed prior to plating (Janetzki *et al.*, 2005).

### 4.2 Immunological Recovery after HAART

HIV infection impairs cell-mediated immunity. The impaired immunity leads to increased number of cases of primary TB and reactivation of TB in HIV-infected people (Hoffmann, Christian, 2007a). An increased rate of TB among HIV infected individuals has been associated with quantitative and qualitative loss of immune function. HAART, through immune restoration, reduces the TB incidence (Santoro-Lopes *et al.*, 2002). Nonetheless, a high rate of TB continues to occur among HIV infected individuals receiving HAART in Sub Saharan African countries (Bonnet *et al.*, 2006; Lawn *et al.*, 2006a). Furthermore, a high rate of mortality and morbidity at initial months of HAART has been reported in this region (Jerene *et al.*, 2006a; Lawn *et al.*, 2005d). The possible reason for this may be explained by the fact that the many of individuals in this region get access to HAART at advanced symptomatic diseases and profound CD4 lymphocytopenia (Geng and Deeks, 2009; Lawn *et al.*, 2006b). This raises the concern that many patients entering ART programmes in sub-Saharan Africa may have limited potential for immune recovery. However, little information is available on the extent of immune recovery after HAART in this region. In this study, we have investigated prospectively recovery of immune response among HIV-infected individuals receiving HAART at ALERT hospital. We focused on recovery of immune parameters in general, such as CD4 cell count, and in particular,

specific immune response to TB using immune parameters such as; *in vitro* TST and *in vivo* IFN- γ ELISPOT assay.

### 4.2.1 Recovery of CD4 cell count

CD4 cell play a fundamental role in the protective response against *M. tuberculosis* through production of cytokines including IFN-γ (Jouanguy *et al.*, 1999), by helping to develop the CD8 cell mediated response (Serbina *et al.*, 2001) and participation in the induction of apoptosis of infected cells (Oddo *et al.*, 1998). Moreover, the remarkable susceptibility of patients with AIDS to TB has demonstrated the critical role of CD4 cell in protective immunity. Thus, assessment of quantitative recovery of CD4 cell may indirectly provide useful information on the extent to which HAART will have effect on tuberculosis control. Furthermore, it may help to provide further insight on the determinants of the reported high rate of early morbidity and mortality among patients who are initiated HAART at very low CD4 cell count (Lawn *et al.*, 2005d) or with profound lymphocytopenia (Jerene *et al.*, 2006b). It may also provide useful information about factors associated with the rate of achieving CD4 cell count above a safer level that decreases the risk of morbidity and mortality (CD4 >200 cells/$mm^3$) or CD4 cell levels that are needed to discontinue prophylactic drugs such as cotrimoxazole (CD4 >350 cells/$mm^3$) (FMOH/HAPCO, 2006). In light of this, in this part we have tried to assess factors associated with recovery of CD4 cell.

In this study, median CD4 cell count incresed from baseline count of 130 cells/$mm^3$ to median cell count of 238 and 228 cells /$mm^3$ at $3^{rd}$ and $6^{th}$ months respectively (Figure 3.7). Moreover, a significant majority of participants had attained CD4 cell count above 200 cells / mm3 (above the zone of severe immunodeficiency); the proportion of study participants with CD4 cell count ≥ 200 cells/$mm^3$ increased from 19.9% at baseline to 60.6% and 64.4% at $3^{rd}$ and $6^{th}$ month respectively (Figure 3.8). Consistent with this finding, several studies have shown the impact of HAART on recovery of CD4 cell numbers among individuals started on HAART in Sub Saharan countries. A meta analysis of 29 studies from 12 African countries has shown the effect of HAART on the reconstitution of CD4 cell counts from a baseline mean CD4 cell count of 141 cells/$mm^3$ to 243.8 and 248.9 cells/$mm^3$ at $3^{rd}$ and $6^{th}$ months respectively (Hammond and Harry, 2008).

In our cohort, although the majority of participants had achieved a CD4 cell count > 200 cells / $mm^3$ after HAART, we found that the likelihood of achieving CD4 cell count above zone of severe immunodeficiency (CD4 > 200 cells / $mm^3$) was significantly affected by the level of CD4 cell count at which HAART was started (Table 3.6). The risk of achieving CD4 cell count >200 cells/$mm^3$ after HAART (at $3^{rd}$ and $6^{th}$ month) was significantly decreased with decresing CD4 cell count. Compared to individuals with baselinc CD4 cell count above 200 cells/$mm^3$, relative risk decreases down CD4 strata; 199 - 100, 50-99, and < 50) [Table 3.7]. This indicates that those individuals with CD4 cell count < 50 cells/$mm^3$ at start of HAART have the least chance of achieving CD4 cell count $\geq$ 200 cells/$mm^3$ at $3^{rd}$ or $6^{th}$ months of treatment. Moreover, our finding, which shows no significant difference on the rate of change in magnitude of CD4 cell count over time between baseline CD4 strata (Table 3.6), indicated that those with low baseline CD4 cell counts at initiation of HAART require a longer period to attain CD4 cell count >200 cells/ $mm^3$. Thus, a prolonged period below the zone of severe immunodeficiency might be more likely to be the cause of the reported high rates of morbidity and mortality during the early months of treatment among individuals receiving HAART in developing countries (Jerene *et al.*, 2006a; Lawn *et al.*, 2005a; 2005d).

As achieving CD4 cell counts $\geq$ 350 cells/$mm^3$ is a criterion to discontinue cotrimoxazole among individuals receiving HAART based on the Ethiopian guideline for ART program (FMOH/HAPCO, 2006), we have also assessed the risk of achieving CD4 cell count $\geq$ 350 cells/$mm^3$ after HAART in a stratified analysis according to baseline CD4 cell count. We found that the risk of attaining CD4 cell count $\geq$ 350 cells/$mm^3$ was significantly associated with baseline CD4 count at which HAART is initiated. The risk of achieving CD4 cell count > 350 cells/$mm^3$ was significantly lower at $3^{rd}$ month, among individuals with baseline CD4 cell strata <50, 50-99 and 100-99 cells/$mm^3$ compared with CD4 count $\geq$ 200 cells/$mm^3$. However, the risk was only significantly lower for those with CD4 cell strata <50 cells/$mm^2$ at $6^{th}$ month (Table 3.8). This indicates that those individual who initiate HAART with CD4 count < 50 cells/$mm^3$ will have very little chance of achieving CD4 count $\geq$ 350 at the $6^{th}$ month. Thus, individuals with CD4 count <50 cells /$mm^3$ at initiation of HAART will have least chance to discontinue cotrimoxazole prophylaxis at $6^{th}$ month. This also has major

negative impact on ART program in Sub Saharan African countries where more than one quarter individuals start HAART with CD4 cell count < 50 cells/mm$^3$ (Colebunders *et al.*, 2005; Lawn *et al.*, 2006b). Individuals who initiate HAART with low CD4 count, in addition to the higher risk of mortality and morbidity during early months of treatment, will also face additional problems associated with prolonged use of cotrimoxazole - pill burden and cotrimoxazole associated side effects such as: itching, rash, fever, Stevens–Johnson syndrome and bone marrow suppression (FMOH/HAPCO, 2006).

As the size and output of the thymus decreases at old age (Guazzi *et al.*, 2002), several studies reported recovery of CD4 cell count is associated with age (Kaufmann *et al.*, 2000a; 2000b; Lawn *et al.*, 2006b). However, in this study, we found no difference in the recovery of CD4 cell count between age groups < 40 and ≥ 40 years. (Table 3.9). The disparity can be explained by a couple of reasons; one possible reason could be that the relatively short follow up period in our study might not allow for sufficient time to demonstrate significant contribution of the thymus to the restoration of CD4 cell count. Previous studies demonstrated two phases of CD4 cell recovery (Pakker *et al.*, 1997). The first phase (rapid recovery starting from 1–2 weeks and extends over the first 2–3 months), represents a redistribution of activated CD45Ro memory cell previously sequestered in lymphoid tissue and a reduction in apoptotic cell death (Carcelain *et al.*, 2001; Lederman, M. M., 2001). A second phase of gradual increase follows, approaching stable CD4 cell counts at 4–6 years. This second phase represents expansion of naive CD45Ra cell as thymic function is restored and is largely responsible for the long-term sustained rise in CD4 lymphocyte count (Pakker *et al.*, 1998). The other possible reason could be explained by variation in the age of study participants. In our study, participants were in the range of 18-55 years old. This could potently mask the effect of age on recovery of CD4 cell as no participant above 55 years of age was included in our study.

Comparison of our findings with previous studies is difficult for several reasons: one the reason is the variability in the stratification of CD4 cell groups employed in previous studies: ( <200, 200-349, 350-500, >500 cells/mm3) (Garcia *et al.*, 2004) and (<50, 51–200, 201– 350, 351–500, and >500 cells/mm3) (Robbins *et al.*, 2009). We could not therefore compare risk analysis and rate of CD4 cell recovery observed among CD4 cell groups with

CD4 cell strata (<50, 50-99 ,100-199 and >200) in our study. However, despite little difference on the way of grouping CD4 strata (<50, 50-99, 100 -149 and >150) and statistical method employed on risk analysis (odd ratio in their study vs relative risk in our study), our finding on risk of recovery of CD4 cell count above 200 cells / $mm^3$ is consistent with the study conducted on recovery of CD4 cell after HAART in South Africa (Lawn *et al.*, 2006b).

The other reason that made our finding difficult to compare with previous studies includes the variability of case definition for immunological failure and being the first (to our knowledge) to use the 350 cut-off value of CD4 cell count for risk analysis. For instance, a case of immunological failure (immunological non-response) was defined in previous studies as patients who failed to attain an absolute CD4 cell count increase from baseline of at least 50 cells/$mm^3$ at 48 weeks (Lawn *et al.*, 2006b). In anther study, patients who failed to attain an increase in CD4 count from baseline of at least 100 cells/ $mm^3$ between 6-12 months of HAART were considered as immunological non responders. Patients who attained CD4 count increases from baseline to at least 100 cells/ $mm^3$ between 6-12 months and thereafter dropped to or below the baseline CD4 cell count were considered to have experienced immunological failure (Dragsted *et al.*, 2004). However, in our study we used the definition recommended by guidelines for management of OI and ART in adolescents and adults in Ethiopia, which defines immunological failure as "fall of CD4 count to pre-therapy baseline (or below) and or 50% fall from the on-treatment peak value (if known) and or persistent CD4 levels below 100 cells/$mm^3$" (FMOH/HAPCO, 2007) [Table 3.10].

Despite the variability in case definition, the observed no effect of baseline CD4 count on proportion of immunological failure among CD4 strata was also consistent with the study conducted by Lawn and his colleagues (2006b). They found an immunological non responder proportion of 9.7% patients at 48 weeks of HAART and the proportion of immunological non responder was independent of baseline CD4 cell strata. However, our finding (Table 3.10) that shows a significant effect of baseline CD4 cell count on the proportion of individuals who achieved CD4 counts > 500 cells/$mm^3$ at $6^{th}$ month was not consistent with their finding. The possible reason for the disparity might be attributed to the relative short study period in our study compared to the duration of their study (24 weeks vs

48 weeks respectively). Nonetheless, our finding is supported by the study on recovery of CD4 cell after HAART, which reported that individuals reaching a CD4 cell count of 500 cells/$mm^3$ or greater at 4 years were characterized by higher baseline CD4 cell count (Kaufmann *et al.*, 2003).

In conclusion, in this study, we observed that there is a recovery of CD4 count above the zone of severe immunodeficiency among the majority of individuals receiving HAART, as measured by an increase in median CD4 count and proportion of patients having CD4 cell count $\geq$200 cells/$mm^3$ after HAART. However, achieving CD4 count above $\geq$ 200 cells/$mm^3$ or $\geq$350 depends on baseline CD4 cell count and may take substantially longer (greater than 6 month) among patients who initiate antiretroviral therapy at lower CD4 cell values. .

### 4.2.2 Recovery of TB specific immune response after HAART

Although protective immune response to TB has not yet well characterized, the central role of Th1 type response in protective immunity has been demonstrated by expression of high levels of IFN-γ among healthy individuals that controlled LTB infection (Demissie, A. *et al.*, 2004). More over, a profound impairment of IFN-γ secretion (Elliott *et al.*, 1999; Zhang *et al.*, 1994), and reduced DTH response in untreated HIV-1-infected individuals (Rangaka *et al.*, 2007; Tegbaru *et al.*, 2006; Wolday *et al.*, 2003), and their increased susceptibility to active tuberculosis could also explain the role of Th1 type response in protective immunity. Thus, previous studies tried to demonstrate restoration of TB specific response using Th1 type responses such as: proliferation of PBMC and DTH response (Schluger *et al.*, 2002; Wendland *et al.*, 1999), intracellular cytokine expression assay (Hengel *et al.*, 2002), and quantitative IFN-γ ELISPOT response (Bourgarit *et al.*, 2006). Most of these studies suggested that immune response to TB may not be fully restored after successful HAART (Schluger *et al.*, 2002; Sutherland *et al.*, 2006) or may fail to restore among individuals who initiated HAART at CD4 cell count below 50 cells/$mm^3$ (Hsieh *et al.*, 2000). This raises the concern that TB specific immune response either restored partially after prolonged time (Schluger *et al.*, 2002) or may becomes irreversible by HAART at advanced HIV disease due to loss of TB specific T cell clone (Hsieh *et al.*, 2000). However, little information is

available in this regard. Thus, we have tried to assess restoration of immune response to tuberculosis *in-vivo* using TST and *in vitro* at a single cell level using IFN-γ ELISPOT assay among individuals receiving HAART at ALERT ART program.

In this study, there was a significant improvement in immune response to tuberculosis following initiation of HAART as observed by increased proportion of TST positive response and quantitative IFN-γ ELISPOT response (median SFC/ million PBMC)[Table 3.11 & Figure 3.9]. The magnitude of quantitative IFN- γ ELISPOT response to mitogen and TB antigens (PPD, ESAT-6 and CFP-10) increased significantly after treatment at 3$^{rd}$ and 6$^{th}$ month compared to baseline. However, the dynamics on an increment in magnitude of response to mitogen or TB antigens were rapid on the first 3 months interval and steady on the second 3 months internal as observed by non significant difference between the response at 3$^{rd}$ and 6$^{th}$ month (Figure 3.9). This confirms that similar pattern of restoration of immune response were followed among our study population as reported elsewhere. Our study suggested rapid increases in immune response with in the first 2–3 months of treatment, all these improvement obtained through one or the combination of proposed mechanisms, which include: redistribution of CD4 memory cell (CD45Ro) from lymphoid tissue and / or a reduction in apoptotic cell death. (Autran *et al.*, 1997; Hengel *et al.*, 2001) and / or restoration of antigen-presenting cells dysfunctions, since IL-12 is a key cytokine driving Th1 responses and is secreted by professional antigen-presenting cells, including macrophages and dendritic cells, which may be dysfunctional in HIV-1-infected individuals (Donaghy *et al.*, 2004). Moreover, the IL-12–IFN-γ axis was shown to be dysfunctional in HIV-1-infected progressors (Chehimi *et al.*, 1994; Marshall *et al.*, 1999).

Some authors suggested that delaying initiation of HAART in chronic HIV infection may lead to long-term impairment of functional immune responses to *M. tuberculosis* by demonstrating as evidence individuals who initiate HAART with CD4 cell count below 50 cells/ mm$^3$ (Hsieh *et al.*, 2000). In contrast, our finding on effect of HAART on restoration of TB specific immune response with baseline CD4 cell stratified analysis indicated, TB specific immune response could be restored among individuals who initiate HAART with CD4 cell count < 50 cells/ mm$^3$ as observed by a significant increment in proportion of TST

response and quntitative ELISPOT response after HAART among this groups (Table 3.11, 3.13, 3.15).

One possible explanation for the disparity could be the difference in methodology used to investigate TB specific immune response. Hsieh and his colleagues (2000), have tried to demonstrate restoration of TB specific immune response using the expression of the lymphocyte activation marker CD69 following an *in vitro* stimulation of PBMC with PPD and their correlation with TST response and level of IFN γ measured with ELISA following an *in vitro* stimulation of PBMC with PPD. CD69 is the earliest activation surface marker on human lymphocyte (Lopez-Cabrera *et al.*, 1993) and barely expressed in T cells with HIV-1–induced dysregulated activation (Autran *et al.*, 1997) and also impaired by HIV infection (De Martino *et al.*, 1999; Krowka *et al.*, 1996). Thus CD69-expressing cells may not be necessarily the antigen specific cells. Moreover, their method, which they had tried to correlate with the expression of CD69, may likely unable to pick the difference in change the level of IFN-γ among group with CD4 < 50 cells/$mm^3$ after HAART. Scince they have emploed ELISA to measere the level of IFN-γ. Whereas, we used quantitative IFN-γ ELISPOT assay which is 200 times more sensitive than ELISA (Tanguay and Killion, 1994).

The other possible explanations might be due to variation on study population and sample size. Hsieh and his colleagues (2000) studded 13 TB HIV co infected individuals who had completed Anti-TB treatment before initiation of HAART. Of which only 5 (Hsieh *et al.*, 2000)] of them had CD4 <50 cells /$mm^3$ at start of HAART. Where as, in our study 161 study participant who had at list one follow up envestigation were included in the ananlysis. Of which 36 of them had baseline CD4 <50 cells /$mm^3$. Moreover, we had tried to control the confunding effect of TB treatment in our study during analysis.

On stratified analysis according to baseline TB history, we found significant increment on proportion of TST reactivity after HAART among individuals with active TB at baseline investigation (Table 3.12). The possible explanation for the observed marked increase in proportion of TST reactivity among this group could be due to the concurrent effect of both Anti-TB treatment and HAART on restoration of TB specific immune response. Since

previous studies have demonstrated impaired immune response to TB among HIV negative individuals with active TB and subsequent improvement after Anti-TB treatment. For instance, an increase in the TST reactivity rate after Anti-TB treatment reported in the study conducted in Ethiopian among individuals either with HIV positive co-infection not receiving HAART or individuals with out HIV infection (Tegbaru *et al.*, 2006). Furthermore, restoration of TB specific immune response has been reported among individuals receiving HAART alone using TST response (Girardi *et al.*, 2002) and PBMC proliferation as a marker (Schluger *et al.*, 2002). This indicates the observed significant increased in TST response amonge this groups may likely represent the contributions of both anti-TB and HAART.

In this study, since the confounding effect of TB treatment on restoration of immune response to TB was observed, we excluded individuals who were receiving anti-TB therapy during study period (patients with prevalent TB at start of HAART or patients who developed TB while receiving HAART) and further analyzed to assess effect of HAART among individuals with different baseline CD4 cell strata (Table 3.13). We observed significant increment in proportion of TST response and median SFC/ million IFN-γ ELISPOT responses to antigens (PPD and ESAT-6) and mitogen (PHA) among individuals with CD4 cells < 50 cells/$mm^3$. This indicates that TB specific T cell repertoire may not be totally lost in HIV infected individuals with CD4 < 50 cells/ $mm^3$ and their levels can still be improved by HAART.

Following the above observation, effect of baseline CD4 cell count on the extent of TB specific immune response restoration after HAART was further assessed. The difference on proportion TST or quantitative ELISPOT responses was compared between groups with enrolment CD4 cell count < 100 and ≥100 cells/$mm^3$. Among the group with enrolment CD4 ≥ 100 cells/$mm^3$ significantly higher proportion TST response was observed before HAART and the response was also higher after HAART but the difference was not statistically significant (Table 3.14 & 3.16). We also observed higher quantitative ELISPOT responses to TB antigens PPD, ESAT-6, and CFP-10 response among group with CD4 cells ≥ 100 cells/$mm^3$ compared to response of those with CD4 < 100 cells/$mm^3$ before and during

HAART. However, the difference was not statistically significant except for quantitative ELISPOT responses to PPD.

Although there was no significant difference on TST or quantitative ELISPOT responses to ESAT-6 and CFP-10 after HAART between CD4 cell groups , the observed higher proportion of TST positive response and significantly higher qualitative ELISPOT response to PPD at $6^{th}$ month among group who initiated HAART with CD4 $\geq$ 100 cells/$mm^3$, indicated that the extent of restoration of TB specific immune response may depend up on baseline CD4 cell count at which HAART initiated or prolonged time may be needed for those who initiated HAART with CD4 < 100 cells/$mm^3$ to achieve the extent of TB specific immune response improvement achieved by those who initiated HAART with CD4 $\geq$ 100 cells/ $mm^3$. However, careful interpretation of this result may be needed as treatment response was not monitored using viral load determination. Further more, we are limited to compare our finding with other studies as to our knowledge this study was the first to assess immune response to TB among individuals receiving HAART at ART program using such methods.

In conclusion, in this study we have observed improvement in immune response to TB among individuals receiving HAART, despite the level of CD4 cell count at which HAART is initiated as evidenced by the following key observation: (i) The proportion of TST positive response increased significantly after HAART in whole cohort and a significant increment was observed among individuals with CD4 cell < 50 $mm^3$ in stratified analysis, (ii) The median IFN- γ spot forming cell increased significantly after HAART in the whole cohort and significant increment in median IFN-γ spot forming cell against PPD and ESAT-6 observed among individuals with CD4 < 50 cell/ $mm^3$ in stratified analysis. Moreover, the observed no effect of baseline CD4 cell level on the rate of immunological failure and on the rate of change in median CD4 level in previous part, further supports indirectly that HAART improves immune response to TB regardless of baseline CD4 cell count.

However, the rate on extent of improvement may depend up on the level of CD4 count at which HAART is initiated as suggested by the following observation (although the observed difference was not statistically significant): (i) the proportion of TST positive response after treatment was also higher among individuals who have started HAART with CD4 $\geq$ 100

cells / $mm^3$; (ii) the median IFN- γ spot forming cells against TB antigens was higher both before and after HAART among individuals with baseline CD4 level $\geq$ 100 cells/$mm^3$ (our study lacks the statistical power to adequately assess these observations except for quantitative ELISPOT response to PPD).

Collectively our result showed HAART improves immune response to tuberculosis even among individuals who have started with profound CD4 cell lymphcytopinea. However, the magnitude of improvement may be affected by baseline CD4 cell count at which HAART is initiated or may take prolonged time for those who initiate HAART with advanced HIV diseases. Although limitations in our study enforced as to carefully interpret the result, these data provide an important basis for the justification of further studies on determinant of TB specific immune response recovery among individuals receiving HAART with larger sample size.

### 4.3 ART associated TB, Unmasking and Paradoxical TB IRIS

Several studies have shown the effect of HAART in reducing the incidence of TB among HIV infected individuals (Badri *et al.*, 2002; Girardi *et al.*, 2000; Jones *et al.*, 2000; Santoro-Lopes *et al.*, 2002). However, despite major reductions, high rates of TB, especially in the initial months of treatment have been reported among individual receiving HAART in sub-Saharan Africa (Lawn *et al.*, 2006a). This high burden of TB during early HAART poses a serious challenge in ART programs in this region. Furthermore, due to the presence of high prevalent TB at start of HAART (Lawn *et al.*, 2006a; Moore D *et al.*, 2006), paradoxical TB IRIS could greatly complicate the delivery of ART, causing substantial morbidity and mortality. Despite the severity of the problem little information is available on the risk factor and incidence rate of TB after HAART. Moreover, information is scarce on contribution of IRIS for clinical presentation of incident TB and incidence of paradoxical IRIS. In the present study we have tried to assess incidence, risk factors and immunological characteristics of ART associated TB, the proportion of TB episodes manifested as Unmasking TB IRIS and incidence of paradoxical TB IRIS.

Due to lack of standard case definition and standard diagnostic criteria, the occurrence of TB after initiation of HAART has been termed collectively as IRIS using simple markers such

as an increase CD4 count as marker (Huruy *et al.*, 2008), and / or decrease in viral load (Puthanakit *et al.*, 2006). However, tuberculosis-associated IRIS frequently develops shortly after initiation of ART and before any measurable increase in peripheral blood CD4 cell count (Meintjes *et al.*, 2008; Phillips *et al.*, 2005). Moreover, it is very likely that CD4 T cell are not the only cellular mediators of IRIS (Dhasmana *et al.*, 2008; Van den Bergh *et al.*, 2006). Thus, some authors and International Network for the study of HIV-associated IRIS (INSHI) (Lawn *et al.*, 2008; Meintjes *et al.*, 2008) strongly disagrees using term tuberculosis IRIS or Unmasking tuberculosis to refer to all episodes of TB during HAART. Accordingly they have suggested that the term ‘‘ART-associated TB’’ be used to refer collectively to all cases of TB presenting during ART and the term ‘‘immune reconstitution disease’ or ‘unmasking tuberculosis-associated IRIS’’ to be used to refer to the subset of ART-associated TB cases in which the effect on disease severity results in exaggerated and overtly inflammatory disease. In light of this, in this study the term ART-associated TB was used for cases of TB that developed during study period and unmasking tuberculosis-associated IRIS for those TB episodes which were consistent with provisional case definition for unmasking tuberculosis-associated IRIS proposed by INSHI (Meintjes *et al.*, 2008).

In this study, high rate tuberculosis was observed among individual receiving HAART at ALERT hospital. The incidence rate was 12.7 cases per 100 person-year observations (Figure 3.10). The observed high incidence was consistent with finding from other high TB burden countries. A study conducted in South Africa has revealed the incidence of TB of 13·4 cases per 100 person-years in the first year of ART (Lawn *et al.*, 2006a). Bonnet *et al* (2006) also reported that the incidence of TB ranged from 4.8 - 17.6 cases per 100 person-years among patients receiving HAART in five high burden countries (Cambodia, Thailand, Kenya, Malawi and Cameroon). In contrast to our study, low incidences of TB were reported among individuals receiving HAART; a study conducted in Arba Minch Hospital, Ethiopia, reported incidence of TB 3.7 cases per 100 person – year (Jerene *et al.*, 2006a). Two other studies also reported relatively lower incidence; in South Africa (incidence rate, 2 cases per 100 person-years) and in Cote d’Ivoire (incidence rate, 4 case 100 person-years). The relative high incidence rate in our study could partly be explained by the relatively

short follow up duration (6th month) compared to median duration of follow up 50 weeks, 16.8 months and 26 months in the other Ethiopian, South Africa and Cote d'Ivoire studies respectively (Jerene *et al.,* 2006a; Kirk *et al.*, 2000; Seyler *et al.,* 2005).

Despite active screening of all study participants for TB prior to HAART initiation in this study, the TB incidence rate in the initial month of HAART was extremely high (37.4 cases/100 PY) and decreased steeply to 17.7 and 12.7 cases/100 PY at 3rd and 6th months respectively. A similar pattern of temporal clustering of cases in the initial months of HAART and subsequent rapid decreases was also observed in ART programmes in resource-limited settings. It is believed that most episodes of early TB likely represent progression of sub clinical TB that was present before ART initiation, either due to persisting immunodeficiency or due to unmasking during ART-induced immune recovery (Lawn *et al.*, 2008; Meintjes *et al.,* 2008). Three possible mechanisms underling the development of TB during the initial months of HAART have been proposed by Lawn and his colleagues (2008). Since immunopathologic host responses to *M. tuberculosis* are central to the clinical presentation of TB, HAART through immune restoration: ''(1) shorten the time for sub clinical TB to become symptomatic (a phenomenon often referred to as ''unmasking''), (2) increased rapidity of initial onset of TB symptoms, and (3) heightened intensity of clinical manifestations''

Although provisional diagnostic criteria of unmasking TB IRIS proposed by International Network for the Study of HIV-associated IRIS (INSHI) is not yet validated, the observed clinical and laboratory parameters in this study indicated that only three cases of TB episodes met the diagnostic criteria of unmasking Tuberculosis IRIS proposed by INSHI. However, in the other 5 TB cases, we did not find any evidence of an exaggerated inflammatory response consistent with the proposed case definition. This indicated that only a portion of TB cases that occur in initial months of HAART are manifested by IRIS. Thus, our data supports proposed idea of lawn *et al* (2008) and INSHI on appropriate use the term IRIS to refer to some but not all cases of incident TB during the initial months of HAART (Meintjes *et al.*, 2008).

Our data show that there was no significant difference in median CD4 count at start of HAART between those who developed TB and those who did not (median (IQR) CD4 cell

count 110 (43-151) count/mm$^3$ and 146 (75-196) count/mm$^3$ (p=0.3068) respectively) [Table 3.20]. A similar finding has been reported elsewhere as well (Seyler *et al.*, 2005). In contrast, other studies reported that low baseline CD4 count was a risk factor for subsequent development of TB during HAART (Girardi *et al.*, 2000; Lawn *et al.*, 2006a). Although our finding is supported by others study, it is not possible to conclude that low baseline CD4 cell count as being not a risk factor to subsequent development of TB during HAART. Because median CD4 cell count among patient who develop TB in this study also lower compared to those who do not developed TB, but our statistical method unable to pick the difference. Thus further investigation is needed to confirm.

In the current study, the median CD4 count at 3$^{rd}$ and 6$^{th}$ month was significantly lower for those who developed TB compared to those who didn't (Table 3.20). Similarly other study also reported significantly lower median CD4 increase among patients who developed TB (Lawn *et al.*, 2005a). The observed significantly lower CD4 count among those who developed TB could partly be explained by the fact that acute TB itself causes CD4 lymphocytopenia (Morris *et al.*, 2003; Zaharatos *et al.*, 2001). In addition, though not significant, in our study almost half 50% (4/8) of those who developed TB had baseline CD4 count < 100 cells /mm$^3$ compared to 31.3% (44/128) who did not develop TB. This could also partly contribute for the difference in reconstitution of CD4 cell count among the groups, as studies demonstrated low baseline CD4 count is associated with poor CD4 cell recovery after HAART (Lederman, *et al.*, 2003; Piketty *et al.*, 1998). However, our study was limited to further support this explanation because the treatment response was not monitored by viral load determination in our study.

The important and unusual finding among identified risk factor for subsequent development of TB following initiation of HAART in our study was male sex (0R, 8.72, 95% CI, 1.35 - 56.3). This result might be attributed to other confounding effect of risk factors to TB, which we didn't document in our study, such as exposure to TB (Recent history of close contact to TB patient) at home or at work. However, similar finding was reported in Ethiopia in a prospective cohort study conducted on HIV-infected individual receiving HAART at Arba Minch hospital (Jerene *et al.*, 2006a). They found that among 6 individuals, who developed TB during HAART, five were male and one was female. Our finding together with Jerene *et*

*al* (2006a) findings may help to generate hypothesis for further studies which will help to explore possible mechanisms that contribute to the events.

The most important finding of baseline predictors that was independently associated with risk of developing TB was TST>5mm indurations (OR, 10.6; 95% CI, 1.78 - 62.5). This result is consistent with the finding reported elsewhere (Girardi *et al.,* 2000). Although tuberculin skin test is not recommended as a screening tool for detecting sub clinical TB/ latent TB in high burden TB countries where there is BCG vaccination and repeated exposure to environmental bacteria, our result indicates TST response as the strongest predictor of occurrence of TB during HAART. Furthermore, available evidences in high TB burden countries have indicated the potential use of TST in HIV infected individuals as screening tool to select candidate for IPT. For example, a recent study conducted in Brazil has reported significantly lower proportion of 1.6 % of TB among TST positive HIV infected individuals who received IPT for six month compared 11.5% who had not received IPT (Golub *et al.*, 2007). On top of this, studies conducted in Kenya, Zambia and Uganda also reported no apparent effect of IPT compared to placebo in reducing incidence of TB among TST-negative HIV infected patients (Hawken *et al.*, 1997; Mwinga *et al.*, 1998; Whalen, *et al.*, 1997). However, the later three studies were conducted in pre HAART era; they should not be considered as evidences presented to support our suggestion. Rather they are presented to show indirect evidence indicating TST negative individuals may not benefit from IPT. Thus our data indicate the old and neglected tuberculin skin test (due to poor sensitivity in HIV infected patient and poor specificity due to several factor in high TB burden countries), may help to predict patients who will develop early TB while taking HAART. Thus our data may be useful as baseline information for further research.

In addition to the challenge due to high burden TB during initial months of HAART, the current ART program in Sub Saharan African countries were facing great clinical challenge by paradoxical TB IRIS (Lawn *et al.*, 2007a). The proportion of paradoxical TB IRIS among patients receiving both Anti-TB and HAART ranged from 8% to 43 % (Breton *et al.*, 2004; Kumarasamy *et al.*, 2004). This wide variation is likely to reflect differences in cohort characteristics, case definitions and differences in the mean time interval between TB diagnosis and ART initiation.

In this study, among 31 TB cases (25 prevalent TB cases at start enrolment and 6 incident TB cases after enrolment who develop TB while taking HAART) who were taking both Anti-TB therapy and HAART, paradoxical TB IRIS was observed in 3 individuals (Figure 3.10). This makes the proportion 9.6 % (3/31) or an incidence rate; 22.5 cases per 100 persons per year. This proportion is relatively low compared to the proportion in previous reports that ranged from 12% to 37%, in high burden countries (Bourgarit *et al.*, 2006; Lawn *et al.*, 2007a). However, comparable proportion (8%) of paradoxical TB IRIS has been reported from India (Kumarasamy *et al.*, 2004) and a study from Tanzania reported no cases at all (Shao H *et al.*, 2006). One possible explanation for the observed low proportion of paradoxical TB IRIS in our study could be due to 50% (13/26) prevalent TB cases were having already completed several months of Anti-TB treatment at enrolment and the median time between initiation of Anti-TB and HAART of all cohort were 46 days (IQR 17-78). Thus many were likely to be at low risk of IRIS, since longer duration of TB treatment before initiating ART may lead to the lower antigen load that elicits IRIS. The other possible reason could be due to small sample size of paradoxical TB I cohort in our study may contribute for discrepancy. Nonetheless, the sample size may not be the only or necessary factor, because the sample size used in several paradoxical TB IRIS studies were in range of 28 – 37 (Bourgarit *et al.*, 2006; Breton *et al.*, 2004; Michailidis *et al.*, 2005; Narita *et al.*, 1998; Wendel *et al.*, 2001), indicating our finding might not be affected by small size if it is compared with the above studies. Thus the observed variation may also partly explained by the fact that our study population have different genetic background which may contributes for variability in risk factors for IRIS., because polymorphism in cytokine gene has been associated with inherited susceptibility with mycobacterial IRIS (Price *et al.*, 2002). However, due to small number (3) paradoxical TB IRIS episodes observed in our study, we were unable to perform further statistical analysis to assess risk factors associated with paradoxical TB IRIS. Thus, based on this background we suggest large prospective cohort study to be conducted to assess possible risk factor and immunological profile of paradoxical IRIS.

In summary, in this study we have observed a high incidence of TB which is 35 fold higher than the incidence of TB of all forms of TB in general population. TB incidence rate in the

initial month of HAART was extremely high (37.4 cases/100 PY) and decreased steeply to 17.7 and 12.7 cases/100 PY at 3rd and 6Th months respectively. Baseline TST response > 5 mm indurations, baseline hemoglobin < 12 gm/dl and being male sex were the strongest predictor of occurrence of TB during HAART. Only 3/8 (37.5%) episodes of TB were presented as unmasking tuberculosis IRIS. The proportion of paradoxical IRIS was 9.6 % (3/31) and the incidence rate was 22.5 cases per 100 persons per year.

**LIMITATIONS OF THE STUDY**

- This study was conducted among HIV infected individuals receiving HAART at ALERT hospital. Thus, the result may not represent the whole population receiving HAART under the national ART program.
- Anti-retroviral treatment response was not monitored by HIV RNA level due to the high cost of viral load determination, thus we were unable to assess the confounding effect of level of HIV RNA on immune response.
- The role of HAART in the restoration of long term (central memory T cells) and defect and recovery of antigen presenting cells (using specific markers) was not assessed in this study; thus we are unable to explore the full potential of HAART in restoring immune response.
- Our study is limited to the analysis of immunological recovery during the first 6 months of HAART only, thus the result does not represent long-term outcomes and sustainability.

## CONCLUSIONS AND RECOMMENDATIONS

### CONCLUSIONS

- Our finding in baseline immune response to tuberculosis showed that the proportion of positive TST response was lower among those with low CD4 cell count whereas the proportion of positive IFN-γ EC ELISPOT response did not vary with level of CD4. Thus, the quantitative IFN-γ EC ELISPOT assay appears to retain diagnostic performance among HIV infected individuals regardless of level of immunodeficiency. However, the observed *in-vitro* anergy and reduced quantitative PHA response with CD4 cell count warrants the needed of further investigation to optimize performance this assay among HIV infected individuals before being implemented in clinical practice.

- HAART restored CD4 cell levels to above the zone of severe immunodeficiency (CD4> 200 cells/$mm^3$) within the first three months of treatment among the majority of individuals in our cohort. The chance of achieving CD4 cell count >200 cells/$mm^3$ at $3^{rd}$ or $6^{th}$ month was associated with baseline CD4 cell level. However, the magnitude of change in CD4 count over time and the proportion of immunological failure were not dependent on baseline CD4 cell count at which HAART is initiated. Thus, those individuals who initiated HAART with the lowest CD4 counts do not have total loss of capacity for immune recovery. Rather, a longer period may be required to attain CD4 cell count > 200 cells/$mm^3$ for those individuals who initiated HAART with the lowest CD4 cell counts.

- We found improvement in immune response to TB among individuals receiving HAART, irrespective of the level of CD4 cell count at which HAART is initiated as evidenced by the following key observations: (i) 1. The proportion of TST positive response increased significantly after HAART in the whole cohort and a significant increment was observed among groups with baseline CD4 cell < 50 cells/$mm^3$ in stratified analysis; (ii) The median number of IFN- γ spot forming cell against TB antigens increased significantly after HAART in the whole cohort and significant

increment in median IFN-γ spot forming cell against PPD and ESAT-6 was observed among groups with baseline CD4 < 50 cells/ $mm^3$ in stratified analysis. Moreover, the observed lack of effect of baseline CD4 cell level on the proportion of immunological failure and on the rate of change in median CD4 level, further supports indirectly that HAART improves immune response to TB regardless of baseline CD4 cell count.

- Although HAART improves immune response to TB, the rate or extent of improvement may depend on the level of CD4 count at which HAART is initiated as suggested by the following observations (although the observed difference was not statistically significant except for the quantitative ELISPOT response to PPD at the $6^{th}$ month): (i) the proportion of TST positive response after treatment was also higher among individuals who have started HAART with CD4 $\geq$ 100 cells / $mm^3$; (ii) the median number of IFN- γ spot forming cell against TB antigens were higher both before and after HAART among individuals with baseline CD4 level $\geq$ 100 cells / $mm^3$.

- We found a high tuberculosis incidence rate (12.7cases/100 PY) during the first 6 months of treatment among individuals receiving HAART at ALERT hospital. The majority (62.5%) cases of TB occurred within the first month of HAART. This could possibly represent the progression of sub clinical TB that was missed at baseline investigations.

- Baseline TST response > 5 mm induration, baseline hemoglobin < 12 gm/dl and being male were the strongest predictors for the occurrence of TB during HAART.

- We found that only 3/8 (37.5%) episodes of TB presented as unmasking tuberculosis IRIS. Thus, our data support the proposal of the International Network for the Study of HIV-associated IRIS (INSHI) on the appropriate use of the term IRIS to refer to some but not all cases of incident TB during the initial months of HAART. The proportion of paradoxical IRIS was 9.6 % (3/31) or the incidence rate was 22.5 cases per 100 persons per year.

**RECOMMENDATIONS**:

- We have demonstrated that the quantitative IFN-γ EC ELISPOT assay retains diagnostic performance among HIV infected individuals regardless of the level of immunodeficiency. Investigation possible factors that contribute for the observed ELISPOT failure should be the second priority. This will enable this test to contribute usefully to the diagnosis of LTBI in HIV infected persons and would usefully contribute to TB control by selecting candidate for IPT.

- Our data on recovery of CD4 cell count indicated that those individuals who have initiated HAART with the lowest CD4 cell levels remained below the relatively more 'safe' CD4 cell levels for a prolonged period, maintaining a longer period for the development of some opportunistic infections. This may undermine the acceptance and impact of delivery of HAART in ART programs due to high rate of morbidity and mortality during the early months. Thus, to reduce this complication at ART service we recommend;

  - ✓ Strategy should be designed to identify patients who need to start HAART as early as possible before getting into profound CD4 lymphocytopenia. Currently, the number individuals receiving HAART in Sub-saharan Africa are much fewer than would be expected from estimates based on HIV prevalence. One of the reasons for this gap could be that many individuals who need to start ART may stay at home, since only 10% of HIV-infected individuals in this region are believed to know their HIV status. Thus, most individuals received a diagnosis of HIV infection only when they presented to the health service with severe immunodeficiency and advanced disease. Our study supports this explanation since the median time interval between time when participants identified their HIV status and were enrolled into the study were 23 (IQR, 10 -112) days.

- We have demonstrated that immune response of HIV infected individuals to tuberculosis can be improved by ART regardless of level of immunodeficiency at which HAART is initiated. However, in order to explore the full potential of HAART in the restoration of immunity, further studies should be conducted to assess immune

response parameters and associated risk factors in order to help design possible alternative strategies to render HIV infected individuals less susceptible to tuberculosis.

- The observed high rate of tuberculosis particularly at the initial month of HAART may potentially be able to affect negatively the contribution of HAART to tuberculosis control through accelerating nosocomial transmission of TB at ART clinics. Thus, the following recommendations were forwarded:
    - ✓ Research priority should be given for investigation of improved diagnostic methods for active TB that are both sensitive and specific in order to decrease the reservoir of patients with subclinical TB before ART initiation.
    - ✓ The potential effect of early TB on nosocomial transmission of TB among patients at ART clinics should be studied.
- Health professionals should be aware that not all of incident TB during the initial months of HAART present as IRIS and this should be considered during patient management.
- Taking this study as a background, we recommend that a prospective cohort study should be conducted to validate the new diagnostic criteria for paradoxical TB IRIS and unmasking TB IRIS in developing countries as proposed by International Network for the Study of HIV-associated IRIS (INSHI).

## FUTURE RESEARCH

Optimization of ELISPOT assay and a large scale evaluation among HIV infected individuals naive to HAART, which includes;

- Fresh PBMC vs frozen PBMC in relations with CD4 cell count
- Fresh PBMC: immediate vs overnight resting
- Freeze-thawed PBMC; immediate vs overnight resting
- Apoptotic markers in relation with quantitative ELISPOT assay

Assessment of immunity to TB before and during HAART:

1. Assessment of defect on antigen presenting cells before and after HAART among HIV infected individuals and comparison of this result with healthy controls.
2. Assessment of both immediate (effecter T cells or memory T cells that have effecter function [$CD62L2^{-}$] ) and long term memory cells (central memory cells [$CD62L2^{+}$]
3. Assessment of the correlation of both the above markers of immune parameters with in vitro model of anti-tuberculosis activity.

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**ANNEX I. DATA ACQUISITION FORM**

**I Baseline, 3rd and 6th months follow up assessment data acquisition form**

Identification of the patient:

Patient study code ☐☐☐

Patient initials ☐☐

Card No_____

Date of examination

dd mm yyyy / dd mm yyyy

/ / / / / / /

Ethiopian calendar / Gregorian calendar

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## Inclusion criteria

1. At least 18 years of age and not older than 55 years of age --------- ---- Yes ☐ No ☐

2. Willing to sign informed consent.---------- Yes ☐ No ☐

3. Resides in Addis Ababa and is available for follow up at ALERT ART center for at least the duration of the study period.---------- Yes ☐ No ☐

NB: all answer above should be yes to qualify

## Exclusion criteria

1. Has initiated HAART before the commencement of the study----------Yes ☐ No ☐

2. Is receiving immunosuppressive treatment such as corticosteroid-------Yes ☐ No ☐

3. Has a known or suspected history of diabetes mellitus or lymphoma, or leukemia, or hemophilia ,or malignant neoplasms ----------Yes ☐ No ☐

4. If female, has evidence of pregnancy or has plan to became pregnant during the study period----------Yes ☐ No ☐

5. Will be unable to attend the schedule visits and to adhere closely to HAART ---------- Yes ☐ No ☐

NB: all answer above ( 1-5) should be No to qualify

# Part one baseline data

## I. Socio-Demographic data

1. Age (years) ☐☐

2. Sex

1. Male ☐ 2. Female ☐

3. Marital status

1. Never married ☐ 2. Married ☐ 3. Separated ☐
4. Divorced ☐ 6 Widow/ widower ☐

4. Education

1- Illiterate ☐ 2- Able to read and write ☐ 3. Primary ☐
4. Secondary ☐ 5. Tertiary ☐

5. Religion

1 Muslim ☐ 2. Orthodox ☐ 3. protestant ☐
4. Catholic ☐ 5. Other ☐

6 Occupation 1. Farmer ☐ 2. Housewife ☐ 3.Government employee ☐
4. Merchant ☐ 5. Daily laborer ☐ 6 House made ☐
7- Other specify ☐ _____

## II. Past Medical/ Treatment History

7. History of any other previous illness? (Opportunistic infection?)

1.Yes ☐ 2. No ☐ 3. Not sure ☐

7.1. If yes, fill the table below:

| Type of Illness | Time of diagnosis | Type of treatment |
|---|---|---|
| | | |
| | | |
| | | |
| | | |

8. Past history of TB 1. Yes ☐ 2. No ☐ 3. Not sure ☐

If yes , ansewer the following

8.1 How long ago (days/weeks/months) _____

8.1 Site of TB. 1, Pulmonary TB ☐ 2. Extra pulmonary ☐

If Extra pulmonary TB, specify the site _____

8.2 TB Smear

1. Not Determined ☐ 2. Postive ☐ 3. Negative ☐

8.2.1 If positive, Date ___/___/___ Site /Health Facility _____

8.3 TB Tx 1. Yes ☐ 2. No ☐ 83 Completed Tx 1. Yes 2. No

8.3.1 If yes Date Tx Started ____/____/__ 8.3.2 Date Completed ____/___/___

8.3.3 Regimen : 1. Not determined ☐ 2. 2SRHZ/6EH ☐

3. 2HRZES/1HRZE/5HRE ☐ 4. 2HRZE/6HE ☐

8.3.4 Post treatment smear 1 Smear +ve ☐ 2 Smear -Ve ☐

9. Recent history of close contact to TB patient

1. Yes ☐ 2. No ☐ 3. Not sure ☐

If yes, how long ago (days/weeks/months) _____-

10. History of HIV test 1. Yes ☐ 2 No ☐

10.1 If yes Date:____/____ /_____ 10.2 Site /Health Facility _____

11. Medication History ( In the past one year) 1. Yes ☐ 2. No ☐ 3. Not sure ☐

| Name the drug | When did the patient start taking the drug | How long did the patient take the drug | last time the patent took the drug | Remark |
|---|---|---|---|---|
| Cotrimoxazole Yes/No | | | | |
| INH Yes/No | | | | |
| Fluconazol Yes/No | | | | |
| Other specify | | | | |

12. Vital Signs Anrtopomtery and functional level

V/s: Temp ( $^{o}$C )_____ PULSE /HR ( /m)_____ BP(mmHg)____ RR (R/m)____

Ant. Height (cm)_____ Weight ( Kg) _____ BMI _____

Fun: status: 1. Working ☐ 2. Ambulatory ☐ 3. Bed ridden ☐

13. Symptoms *( Mark all that apply)*

| | | |
|---|---|---|
| 1. Chronic Cough ☐<br>2. Dyspenia ☐<br>3. Hemoptysis ☐<br>4. Chronic Fatigue ☐<br>5. Weight loss 0 ____% body wt ☐<br>6. Flu-like ( URTI) ☐ | 7. Night Sweat ☐<br>8. Fever > 1 month ☐<br>9. Desophagia and /or odynophagia ☐<br>10. Nausea and / or Vomiting ☐ | 11. Abdominal pain ☐<br>12. Numbness/ Tingling ☐<br>13. Persistence Headache ☐<br>14. Mental Confusion ☐<br>15. Chronic Diarrhea ☐<br>16. STI Symptoms ☐ |

14. General appearance of patient at presentation:

_____

15. Physical examination

| Physical Exa | Normal | Abnormal | Specify abnormal finding |
|---|---|---|---|
| HEENT | | | |
| lymph nodes | | | |
| Chest | | | |
| Heart | | | |
| Abdomen | | | |
| Genitourinary System | | | |
| Musculo-skeletal system | | | |
| Skin | | | |
| Nervous System | | | |

16. Other finding

_____

_____

## Evaluation for TB

17. ZN stain for AFB

16.1 Sputum: Spot _____1.Positive 2. Negative 3 Not available

16 if positive, 1. +1 ☐ 2. +2 ☐ 3 . +3 ☐

Morning____1.Positive 2. Negative 3 Not available

16 if positive, 1. +1 ☐ 2. +2 ☐ 3 . +3 ☐

Spot _____ 1.Positive 2. Negative 3 Not available

16 if positive, 1 +1 ☐ 2. +2 ☐ 3 . +3 ☐

16.2 Other sample: specify _____

1.Positive ☐ 2. Negative ☐ 3 Not available ☐

18. Culture

17.1 Sputum

1.Positive ☐ 2. Negative ☐ 3 Not available ☐

17.2 Other sample: specify _____

1.Positive ☐ 2. Negative ☐ 3 Not available ☐

19. Abnormal chest radiographic features 1. Yes ☐ 2.No ☐

If yes, specify _____

20. Pathology finding (if needed)

1. Yes ☐ 2.No ☐ 3. Not done ☐

If yes, specify _____

21. Evidence of active TB?

1. Present ☐ 2.Absent ☐

If present,

20.1 Site of TB. 1. Pulmonary TB ☐ 1.1. smear +ve ☐ 1.2 Smear -Ve ☐

2. Extra pulmonary ☐

If Extra pulmonary TB, specify the site _____

20.2 Planned Regimen 1. 2SRHZ/6EH ☐ 2. 2HRZES/1HRZE ☐

3. 2HRZES/1HRZE/5HRE ☐ 4. Other _____

22 . Clinical WHO stage of HIV/AIDS 1. Stage I ☐ 2. Stage II ☐

3. Stage III ☐ 4. Stage IV ☐

23. ART regimen planned to initate :

1. 1a (30) = d4t (30)-3TC-NVP ☐ 2. 1a(40) = d4t (40)-3TC-NVP ☐

3. 1b(30) = d4t (30)-3TC-EFV ☐ 4 . 1b(40) = d4t (40)-3TC-EFV ☐

5. 1c = AZT- 3TC-NVP ☐ 6. 1d = AZT - 3TC-EFV ☐

24. Any planned treatment for other conditions

1. Yes ☐ 2.No ☐

If yes, fill the table below

| Resons or conditions for Tx | Type of Tratment | Duration |
|---|---|---|
| 1.<br>2.<br>3<br>4. | | |

## Laboratory data

25. CD4 and CD8 cell count

| Lab Tests | Result | | |
|---|---|---|---|
| | **0 (baseline)** | **3rd month** | **6th month** |
| CD4 Count (cell/$mm^3$) | | | |
| CD3 Count (cell/$mm^3$) | | | |
| CD8 Count (cell/$mm^3$) | | | |
| CD4/ CD8 Ratio | | | |

26. Hematology

| Lab parameters | | Result at Week | | | | | |
|---|---|---|---|---|---|---|---|
| | | **0** | **2nd** | **4th** | **8th** | **12th** | **24th** |
| TWBC | | | | | | | |
| TLC | | | | | | | |
| DIFF.C | Neutrophil | | | | | | |
| | Lymphocyte | | | | | | |
| | Eosinophil | | | | | | |
| | Basophil | | | | | | |
| | Monocyte | | | | | | |
| Hemoglobin | | | | | | | |
| HCT | | | | | | | |
| MCV | | | | | | | |
| MCHC | | | | | | | |
| MCH | | | | | | | |
| Platelet | | | | | | | |

27. Clinical chemistry

| Lab Tests | | Result at Week | | |
|---|---|---|---|---|
| | Normal Range | 0 | 12h | 24th |
| SGOT | | | | |
| SGPT | | | | |
| AP | | | | |
| Bilirubin | | | | |
| BUN | | | | |
| Creatinine | | | | |
| Amylase | | | | |
| Glucose | | | | |

28. RPR/VDR ***(only at baseline)***

Result _____

29. Stool examination

| At baseline | At 3rd month | At 6th month |
|---|---|---|
| Any finding? Yes/No<br>If yes, list below | Any finding? Yes/No<br>If yes, list below | Any finding? Yes/No<br>If yes, list below |
| | | |

30. Urine analysis *( write only if abnormal result )*

| Type Test | | Value at: | | |
|---|---|---|---|---|
| | | Baseline | 3rd month | 6th month |
| Chemical test | PH | | | |
| | Protein | | | |
| | Glucose | | | |
| | Ketonens | | | |
| | Bilirbin | | | |
| | Blood | | | |
| | Nitrate | | | |
| | Urobilinogen | | | |
| Microscopy | WBC/ HPF | | | |
| | RBC/HPF | | | |
| | Cast<br>Specify | | | |
| | Crystal<br>Specify | | | |

31. Skin test and ELISPOT assay result ( *to be filled by principal investigator)*

| TESTS | | Months | | |
|---|---|---|---|---|
| | | 0 | 3 | 6 |
| Skin test | PPD | | | |
| | Candida/mumps (Optional ) | | | |
| ELISPOT | PPD | | | |
| | PHA | | | |
| | ESAT-6/ CFP-10 | | | |

3. Viral load result ( *to be filled by principal investigator)(optional)*

| Lab Tests | **Result** | |
|---|---|---|
| | **0 (baseline)** | **6th month** |
| Plasma HIV-1 RNA level | | |

If patient is excluded from the study or is lost to follow up, please indicate reasons:

_____ _____ _____

Name of the physician DATE Signature

## Part two Clinical Data at third month

### General condition and physical Examination

1. Vital Signs Anrtopomtery and functional level

Temp ( $^{o}$C )_____ PULS /HR ( /m)_____ BP(mmHg)____ RR (R/m)____

Weight ( Kg) _____ BMI _____

Fun: status: 1. working ☐ 2. Ambulatory ☐ 3. Bed ridden ☐

2. General appearance of patient at presentation:

_____

3. Physical examination

| Physical Exa | Normal | Abnormal | Specify abnormal finding |
|---|---|---|---|
| HEENT | | | |
| lymph nodes | | | |
| Chest | | | |
| Heart | | | |
| Abdomen | | | |
| Genitourinary System | | | |
| Musculo-skeletal system | | | |
| Skin | | | |
| Nervous System | | | |

4. Other finding

---

5. Adherence to HAART
   1. Good ☐ 2. Fair ☐ 3 Poor ☐

   If poor/ fair adherence, specify the reason

   _____

   _____

6. ART drug being taken by the patient currently

   1. 1a(30) = d4t (30)-3TC-NVP ☐ 2. 1a(40) = d4t (40)-3TC-NVP ☐

   3. 1b(30) = d4t (30)-3TC-EFV ☐ 4 . 1a(40) = d4t (40)-3TC-EFV ☐

   5. 1c = AZT- 3TC-NVP ☐ 6. 1d = AZT - 3TC-EFV ☐

7. Is there any change in ART regimen from the original ( start )?

   1. Yes ☐ 2.No ☐

   If yes , 7.1 Since when (Date) ____/_____/ _____

   7.2 Specify the reason _____

8. Any ART related side effects ( clinical and/or Lab evidence )

   1. Yes ☐ 2.No ☐

   If yes, specify _____

   _____

9. Any other drugs being taken by the patient currently. 1. Yes ☐ 2.No ☐
   If yes, fill the table below

| Chemical Name of Drug | Dose (per day) | How long ago did the patient start taking the drug (in days) | How long the drug planned to be taken | Any related side effects ( clinical and Lab evidence ) |
|---|---|---|---|---|
| | | | | |
| | | | | |

8. Any opportunistic infection currently diagnosed ? . 1. Yes ☐ 2.No ☐

2.1. If yes fill the table below:

| Type of Illness | Time of diagnosis | Planed treatment |
|---|---|---|
| | | |
| | | |
| | | |
| | | |

If the study participant is excluded from the study or is lost to follow up, please indicate reasons:

_____ _____

**Name of the physician** **Signature**

## Part three  cliniacl data at six month follow up

### General condition and physical Examination

1. Vital and functional level

Temp ( $^{o}$C )_____ PULS /HR ( /m)_____ BP(mmHg)____ RR (R/m)____

Weight ( Kg) _____ BMI_____

Fun: status: 1. working ☐ 2. Ambulatory ☐ 3. Bed ridden ☐

2. General appearance of patient at presentation:

_____

_____

3. Physical examination

| Physical Exa | Normal | Abnormal | Specify abnormal finding |
|---|---|---|---|
| HEENT | | | |
| lymph nodes | | | |
| Chest | | | |
| Heart | | | |
| Abdomen | | | |
| Genitourinary System | | | |
| Musculo-skeletal system | | | |
| Skin | | | |
| Nervous System | | | |

4. Other finding

5. Adherence to HAART

1. Good ☐ 2. Fair ☐ 3 Poor ☐

If poor/ fair adherence, specify the reason

_____

_____

6. ART drug being taken by the patient currently

1. 1a(30) = d4t (30)-3TC-NVP ☐ 2. 1a(40) = d4t (40)-3TC-NVP ☐

3. 1b(30) = d4t (30)-3TC-EFV ☐ 4 . 1a(40) = d4t (40)-3TC-EFV ☐

5. 1c = AZT- 3TC-NVP ☐ 6. 1d = AZT - 3TC-EFV ☐

7. Is there any change in ART regimen from the original?

1. Yes ☐ 2.No ☐

If yes , 7.1 Since when _____

7.2 Specify the reason _____

8. Any ART related side effects ( clinical and/or Lab evidence )

.

1. Yes ☐ 2.No ☐

If yes, specify _____

_____

9. Any other drugs being taken by the patient currently. 1. Yes ☐ 2.No ☐

If yes, fill the table below

| Chemical Name of Drug | Dose (per day) | How long ago did the patient start taking the drug (in days) | How long the drug planned to be taken | Any related side effects ( clinical and Lab evidence ) |
|---|---|---|---|---|
| | | | | |
| | | | | |
| | | | | |
| | | | | |

10. Any opportunistic infection currently diagnosed ? . 1. Yes ☐ 2.No ☐

2.1. If yes fill the table below:

| Type of Illness | Time of diagnosis | Planed treatment |
|---|---|---|
| | | |
| | | |
| | | |
| | | |

If the study participant is excluded from the study or is lost to follow up, please indicate reasons:

_____

_____

_____

_____

_____

_____ _____

Name of the physician Signature

## ANNEX II. INFORMATION SHEET FORM

a) English Version

**Title** **Assessment of Specific Immune Response to Tuberculosis in HIV Infected Patients before and during Highly Active Antiretroviral Therapy HAART)**


**Principal Investigator: Tesfaye Berhanu ( BSc), AAU, AHRI**

**Supervisors**: **Daniel Asrat (MD, PhD), AAU**

**Yimtubezinash Woldeamanuel (MD, PhD), AAU**

**Abraham Aseffa (MDPhD), AHRI**

**Co- Investigators**: **Asfawessen G/Yohans (MD, Internist), ALERT**

**Selamawit Ejigu (MD) ALERT**

**Jemal Hussein (MD Phatologist), AHRI**

**Lawrence Yamuah, (PhD), AHRI**

**Kidist Bobosha (BSC, MSc). AHRI**

**Markos Abebe (Msc, PhD), AHRI**



**Name of sponsor: Armauer Hansen Research institute (AHRI)**

**Addis Ababa University, School of Graduate Studies (AAU)**


## Purpose of the study

Data from 2003 indicates HIV/ AIDS accounted for an estimated 38% or 54,000 of all new TB cases in Ethiopia. Tuberculosis is the major cause of illness and death in HIV infected Patients. The main control strategies are improving the body defense of People living with HIV /AIDS by antiretroviral therapy and preventing the development of active tuberculosis by TB preventive treatment. For

effective implementation and better management of TB/HIV co-epidemic in our country, baseline information on effect of HAART to the body defense, blood viral load, and clinical course of PLWHA is needed. Therefore, the aim of this study is to assess how fast and how well body defense to tuberculosis is rebuilt with respect to body defense during initiation of HAART. These investigations will help to recognize how well HAART works and to determine the magnitude of improvement of body defense and how fast it changes. This will help physicians manage HAART better and improve tuberculosis control at large

**Procedure**

To perform this study, volunteer participants who have not previously received HAART will be recruited from ALERT ART center and evaluated for enrollment criteria. After enrollment and initiation of HAART study participants will be followed up to 6 months according to the national guideline. Blood samples will be collected during their visit to assess element of body defense to tuberculosis. In addition, the participant will receive injection in to the skin to see if their body can show a response. A positive response is said to occur when the skin becomes read and a firm swelling is seen or felt at the site of injection. The size of this firm swelling will be measured on the third day (48 hours). Skin test is negative in patients who have low body defense or person who have never been exposed to TB.

If you agree to participate in the study, you will be tested for element of body defense to tuberculosis in your blood and with (PPD) skin test where you will be injected with purified parts of the organism into the skin to see how strongly your body reacts. About 20 ml of blood (about four teas spoonfuls) will be taken from you for these investigations at enrollment, 3$^{rd}$ and 6$^{th}$ months of following initiation of HAART. In addition, you will be requested to give blood and other specimen for routine laboratory tests needed to monitor patients receiving HAART

*Risks*

There are some risks associated with this study. You may experience a brief moment of physical discomfort during PPD skin test, collection of blood sample and inflammatory response in the skin. In addition you need to return to the clinic so that your test can be read on the third day. However, the reading itself will take less than 10 minutes of your time.

*Benefits*

Subjects enrolled in this study will benefit in several ways. All subjects will have more frequent laboratory investigation on clinical chemistry tests and CD4 cell counts. In addition, the treatment response will also assessed by plasma viral load determination. These all provide better information on course of clinical condition that benefits the participants. Moreover, information obtained from this study provides improved understanding for better management of HAART that benefits the community.

*Incentives*

You will not be provided any payment to take part in the research however you will be reimbursed 15 Birr per day for your transport cost.

Confidentiality

If you decide to take part in this study any personal information about you will be kept private, the nature of the questionnaire is private, and your name will not appear in any laboratory test or other paper. Information about you will be filed with code. Only the physician will know the codes.

**Rights to refuse or withdraw**

You have full rights to refuse taking part in the study. You also have the right to withhold information, refuse cooperation or to drop out of the study at any time you want without any need to explain the reason to anyone, and your actions will have no effect on the overall management of your disease. You have the right to ask and get clarifications at any time.

Whom to contact

If you don't understand or if you have any questions about the information given above you may contact the principal investigator:

Tesfaye Berhanu Tel. 0916826340 or tesbelay@yahoo.com

b) Amharic Version

# የሳንባ ቲቢ በሽታ ሊያመጣ የሚችለውን ተውሳክ የሚከላከለው የሰውነታችን ደም ህዋስ የጸረ ኤች አይ ቪ መድሐኒት በሚወሰድበት ወቅት የሚናረውን ለውጥ ለማወቅ የሚደረግ ጥናት

## ማብራሪያ ቅጽ

**የተመርማሪው ስም** ተስፋዬ ብርሐኑ

**ሱፐርቫይዘሮች** ዶ/ር ዳንኤል አስራት

ዶ/ር ይምጡበዝናሽ ወ/አማኑኤል

ዶ/ር አብርሃም አሰፋ

**ረ/ተመራማሪዎች** ዶ/ር አስፋወሰን ገ/ዮሀንስ

ዶ/ር ሰላማዊት እጅጉ

ዶ/ር ጀማል ሁሴን

ዶ/ር ሎረንስ ያሙሀ

ወ/ሮ ቅድት ቦቦሻ

## ምርምር የሚደረግበት ቦታ

አርማወር ሀንሰን የምርምር ማዕከል እና አለርት ሆስፒታል

## ጥናቱን ስፖንሰር የሚያደርገው

አርማወር ሀንሰን የምርምር ኢኒስቲትዩት እና አዲስ አበባ ዩኒቨርስቲ

## የጥናቱ ዓላማ

የ2003 መረጃ እንደሚያመለክተው በኢትዮጵያ 38% (ሰላሳ ስምንት በመቶ) ለሚሆነው የሳንባ ቲቢ በሽታ መንስኤው ኤች አይ ቪ ኤድስ መሆኑን ያረጋግጣል። ሳንባ ቲቢ በሽታም በኤች አይ ቪ ኤድስ ምክንያት ሊከሰት ለሚችለውን ህመምና ሞት ዋነኛው ምክንያት ነው። ይህንንም በሽታ ለመቆጣጠር በዋነኛነት ከቫይረሱ ጋር ለሚኖሩ ሰዎች የፀረ ኤች አይ ቪ ኤድስ መድሀኒት በመስጠት የሰውነትን በሽታ የመከላከል ብቃት መጠበቅ ወይም መልሶ መገንባትና የሳንባ ቲቢ በሽታን ለመከላከል ለእነዚሁ ህሙማን የፀረ ሳንባ ቲቢ መድሐኒት እንደ መከላከያ መስጠትን ያካትታል። እነዚህን መቆጣጠሪያ መንገዶች በሥራ ላይ በብቃት ለማዋል የአገራችን የኤች አይ ቪ ኤድስ ህሙማን በደማቸው ውስጥ ያለውን ቫይረስ መጠን፣ የጤንነት ሁኔታ እንዲሁም የሰውነታቸው በሽታን የመቋቋም ብቃት በተለይም የሳንባ ቲቢ በሽታን የሚከላከለው የደም ህዋስ በፀረ ኤች አይ ቪ ኤድስ መድሀኒት ምን ያህል ለውጥ ሊያመጣ እንደሚችል መረጃ የስፈልጋል። ስለዚህም የዚህ ጥናት ዋነኛ ዓላማው በፀረ ኤች አይ ቪ መድሐኒት የሳንባ ቲቢ በሽታን የሚከላከልው የደም ህዋስን በምን ያህል መጠንና ፍጥነት መልሶ ሊገነባ እንደሚችል ለማወቅ ሲሆን የጥናቱ ውጤም የፀረ ኤች አይ ቪ ኤድስ መድሀኒትን የበለጠ ውጤታማ ብሆነ መልኩ ለመጠቀምና የሳንባ ቲቢ በሽታን ለመቆጣጠር ይረዳል።

## ቅድመ-ተከተል

ይህንን ጥናት ለማከናወን ፍቃደኛ የሆኑና ከዚህ ቀደም የፀረ ኤች አይ ቪ ኤድስ መድሀኒት መውሰድ ያልጀመሩ የኤች አይ ቪ ኤድስ ህሙማን ከአለርት የፀረ ኤች አይ ቪ የህክምና ማዕከል በጥናቱ መስፈርት መሰረት ይመለመላሉ ። የተመለመሉትም የጥናቱ ተሳታፊዎች የፀረ ኤች አይ ቪ መድሀኒት ከጀመሩ በኋላ ለ6 ወር ክትትል ይደረግላቸዋል። በዚህም የክትትል ወቅት ከተሳታፊዎች የደም ናሙና ተወስዶ የነጭ የደም ህዋስ በሳንባ ቲቢ በሽታ ላይ ያለዉ የመከላከል ብቃት ይዳሰሳል። ከዚህም በተጨማሪም ለተሳታፊዎች ከሳንባ ቲቢ ተውሳክ የተወሰደ የተጣራ ፕሮቲን በቆዳቸው ላይ በመርፌ ተሰጥቶ በቆዳ ላይ ቀላ፣ ጠንከር ያለ እብጠት ሲታይ ፖዘቲቭ ምላሽ ይባላል ይህም በሶስተኛው ቀን መጠኑ ይለከል። የሰውነት በሽታን የመከላከል ብቃት እጂግ በጣም የተዳከመበት ወይም ለሳንባ ቲቢ አስቀድሞ ያልተጋለጠ ሰው ይህ ምላሽ አይኖረውም።

እርስዎ በዚህ ጥናት ፍቃደኛ ሆነው ከተሳተፉ የሰውነትዎ ሳንባ ቲቢን በሽታ የመከላከል ብቃት፤የፀረ ኤች አይ ቪ መድሀኒት ሳይጀምሩ በፊት ከነበረው ጋር ለማስተያየት መድኃኒቱን ከጀምሩ በኋላ ያለው ለውጥ፤በቆዳዎና በደምዎ ህዋስ ላይ በሚደረግ ጥናት ይዳሰሳል። ለዚህም ልክ መድኃኒቱን ሊጀምሩ ሲሉ፣ ከጀመሩ በኋላ ደገሞ በ3 እና በ6ኛው ወር ላይ በቆዳዎ ላይ ከሳንባ ቲቢ ተውሳክ የተወሰደ የተጣራ ፕሮቲን እየተወጋና አራት የሻይ ማንኪያ የሚያህል ደም በእነዚህ ጊዜያት እየተወሰደ የሰውነትዎ የነጭ የደም ህዋስ የሳንባ ቲቢን በሽታ የመከላከል ብቃት(ምላሽ) በፀረ ኤች አይ ቪ መድሀኒት ምን ያህል መልሶ ሊገነባ እንደሚችል ጥናት ይደረጋል።

## ሊከሰቱ የሚችሉ ስጋቶች ወይም ምቾቶች መጓደል

ከጥናቱ ጋር በተያያዘ ሊከሰቱ ከሚችሉ ችግሮች መካከል በቆዳ ላይ በሚደረገው ምርምራ የተጣራ የቲቢ ህዋስ ፕሮቲን በሚወጋበት ወቅት በመርፊው አማካይነት ከሚሰማው ጊዜያዊ ህመምና በቆዳ መቆጣት የተነሳ ሊከሰት ከሚችል መጠነኛ የሆነ ህመም ውጪ ጉዳት የለውም ። ውጤቱንም ለማንበብ በሶስተኛው ቀን ተመልሰው ወደ ክሊኒክ እንዲመጡ ይደረጋል። ነገር ግን ውጤቱን ብቻ ለማንበብ የሚባክነው ጊዜዎ ከ 10 ደቂቃ አይበልጥም።

## ጥቅም

በዚህ ጥናት ላይ ለመሳተሰፍ የተመረጡ ግለሰቦች በተለያየ መንገድ ጥቅሞች ያገኛሉ። በሙሉ በጥናቱ ላይ የሚሳተፉ ግለሰቦች ተደጋጋሚ የላቦራቶሪ ምርመራ (በክሊኒካል ኬሚስትሪና CD4 በመባል የሚታወቀውን የነጭ የደም ህዋስ መጠን) ውጤት ያገኛሉ ። በተጨማሪም የመድሀኒቱ ውጤታማነት በደም ውስጥ የሚገኘውን ኤች አይ ቪ ቫይረስ መጠን በመለካት ምርመራ ይደረግል ። እነኚህ ተጨማሪ ምርመራዎች በአጠቃላይ ጥናቱ ላይ የሚሳሰተፉ ግለሰቦችን የጤንነት ክትትል ሁኔታን የተሻለ በማድረግ ለተሳታፊዎቹ ጥቅምን ይሰጣል። ከዚህ በበለጠ መልኩ ከጥናቱ የሚገኝ ው ውጤት የዕድሜ ማራዘሚያ መድሀኒት በአግባቡ ከመጠቀም አንፃር የተሻለ መረጃ በማቅረብ ለባለሞያው፣ለበሽተኛውና ለህብረተሰቡ የላቀ ጥቅም ይሰጣል።

## ማበረታቻ

በዚህ ጥናት ውስጥ ለሚሳተፉ የተለየ ማበረታቻ አይሰጥም። ነገር ግን ለዚህ ጥናት በሚመላለሱበት ወቅት ያባከኑትን ጊዜና የሚመለለሱበትን የትራንስፖት ወጪ ለመካስ በቀን 15 ብር ይታሰብሎታል።

## ሚስጥር ስለመጠበቅ

በዚህ ጥናት ላይ ለመሳተፍ ከወሰኑ የእርስዎ የግል መረጃ ሁሉ ሚስጢራዊ ይደረጋል። የመጠይቁም ተፈጥሮ ሚስጢራዊ ሲሆን ስምዎም በማንኛውም ወረቀት ወይም መዝገብ

ላይ አይሰፍርም። ስለዚህ ሁሉም መረጃዎች በኮድ (በሚስጢር ቁጥር) ይሰፍራሉ። ይህንንም መረጃ የሚያቀው ሀኪሙ ብቻ ነው።

## በጥናቱ ያለመሳተፍ ወይም እራስን የማግለል መብት

በዚህ ጥናት ላይ ያ<u>ል</u>መሳተፍ ሙሉ መብት የተጠበቀ ነው። በጥናቱ ከተሳተፉም በማንኛውም ጊዜ ምንም ዓይነት ምክንያት ሳያቀርቡ ወይም እንዲያቀርቡ ሳይገደዱ ከጥናቱ የመውጣት መብት አሎ-ዎት። ይህም እርምጃ በእርስዎ የህክምና ክትትል ላይ ምንም ዓይነት ተጽእኖ አያመጣም። በተጨማሪም በማንኛውም ሰዓት ማብራሪያ የመጠየቅ መብት አሎዎት።

## መረጃ ለማግኘት

ስለ ጥናቱ ጥያቄ ወይም ተጨማሪ ማብራሪያ ካስፈለግዎት በሚከተለው አድራሻ ይጠይቁ።

**ተስፋዬ ብርሐኑ** አ.አ.ዩ. / አህሪ

ስ. ቁ. **0916-826340**

## ANNEX III. CONSENT FORM

a) English Version

**Patient Code No: _____**

I have been informed that I have HIV virus in my blood after I had been counseled and tested. After the test result was given to me, I was counseled and have understood the benefit of treatment and agreed to start HAART. Then I have been requested to participate in a research project that aims at assessment of body defense to tuberculosis while taking HAART. This requires that I will be tested for elements of body defense to tuberculosis in my blood and with (PPD) skin test where I will be injected with purified parts of the organism into the skin to see how strongly my body reacts. About 20 ml of blood (about four teas spoonfuls) will be taken from my arm for these investigations at enrolment, 3rd, and 6th months following initiation of HAART.

I have been informed that all information I will be giving to the physician will be kept confidential and that the nature of the questionnaire is private. My name will not appear in any laboratory test or other paper. I understand that the risks I am exposed to will be the risk from blood drawing and skin injection. I will have to come again on the 3rd and 6th months following initiation of HAART to provide blood samples and undergo skin testing for this study. Extra investigation may be required by the physician for my care depending on how my body responds to the drugs. The investigations will help to recognize how well HAART works and to determine the magnitude of improvement of body defense and how fast it changes. This will help physicians manage HAART better. I also know that I have the right to withhold information, refuse cooperation or to drop out of the study at any time I want without any need to explain the reason to anyone, and that my actions will have no effect on the overall management of my disease. I know that I have the right to ask and get clarifications at

any time. I have asked for clarification and I have received satisfactory responses in a language I understood. I have been given sufficient time to respond. Therefore

1. To participate in the research . I agree: I ☐ I disagree ☐

2. Moreover the specimens leftover from the test to be used for further additional investigations on HIV and body defense: I agree ☐ I disagree ☐

Signature of patient Date

_____ _____

Signature of witness Date

_____ _____

Signature of the physician who receive consent Date

_____ _____

b) Amharic Version

# <u>የስምምነት ቅፅ</u>

እኔ በመለያ ቁጥር ---------- የተመዘገብኩ ግለሰብ የደም ናሙና ከመስጠቴ በፊት የቅድመ ምርመራ ምክር እንዲሁም የደም ናሙና ከሰጠሁ በኋላ የድህረ ምርመራ ምክር የተደረገልኝ ሲሆን ፤ በምርመራውም ውጤት የኤች አይ ቪ ቫይረስ በደሜ ውስጥ እንደተገኘ ተነግሮኛል፡፡ ውጤቱንም ከተረከብኩ በኋላ የፀረ ኤድስ /የእድሜ ማራዘሚያ/ መድኃኒት እንድጀምር ተጠይቄ ጥቅሙን በመረዳቴ ለመጀመር ተስማምቻለሁ፡፡ በመቀጠልም የጸረ ኤች አይ ቪ መድሐኒት በሚወስዱ ሕሙማን ላይ ያተኮረ ጥናት ፡ አላማውም የሳንባ ቲቢ በሽታ ሊያመጣ የሚችለውን ተውሳክ የሚከላከለውን የሰውነታችንን የደም ህዋስ የጸረ ኤች አይ ቪ መድሐኒት በሚወሰድበት ወቅት የሚናረውን ለውጥ ለመዳሰስ በሚደረግ ጥናት እንድሳተፍ ተጠይቄአለሁ፡፡ በዚህ ጥናት ሂደት ውስጥ ይህንን መድሃኒት ለሚወስዱ የቫይረሱ ተሸካሚች ከሚደረገው የደም ምርመራ በተጨማሪ ሌሎች ምርመራዎች ማለትም ከሳንባ ቲቢ ተውሣክ የተወሰደ /በሽታ የማያመጣ/ የተጣራ ፕሮቲን በቆዳዬ ላይ እየተወጋ ጥናት እንደሚደረግና በተጨማሪም የደም ናሙና ተወስዶ የሰውነቱ ነጭ የደም ህዋስ ይህንን በሽታ በምን ያህል ብቃት እንደሚከላከል ምርመር እንደሚደረግ ተነግሮኛል፡፡ ስለዚህም አራት የሻይ ማንኪያ የሚያህል ደም መድኃኒቱን ልጀምር ስል ፣ከጀመርኩ በኋላ ደገሞ በ3ኛውና በ6ኛው ወር ላይ ለመስጠት እጠየቃለሁ፡፡

የሰጠሁት ቃለ ምልልስ በሚስጥር እንደሚያዝ ፤ የመጠይቁም ተፈጥሮ ሚስጥራዊ እንደሆነና በማንኛውም ላቦራቶሪ መዝገብ ወይም ወረቀት ላይ ስሜ እንደማይሰፍር ተነግሮኛል፡፡ በምርምሩ የተነሳ ሊደርስብኝ የሚችል ጉዳት ቢኖር በቆዳዬ ላይ ሊደረግ የሚችለው ምርምራ የሚያ ስከትለው ጊዚያዊ የማቃጠል ስሜትና ለምርምራ የደም ናሙና በሚወሰድበት ወቅት በመርፊው አማካይነት ሊመጣ የሚችል መጠነኛ ህመም እንደሆነ ተረድቻለሁ፡፡ መድኃኒቱን ልክ እንደጀመርኩ ፣ በ3ኛውና በ6ኛው ወር ለምርመራ እንደምመላለስና ከዚህ በተጨማሪም ለምወስደው የፀረ-ኤች አይ ቪ መድኃኒት ሰውነቴ ያለውን ምላሽ ለማወቅ በሀኪም ምርመራ እንደሚደረግልኝ አውቄያለሁ፡፡ ይህም ምርመራ የጸረ ኤች አይ ቪ መድኃኒት በሰውነታችን ውስጥ የሚገኘውን

የሳንባ ቲቢ መከላከያ ነጭ የደም ህዋስ እንዴት እና በምን ያህል ፍጥነት እንደሚያሻሽል ለማወቅ እንደሚረዳ ተረድቻለሁ፡፡ ይህም ሀኪሞች የሳንባ ቲቢ በሽታን ለመቆጣጠር ብሎም የፀረ-ኤች አይ ቪ መድኃኒትን የሚወስዱ ህሙማንን በተሻለ ለመርዳት ያስችላቸዋል መይም ይረዳቸዋል፡፡

በዚህ ምርምር ሂደት ውስጥ መንገር ያልፈለኩትን ነገር እንድነግር እንደማልገደድ ፤ ከጥናቱ ራሴን ማግለል በፈለኩ ጊዜ ማቋረጥ እንደምችልና ይህን ማድረጌ ደግሞ በህክምና አሰጣጡ ሂደት ላይ ምንም አይነት ተጽእኖ እንደማይኖረው የተገነዘብኩ ሲሆን በጥናቱ ውስጥ እንደታቀፍኩ ማንኛውንም ተገቢ ጥያቄ ጠይቄ ለጥያቄውም ተገቢውን ምላሽ እና ማብራሪያ በሚገባኝ ቋንቋ ያገኘሁ ስለሆነ

1. በዚህ ጥናት ለመሳተፍ እስማማለሁ ☐ አልስማማም ☐

2. ከምሰጠው የደም ናሙና ውስጥ የሚቀረው ለተለያዩ ምርመራዎች ማለትም፡- የኤች አይ ቪ እና በሰውነት የመከላከል አቅም ላይ በሚደረጉ ጥናቶች ላይ ቢውል ፈቃደኛ ለመሆን፡ እስማማለሁ አልስማማም ☐ ☐

| | |
|---|---|
| የህሙማን ፊርማ | ቀን |
| --- | --- |
| የምስክር ፊርማ | ቀን |
| --- | --- |
| የስምምነት ተቀባይ ፊርማ | ቀን |

## ANNEX IV WHO CLINICAL STAGING OF HIV/AIDS

# REVISED WHO CLINICAL STAGING OF HIV/AIDS
## FOR ADULTS AND ADOLESCENTS

(Interim African Region version for persons aged 15 years or more with positive HIV antibody test or other laboratory evidence of HIV infection)[b]

TABLE 1. REVISED WHO CLINICAL STAGING OF HIV/AIDS FOR ADULTS AND ADOLESCENTS

**Primary HIV infection**

- Asymptomatic
- Acute retroviral syndrome

**Clinical stage 1**

- Asymptomatic
- Persistent generalized lymphadenopathy (PGL)

**Clinical stage 2**

- Moderate unexplained weight loss (<10% of presumed or measured body weight)
- Recurrent respiratory tract infections (RTIs, sinusitis, bronchitis, otitis media, pharyngitis)
- Herpes zoster
- Angular cheilitis
- Recurrent oral ulcerations
- Papular pruritic eruptions
- Seborrhoeic dermatitis
- Fungal nail infections of fingers

**Clinical stage 3**

***Conditions where a presumptive diagnosis can be made on the basis of clinical signs or simple investigations***

- Severe weight loss (>10% of presumed or measured body weight)
- Unexplained chronic diarrhoea for longer than one month
- Unexplained persistent fever (intermittent or constant for longer than one month)
- Oral candidiasis
- Oral hairy leukoplakia
- Pulmonary tuberculosis (TB) diagnosed in last two years
- Severe presumed bacterial infections (e.g. pneumonia, empyema, pyomyositis, bone or joint infection, meningitis, bacteraemia)
- Acute necrotizing ulcerative stomatitis, gingivitis or periodontitis

***Conditions where confirmatory diagnostic testing is necessary***

- Unexplained anaemia (<8 g/dl), and or neutropenia (<500/$mm^3$) and or thrombocytopenia (<50 000/ $mm^3$) for more than one month

**Clinical stage 4**

***Conditions where a presumptive diagnosis can be made on the basis of clinical signs or simple investigations***

- HIV wasting syndrome
- Pneumocystis pneumonia
- Recurrent severe or radiological bacterial pneumonia
- Chronic herpes simplex infection (orolabial, genital or anorectal of more than one month's duration)
- Oesophageal candidiasis
- Extrapulmonary TB
- Kaposi's sarcoma
- Central nervous system (CNS) toxoplasmosis
- HIV encephalopathy

***Conditions where confirmatory diagnostic testing is necessary:***

- Extrapulmonary cryptococcosis including meningitis
- Disseminated non-tuberculous mycobacteria infection
- Progressive multifocal leukoencephalopathy (PML)
- Candida of trachea, bronchi or lungs
- Cryptosporidiosis
- Isosporiasis
- Visceral herpes simplex infection
- Cytomegalovirus (CMV) infection (retinitis or of an organ other than liver, spleen or lymph nodes)
- Any disseminated mycosis (e.g. histoplasmosis, coccidiomycosis, penicilliosis)
- Recurrent non-typhoidal salmonella septicaemia
- Lymphoma (cerebral or B cell non-Hodgkin)
- Invasive cervical carcinoma
- Visceral leishmaniasis

## ANNEX V. CRITERIA FOR INITIATION OF ART IN ADULTS AND ADOLESCENTS

| CD4 count not available | CD4 count available |
|---|---|
| WHO clinical stage IV and III irrespective of Total Lymphocyte Count (TLC) | WHO clinical stage IV, irrespective of CD4 count |
| WHO Clinical stage II if TLC $\leq$1200/ mm$^3$ | WHO clinical stage III, if CD4 cell counts $\leq$ 350/mm$^3$ [a] |
| Do not treat WHO clinical stage I, in absence of CD4 count | All WHO clinical stages, if CD4 cell counts $\leq$ 200/ mm$^3$ |

WHO clinical staging in adults and adolescents is ANNEX IV

TLC is only useful in deciding when to initiate ART in symptomatic patients with WHO clinical stage II disease.

The use of CD4 cell count to guide treatment decision is advisable. For example, pulmonary TB may occur at any CD4 level and other conditions may be mimicked by non - HIV aetiologies.

**ANNEX VI. ELISPOT ASSAY DATA TEMPLATE**

Date _____

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Med | PHA | PPD | ESAT-6 | CFP-10 | Blank | Med | PHA | PPD | ESAT-6 | CFP-10 | Blank | |
| B | Med | PHA | PPD | ESAT-6 | CFP-10 | Blank | Med | PHA | PPD | ESAT-6 | CFP-10 | Blank | |
| C | Med | PHA | PPD | ESAT-6 | CFP-10 | Blank | Med | PHA | PPD | ESAT-6 | CFP-10 | Blank | |
| D | Med | PHA | PPD | ESAT-6 | CFP-10 | Blank | Med | PHA | PPD | ESAT-6 | CFP-10 | Blank | |
| E | Med | PHA | PPD | ESAT-6 | CFP-10 | Blank | Med | PHA | PPD | ESAT-6 | CFP-10 | Blank | |
| F | Med | PHA | PPD | ESAT-6 | CFP-10 | Blank | Med | PHA | PPD | ESAT-6 | CFP-10 | Blank | |
| G | Med | PHA | PPD | ESAT-6 | CFP-10 | Blank | Med | PHA | PPD | ESAT-6 | CFP-10 | Blank | |
| H | Med | PHA | PPD | ESAT-6 | CFP-10 | Blank | Med | PHA | PPD | ESAT-6 | CFP-10 | Blank | |
| | | | | | | | | | | | | | |

Sample code Date No Cell/ well Remark Sample code Date No Cell/ well Remark

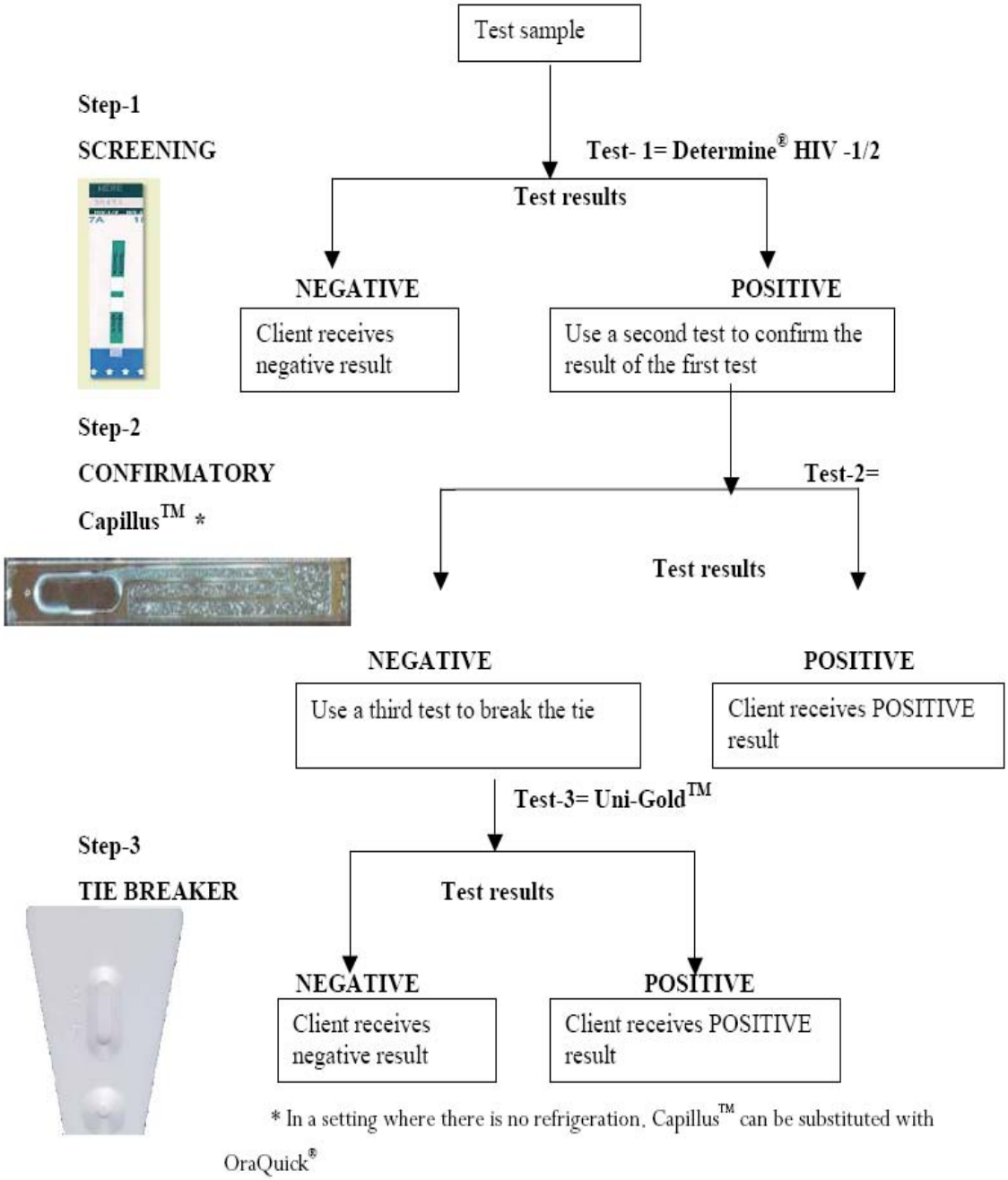
AB1_____ _____ _____ _____ AB7_____ _____ _____ _____

CD1_____ _____ _____ _____ CD7_____ _____ _____ _____

DE1_____ _____ _____ _____ DE7_____ _____ _____ _____

GH1_____ _____ _____ _____ GH7_____ _____ _____ _____

## ANNEX VII. RAPID HIV TEST ALGORITHM




* In a setting where there is no refrigeration, Capillus™ can be substituted with OraQuick®

Developed by the Ethiopian Health & Nutrition Research Institute in collaboration with the Ministry of Health and U.S. Centers for Disease Control and Prevention

DECLARATION

I the undersigned declare that this thesis is my original work. It has not been presented for a degree in this or any university and all the source materials used for this thesis have been duly acknowledged.

Name of the candidate Tesfaye Berhanu Belay

Signature ----------

Place Addis Ababa

Date -------/-------/----------

This thesis has been submitted for examination with my approval as university advisor.

1. Dr. Daniel Asrat (MD, MSc, PhD)

Signature_____ date _____

2. Dr. Yimtubezinash Weldeamanuel (MD, MSc, PhD)

Signature_____ date _____

Date and place of submission: Department of Microbiology, Immunlogy and Parasitology

Faculty of Medicine, Addis Ababa University

Addis Ababa, Ethiopia

March 2009**a**