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Addis Ababa University

**ADDIS ABABA UNIVERSITY, COLLEGE OF HEALTH SCIENCE, SCHOOL OF GRADUATE STUDIES, DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND PARASITOLOGY**

# Arginase activity in the blood of patients with HIV, TB and HIV/TB co-infections.

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## List of abbreviations

| | |
|---|---|
| AAU | Addis Ababa University |
| AFB | Acid fast bacilli |
| AIDS | Acquired immunodeficiency syndrome |
| AMCase | Acidic mammalian chitinase |
| ARG | Arginase |
| ART | Antiretroviral Therapy |
| ASL | Argininosuccinate lysate |
| ASS | Argininosuccinate synthase |
| BD | Becton Dickinson |
| BMI | Body mass index |
| Bp | Base pair |
| CAT | Cationic amino acid transporter |
| CD4 | Cluster of Differentiation four |
| cdk | Cyclin-dependent kinase |
| cDNA | Complementary deoxyribonucleic acid |
| CNR | Case notification rate |
| CPU | Carbomyl phosphate synthase |
| DERC | Departmental Ethical Review and Research Committee |
| dH2O | Distilled Water |
| DMIP | Department of Medical Microbiology, Immunology and Parasitology |
| EBP | Enhancer binding protein |
| EDTA | Ethylene diamine tetra-acetate |
| HCL | Hydrochloric acid |
| HIV | Human Immune Virus |
| HNF | Hepatocyte nuclear factor |
| $H_3PO_4$ | Phosphoric Acid |
| $H_2SO_4$ | Sulpheric Acid |
| IDO | Indoleamine 2 3-dioxygenase |
| IFN | Interferon |

| | |
|---|---|
| IL | Interleukin |
| iNOS | Inducible nitric oxide synthase |
| IQR | Inter quartile range |
| ISPF | Isonitrosopropiophenone |
| LPS | Lipopolysaccharide |
| LXR | Liver X receptor |
| M | Molar |
| MSC | Myeloid suppressor cell |
| MTB | *Mycobacterium tuberculosis* |
| NO | Nitric oxide |
| OAT | Ornithine aminotransferase |
| ODC | Ornithine decarboxylase |
| OI | Opportunistic infection |
| OTC | Ornithine transcarbamoylase |
| PAMP | Pathogen-Associated Molecular Patterns |
| PBS | Phosphate buffered saline |
| PBMC | Peripheral blood mononuclear cells |
| PKAI | Protein Kinase A Type I |
| PMN | Polymorphonuclear leukocytes |
| PTB | Pulmonary tuberculosis |
| SPSS | Statistical Package for Social Sciences |
| STAT | Signal transducer and activator of transcription |
| TCR | T cell receptor |
| Th | T helper |
| TNF | Tumour necrosis factor |
| V | Volume |
| WHO | World health organization |

**ABSTRACT**

**Background**: HIV/AIDS and TB remain a major global public health problem and their global distribution is heavily skewed toward low income and emerging economics. Africa, and more specifically Sub-Saharan Africa, faces the worst epidemic of the two diseases since the advent of the antibiotic era. Both TB and HIV have profound effects on the immune system, as they are capable of disarming the host's immune responses through mechanisms that are not fully understood. The catabolism L-arginine by arginase has emerged as a potent mechanism for the regulation of immune responses.

**Objectives:** To measure arginase activity in the blood of patient with HIV, TB and HIV/TB co-infected patients at Zewditu Memorial and St. peter's Hospital, Addis Ababa, Ethiopia.

**Methodology:** A cross-sectional study was conducted from April 2014 to October 2014.Venous blood was collected from patients before initiation of treatment and controls in BD Vacutainer EDTA tubes. Isolation of peripheral blood mononuclear cells (PBMC) is performed by density gradient centrifugation on Histopaque®-1077(Sigma) where as Plasma was obtained after centrifugation of a blood at 1800 rpm for 10 minutes. Arginase enzyme activity was determined using colorimetric assay based on color formed when urea produced is heated in acid with α-isonitrosopropiophenone. Data were evaluated by using GraphPad Prism version 6.05 and the differences were considered statistically significant at $p < 0.05$.

**Result:** Increased arginase activity was observed in PBMC of HIV, TB and HIV/TB co-infected patients than in PBMC of healthy controls and similarly higher arginase level also measured in plasma of TB and HIV/TB co-infected patients than in plasma of healthy control. Moreover, a CD+4 T cell counts of HIV and HIV/TB co-infected patients and BMI of HIV, TB and HIV/TB patients were negatively associated with PBMC arginase activity.

**Conclusion:** Our results suggest that arginase activity is become higher during HIV, TB and HIV/TB co-infections.

**Keywords:** PBMC, plasma, HIV, TB, HIV/TB patients, Arginase

# 1. INTRODUCTION

## 1.1. Background

Tuberculosis is an infectious bacterial disease caused by Mycobacterium Tubercle, an Acid Fast Bacilli as Koch stated and it is spread by aerosolization of droplet nuclei bearing *Mycobacterium tuberculosis* particles released from the lung of patient with cavitary pulmonary or laryngeal disease. It is also transmitted by consumption of raw milk containing *Mycobacterium bovis* (Mathema et al., 2006, Subba et al., 2009). Human immunodeficiency virus (HIV) is the virus that causes HIV infection. During HIV infection, the virus attacks and destroys the infection-fighting CD4 cells of the body's immune system. Loss of CD4 cells makes it difficult for the immune system to fight infections (U.S Department of Health and Human Services, 2012). Acquired Immunodeficiency Syndrome, or AIDS, occurs when the immune system is weakened by HIV to the point where a person is susceptible to any number of opportunistic Infections (OIs) or diseases. Having AIDS is defined as presenting with HIV and one or more OIs (U.S Department of Health and Human Services, 2012). HIV-induced progressive CD4 depletion is associated with increased risk of TB, disseminated TB and death (Geldmacher et al., 2012). Other cells are also directly or indirectly affected by the virus, including CD8+ T cells (Roederer et al., 1995), monocytes (Zhu et al., 2002), macrophages (Meltzer et al., 1990), B lymphocytes (Moir et al., 2001), neutrophils (Gabrilovich et al., 1993) and dendritic cells (Macatonia et al., 1990). Dysfunction of these cells plays a major role in particular aspects of HIV pathogenesis.

It is estimated that around two billion people around the world are currently infected with the bacillus but yet only 10% of infected immunocompetent individuals are likely to develop symptomatic TB during their lifetime when compared to 50% in individuals that are immunologically weakened by concurrent HIV infection (Vynnycky and Fine, 2000). The risk of developing the clinical manifestations of the disease is greatly increased by HIV co-infection (Swaminathan et al., 2010). This strong association between HIV and TB in sub-Saharan Africa is responsible for the massive increase in the incidence of TB observed in the region in the last 20 year (Keshinro and Mukadi, 2006). The depletion of CD4+ T cells, which is a main feature of AIDS, is certainly an important contributor to the increased risk of reactivation of latent TB and susceptibility to new *M. tuberculosis* infection. There is also some evidence that CD8+ T cells play a role in the control of latent TB (Lewinsohn et al., 2003). Other mechanisms reported to

facilitate *M. tuberculosis* infection and disease in individuals with HIV are up-regulation of *M. tuberculosis* entry receptors on macrophages, HIV manipulation of macrophage bactericidal pathways, deregulated chemotaxis, and a tipped Th1/Th2 balance (Rosas-Taraco et al., 2006). It has also been shown that HIV impairs TNF-mediated macrophage apoptotic response to *Mycobacterium tuberculosis* and thus facilitates bacterial survival (Patel et al., 2007)

TB remains a major global health problem and HIV infection has contributed to a significant increase in the worldwide incidence of TB. Both HIV/AIDS and TB disease causes ill-health among millions of people each year and ranks as the first and second leading cause of death from an infectious disease worldwide respectively (WHO, 2013). Every year, more than 1.5 million people die of tuberculosis (13% HIV positive), and 9 million new cases are reported. TB disease is the most frequent co-infection in HIV infected patients, thought to have caused a third to a half of all acquired immune deficiency, particularly in sub-Saharan Africa and South East Asia, areas of world where HIV infection is expanding most rapidly (WHO., 2014).

TB and HIV-1 often occur in the same geographic area, in the same person, and even in the same cell. It is not surprising, therefore, that they have important and bidirectional interactions. The impact of HIV on TB is profound. The immunosuppression of HIV dramatically increases the risk of reactivation of a latent focus of infection and progression of primary infection. The rapid progression of primary infection to infectious TB catalyzes the spread of the strains of *M. tuberculosis* that are prevalent in the community, including multi-drug-resistant TB. HIV-1 also alters the clinical expression of TB, as there is less cavitary disease and more atypical, disseminated, and extrapulmonary manifestations (Ellner, 1997). Tuberculosis can occur early in the course of HIV infection and throughout all stages of HIV infection. The risk of TB increases soon after infection with HIV (Gagneux et al., 2006). Although TB can be a relatively early manifestation of HIV infection, it is important to note that the risk of developing TB, and of disseminated infection, increases as the CD4 cell count decreases. Even with effective immune reconstitution with ART, the risk of TB generally remains elevated in HIV-infected patients above the background risk of the general population, even at high CD4 cell counts (Moore et al., 2007, Van Rie et al., 2011). In the individual host, the two pathogens, MTB and HIV, potentiate one another, accelerating the deterioration of immunological functions (Pawlowsk et al., 2012). However, our knowledge about the mechanisms of interaction of the two pathogens and the

relationship between the immunological abnormalities at a cellular level and the overall immunopathogenesis is still unclear and has many gaps that need to be filled in order to develop preventive measures against the two diseases (Walker et al., 2013).

L-arginine is a semi-essential amino acid involved in multiple areas of human physiology and metabolism. It is not considered essential because humans can synthesize it *de novo* from glutamine, glutamate, and proline. However, dietary intake remains the primary determinant of plasma arginine levels, since the rate of arginine biosynthesis does not increase to compensate for depletion or inadequate supply (Castillo et al., 1994, Castillo et al., 1993). T lymphocytes depend on arginine for multiple key biological processes, including proliferation, the expression of the TCR complex and the CD3ζ peptide, and the development of memory (Bronte et al., 2003b, Ochoa et al., 2001b).

Arginase is an enzyme that catalyses the conversion of L-arginine in to ornithine and urea. Arginase, can be up regulated in murine macrophages, dendritic cells and neutrophils , however, human neutrophils constitutively express arginase (Munder, 2009). There are two iso-forms of arginase in mammals, arginase I and arginase II (Morris, 2009, Morris, 2012). The catabolism of L-arginine by arginase can regulate the availability of L-arginine and therefore the efficiency of T cell responses: increased catabolism of L-arginine by arginase results in the depletion of L-arginine from the microenvironment; since L-arginine is essential for efficient T cell activation, this decrease in L-arginine results in impaired T cell responses (Munder, 2009). In addition, the catabolism of L-arginine by arginase results in the production of ornithine, which is further catabolised into polyamines that are crucial for cell division; and into proline, which is the building block for collagen synthesis. However, in some cases, the degradation products of arginine have a beneficial effect on intracellular microbes. For example, Mycobacterium enhances arginine transport in infected macrophages and acquires the metabolites necessary for bacterial growth (Peteroy-Kelly et al., 2003). Therefore, arginase enzyme can be considered as a moonlighting enzyme that acts as a double edged sword in immunity.

L-arginine is also the substrate for nitric oxide synthase (NOS), that catabolises L-arginine into nitric oxide (NO), a molecule critical for the regulation of vascular homeostasis, neurotransmission and the killing of many pathogens (Morris, 2004b). Therefore, by competing

for the shared substrate L-arginine, increased arginase activity also modulates the production of NO. Consequently, deficiencies in L-arginine metabolism can disrupt many cellular and organ functions.

Increased arginase activity is common to several pathological and physiological conditions: it has been prominently described in cancer (Nagaraj and Gabrilovich, 2010, Ochoa et al., 2007), but also in asthma, myocardial infarction, pregnancy and infectious and autoimmune diseases (Munder, 2009). It has also been shown that increased arginase activity is a marker of disease severity in HIV seropositive (HIV+) patients (Cloke et al., 2010b), in patients with visceral leishmaniasis and HIV co-infection (Takele et al., 2010) and in patients with visceral (Abebe et al., 2013) and cutaneous leishmaniasis (Abebe et al., 2012).

So Arginase enzyme can be considered as immunologically vital enzyme as it has a potential of causing immune suppression by starving L- arginine (Bronte and Zanovello, 2005). L-arginine is important amino acid for the normal function of T cell and its depletion might bring dysregulated T cell response (Rodriguez Pc et al., 2002). Therefore this study is to measure level of arginase enzyme in the blood of HIV, TB and HIV/TB co-infected patients which could have important implication for the better understanding of the pathogenesis of HIV, TB and HIV/TB infection from these enzyme points of view and also it is usefull for the advancement of knowledge about progressive immune dysfunctions in HIV, TB and HIV/TB co-infection, this may open up new therapeutic avenues to target dysregulated T cells responses..

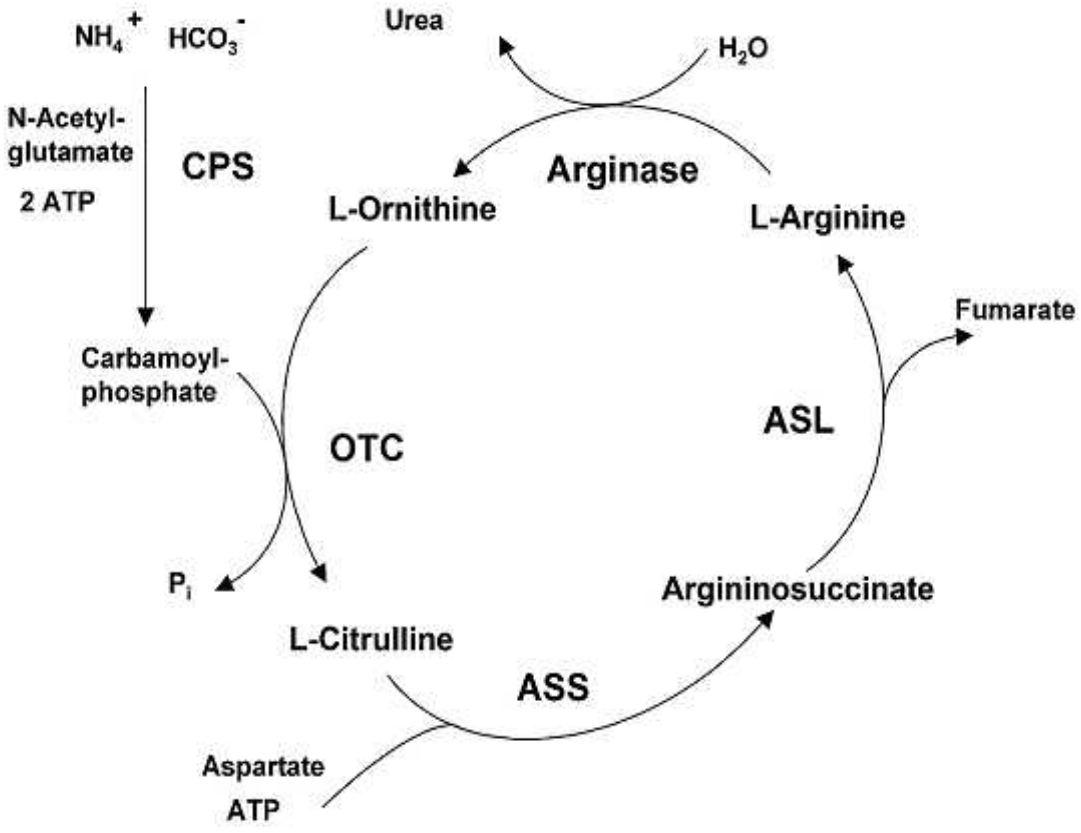
## 1.2 Arginase

### 1.2.1 What is arginase? Types of arginase

Arginase is the final enzyme of the urea cycle in the liver and is the key enzyme for the removal of highly toxic ammonium ions from the body. To this aim, L-arginine, which is synthesized from L-citrulline by the subsequent actions of arginine succinate synthase and argininosuccinate lyase, is converted into L-ornithine and urea by the action of arginase. Besides in liver, arginase is also expressed in cells and tissues that lack a complete urea cycle, including the airways and the lung (Jenkinson et al., 1996, Que et al., 1998, Meurs et al., 2000). In other word, arginase is a binuclear $Mn^{+2}$ metalloenzyme that catalyze the conversion of L-arginine to L-ornithine and urea (Mendz and Hazell, 1996). The enzyme is widely present throughout the evolutionary spectrum including

bacterium, plant, yeast, and vertebrates, and is important for L-arginine homeostasis. All arginases exhibit higher catalytic activity with $Mn^{+2}$ as a metal co-factor except the *Helicobacter pylori*, which shows higher activity with $Co^{+2}$ (Sekowska et al., 2000).

Arginase exists in two isoform, liver-type arginase I and non hepatic-type arginase II which are encoded by different gene. Properties of these isoform are summarized in Table 1. Arginase I is expressed almost exclusively in the cytosol of the liver cells under normal conditions and catalyze the last step of urea synthesis which detoxify ammonia in mammal (Figure 1). The enzyme was purified from livers of various mammals, and the crystal structure of the trimeric rat enzyme was revealed. cDNAs for the rat and human enzyme were isolated, and the predicted polypeptide chains are composed of 323 and 322 amino acid residues (Kawamoto et al., 1987, Haraguchi et al., 1987). The rat and human genes are ~12-kbp and ~ 11.5-kbp long, respectively, and consist of 8 exons (Ohtake et al., 1988). In the promoter region there are 2 binding sites for CCAAT/enhancer binding protein (C/EBP), 1 at position around -90 bp and the other around -55 bp (Gotoh et al., 1994, Chowdhury et al., 1996). Binding of C/EBP family members to the region around -55 bp stimulates the promoter activity. Hepatocyte nuclear factor-4 (HNF-4) represses the promoter activity without directly binding the promoter region, and the region overlapping with the C/EBP binding site at ~55 bp is responsible for the HNF-4 repression (Chowdhury et al., 1996).


$NH_4^+$
$HCO_3^-$
Urea
$H_2O$
N-Acetyl-
glutamate
2 ATP
CPS
Arginase
L-Ornithine
L-Arginine
Fumarate
Carbamoyl-
phosphate
OTC
ASL
$P_i$
Argininosuccinate
L-Citrulline
ASS
Aspartate
ATP

al., 2006). This study suggests that the regulation of arginase II contributes to the immunomodulatory effects of LXRs.

TABLE 1.2.1 Properties of mammalian arginase I and II

| | Arginase I | Arginase II |
|---|---|---|
| **Amino acid residues** | **322, 323** | **332** |
| **Size** | **34,700 Da** | **~36,100 Da** |
| **Subunit structure** | **Trimer** | **Trimer** |
| **Km (arginine)** | **5-9 mM** | **7 Mm** |
| **pH optimum** | **9.7-9.9** | **9.4-10.0** |
| **Cofactor** | **$Mn^{2+}$** | **$Mn^{2+}$ ?** |
| **Inhibitors** | **NG-hydroxy-L-arginine** | **NG-hydroxy-L-arginine** |
| | **Valine** | |
| **Abundant tissues** | **Liver** | **Small intestine** |
| | **Erythrocytes** | **Kidney** |
| | **Submaxillary gland** | **Lactating mammary gland** |
| **Intracellular localization** | **Cytosol** | **Mitochondrial matrix** |
| **Location on chromosome** | **6q23** | **14q24.1-24.3** |

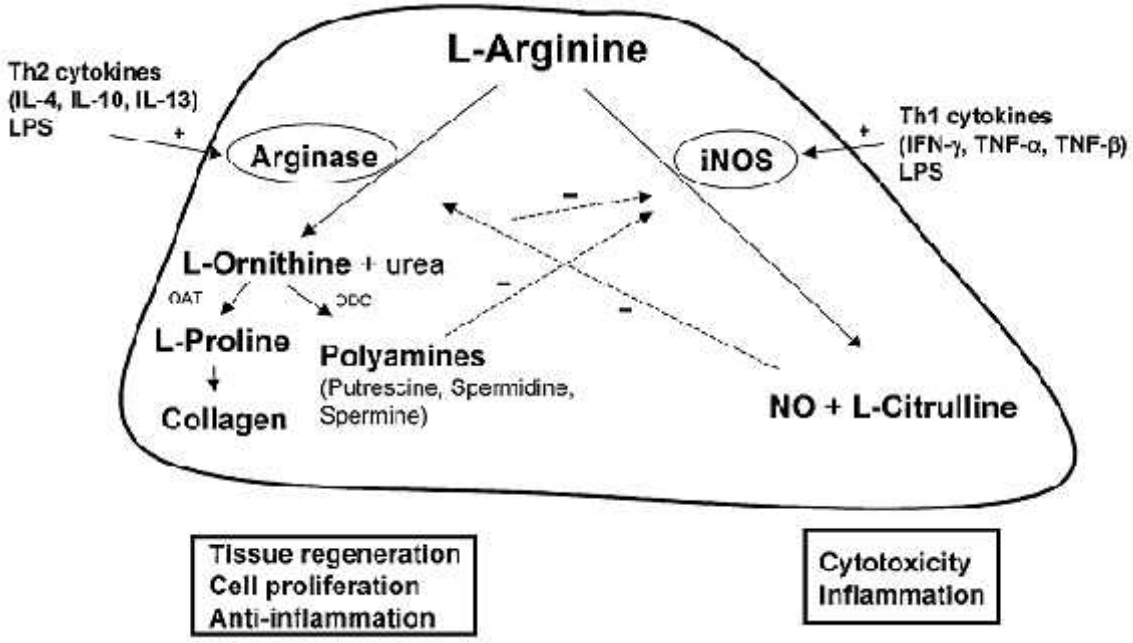
All arginases have conserved motifs GGDHS, SxDxDxxDP, and DAHxD. These are important for recognition of the metal ions and thus form a bimetallic cluster at the active site. An intact bimetallic cluster is found to be essential for catalysis. A water molecule bridged (pKa ~7.9 in rat liver arginase) between the two metal ions has been suggested to be important for catalytic activity. The protein exists as an oligomer; eukaryotic proteins are primarily trimeric, whereas the bacterial counterparts are hexameric. Each monomer contains one active site. Mutational studies showed that the monomer exhibits much lower activity than the oligomer. The amino acids sequence alignment revealed a significant difference at the N and C-terminal sites of eukaryotic arginases than the bacterial counterpart such as Bacillus caldovelox and Bacillus subtilis, which lacks the C-terminal motif. The C-terminal S-shaped motif in human and rat liver arginases appears to be important for oligomerization. Although, the bacterial arginases do not have the C-

terminal motif, these proteins exist as a hexamer (Lavulo et al., 2001). This suggests that the mechanism of oligomerization in the bacterial arginases is different from the mammalian counterpart.

### 1.2.2 Arginase and inducible nitric oxide synthase (iNOS)

Arginase and iNOS use arginine as a common substrate and may compete with each other for this substrate. The metabolism of arginine by iNOS or arginase 1 produces radically different biological products (Bernard et al., 2001). iNOS generates NO in large quantities and under physiologic conditions plays an important role in killing parasites, bacteria, viruses, and cancer cells and producing vasodilatation (Bogdan, 2001). However, excessive amounts of NO can also produce a cytotoxic effect in the host and can lead to immune suppression of T cells (Goni et al., 2002) and to pathological findings (Noël et al., 2004). Arginase 1 generates ornithine and urea. Ornithine is a precursor of different products, including polyamines and proline, and thus may play an important role in cell proliferation (Bronte et al., 2003a). As shown by Table 1 Classic pro-inflammatory cytokines IL-1, TNF-α, IFN-γ, and IL-2 induce iNOS. In turn, humoral anti inflammatory cytokines IL-4, IL-10, IL-13, and TGF-β induce arginase1 expression. Endotoxin appears to induce both iNOS and arginase 1 (Bronte and Zanovello, 2005). Upon induction, iNOS exerts a regulatory effect on arginase activity through the production of hydroxy-L-arginine, an intermediate product in the generation of NO. Arginase 1 in turn regulates NO through depletion of arginine availability (Morris, 2004a). Because expression of arginase 1 and iNOS is reciprocally influenced by Th1 and Th2 cytokines, activation of each may be restricted to separate subsets of myeloid-derived cells, termed alternative activation (Gordon, 2003). It has been shown, however, that both enzymes are produced in CD11b1IL-4 receptor α+MSC and that MSC produce both Th1-type and Th2-type cytokines (IFN-γ and IL-13) (Gallina et al., 2006). Therefore, the designation ‘‘alternatively activated’’ may not be applicable or relevant in the characterization of MSC. The determination that MSC produce both arginase 1 and iNOS provides insights that can be potentially translated to the clinic (Frey, 2006)

| Enzyme | Cytokine stimulation | Other stimulation |
|---|---|---|
| iNOS | IL-1, TNF-~, IFN-~, IFN- , IFN-¥ | Endotoxine, lipopolysaharide |
| Arginase I | IL-4, IL-6, IL-10, IL-13, TGF- | PGE1, PGE2, catecholamine |



role of macrophage arginase in inflammation is also clearly demonstrated by the fact that the murine enzyme is inducible by pathogen-derived macro-molecules like lipopolysaccharide (LPS) or lipoproteins (Corraliza Im et al., 1995), by a broad range of inflammatory stimuli (Hrabak et al., 2006) and in rat alveolar macrophages by the reactive oxygen product hydrogen peroxide (H2O2) (Matthiesen et al., 2008). The induction of arginase I in murine macrophages by oxidized and acetylated lipoproteins was demonstrated (Gallardo-Soler et al., 2008) connecting macrophage alternative activation and an inflammatory phenotype with lipid metabolism and vascular atherosclerosis. Arginase expression is regulated by glucocorticoids in a cell type and species-specific way. While rat liver arginase is induced by glucocorticoids via the binding of the transcription factor CCAAT/enhancer binding protein β (C/EBPβ) to the arginase I enhancer (Gotoh et al., 1997), both the LPS-induced up-regulation of rat alveolar macrophage arginase (Klasen et al., 2001) as well as the IL-4/IL-13-mediated up-regulation of rat airway fibroblast arginase (Lindemann and Racke, 2003) are inhibited by dexamethasone. The constitutive expression of arginase I in human polymorphonuclear granulocytes (PMN) is not modulated in vitro by a variety of pro- and anti-inflammatory stimuli including glucocorticoids (Munder M et al., 2005) .

Several groups have addressed the intracellular signaling mechanisms responsible for arginase induction in murine cells. Th2-mediated induction of arginase I is regulated by the coordinated action of the transcription factors PU.1, signal transducer and activator of transcription (STAT)-6 and C/EBPb at an enhancer 3 kilobases (kb) upstream of the basal promotor (Pauleau et al., 2004). The cAMP-mediated induction of murine arginase I (Corraliza et al., 1997) is mediated by Protein Kinase A Type I (PKAI) (and not PKA-II or EPAC) involving histone deacetylation, while arginase I induction by modified lipoproteins is mediated via Peroxisome Proliferator-Activated Receptor (PPARγ and PPARδ) (Gallardo-Soler A et al., 2008). The Src-homology 2 (SH2)-containing inositol-5′-phosphatase SHIP1 restrains the inducibility of murine macrophage arginase I as macrophages are skewed towards an M2 phenotype with high levels of arginase I in SHIP1–/– mice (Rauh et al., 2004).

Based on the above regulatory principles and the different metabolic pathways associated with up-regulated immune cell arginase, it was hypothesized that the enzyme might causally be involved in disease pathogenesis because of (i) suppression of NO-mediated cytotoxicity via L-arginine

consumption, (ii) enhanced collagen synthesis and fibrosis via proline generation and (iii) enhancement of cellular proliferation via polyamine generation. Consecutively, arginase expression was analyzed in various disease models. In vivo, the enzyme was demonstrated in murine inflammatory cell infiltrates in experimental glomerulonephritis (Waddington et al., 1998), schistosomiasis (Hesse et al., 2001), trypanosomiasis (Gobert Ap et al., 2000), leishmaniasis (Kropf et al., 2005), autoimmune encephalomyelitis (Xu et al., 2003), asthma (Zimmermann et al., 2003), several viral (Wang et al., 2005) and bacterial (Gobert et al., 2002) infections, lung fibrosis (Liu et al., 2005), sepsis (Carraway et al., 1998), and trauma (Makarenkova et al., 2006). The reciprocal regulation of iNOS and arginase that was found in vitro is replicated in various inflammatory pathologies in vivo. In murine disease models that follow the Th1/Th2 paradigm with regard to disease susceptibility or resistance iNOS is induced in the context of a Th1-dominated resistant phenotype while macrophage arginase is up-regulated during Th2-mediated disease progression (e.g. Leishmaniasis; (Kropf et al., 2005)). In chronic murine schistosomiasis, arginase is similarly induced in Th2 granuloma-associated macrophages and is critical for enhanced granuloma size and fibrosis (Hesse et al., 2001). On the other hand, arginase I expressing alternatively activated macrophages protect the host during acute schistosomiasis by reducing massive Th1-mediated immune pathology and iNOS activity, as demonstrated by 100% mortality of mice with a macrophage/neutrophil – targeted deletion of the IL4Rα chain (i.e. absence of alternatively activated macrophages) during acute infection with *Schistosoma mansoni* (Herbert et al., 2004). Genes of arginine metabolism like cationic amino acid transporter (CAT)-1, arginase I and arginase II are highly up-regulated in the lung in different murine asthma models (Zimmermann et al., 2003) and arginase has been linked pathogenetically with bronchial hyperreactivity by the depletion of L-arginine and consecutively reduced synthesis of the bronchodilatory agent NO (Meurs et al., 2002).

While myeloid cell arginase I is induced in a variety of infectious diseases via a Th2 driven adaptive immune response, it became clear that arginase I can also be directly up-regulated in macrophages via Pathogen-Associated Molecular Patterns (PAMP) so that the nature of the pathogen dictates the type of the evolving innate immune response. Chitin, a widespread polymer of N-acetyl-β-D-glucosamine and part of, for example, fungal cell walls and helminths, leads to arginase I expression and the acquisition of an alternative activation phenotype of resident murine

macrophages during infection with *Nippostrongylus brasiliensis* (Reese et al., 2007). The ensuing, leukotriene B4-mediated tissue infiltration of IL-4 expressing innate immune cells (eosinophils/basophils) then leads to the Th2-mediated, STAT6-dependent induction of chitinase-like proteins acidic mammalian chitinase (AMCase), Ym1 and Ym2, which are characteristic of alternatively activated macrophages. Interestingly, chitin degradation by AMCase is able to abrogate Th2 inflammatory cell infiltration so that a possible feedback inhibition of chitin-induced allergic or helminth-induced innate immune response by AMCase is likely (Reese et al., 2007). Another pathogen with direct arginase-inducing potential is *Mycobacterium tuberculosis*, which induces arginase I in murine macrophages through the toll like receptor (TLR) – MyD88 pathway. This induction is independent of STAT6 but involves the up-regulation of C/EBPβ and binding of this transcription factor to the C/EBPβ site within the upstream enhancer of the murine arginase I gene (Pauleau et al., 2004). In the case of *Mycobacterium bovis* (BCG) infection, it has been demonstrated that host urea production slowly increases with the infection time. After 24 hours and 72 hours of infection, there was a significant induction in J774.1 macrophage arginase activity. However, the replication of intracellular BCG increased when arginase activity was inhibited. Hence, it is indicative that the enhanced growth of BCG might be due to increased availability of the intracellular arginine pool to the bacteria in the arginase-blocked condition (Talaue et al., 2006).

The analysis of the role of myeloid cell-associated arginase during infection is further complicated by the fact that various pathogens express an enzymatically active arginase themselves (Mcgee et al., 2004). In parallel to the myeloid cell-expressed arginase of the host, the pathogen-encoded arginase might contribute, for example, to the down-regulation of T lymphocyte functions in the context of *Helicobacter pylori* infection (Zabaleta et al., 2004) or to the down-regulation of macrophage microbicidal activity by reducing NO production via L-arginine depletion in *Leishmania mexicana* infection (Gaur et al., 2007). Another critical component in the system of arginase-mediated L-arginine metabolism clearly is the capacity and regulation of L-arginine transport via the cell membrane. The transport system y+ is selective for the transport of cationic amino acids like L-arginine (Closs et al., 2004). Murine macrophages express CAT-1 constitutively and up-regulate CAT-2 upon classical (IFN-γ) or alternative (IL-4, IL-10) activation (Rodriguez et al., 2003, Yeramian et al., 2006) so that enhanced catabolism via induced iNOS or arginase, respectively, is met by coordinated increased cellular uptake capacity. On the other hand,

murine infections with the helminth parasite *Schistosoma mansoni* or the protozoan pathogen *Toxoplasma gondii* are exacerbated in the absence of CAT-2, demonstrating a crucial regulatory role of L-arginine membrane transport for immune responses (Thompson et al., 2008). The expression and regulation of L-arginine transport systems in cells of the human immune system is largely unresolved so far.

### 1.2.4 Arginase in the human immune system

In human blood, the main arginase-expressing cell types are neutrophils (Munder M et al., 2005), myeloid suppressor cells (Rodriguez et al., 2009) and alternatively activated macrophages (Kropf et al., 2007). Arginase was detected in the peripheral blood mononuclear cell (PBMC) fraction after injury (Ochoa et al., 2001a), inflammatory synovial fluid macrophages (due to arginase II) of patients with arthritis (Corraliza and Moncada, 2002), inflammatory cells of bronchoalveolar lavage fluid of asthmatic patients (Zimmermann et al., 2003), psoriatic lesions (Bruch-Gerharz et al., 2003), in activated monocytes of patients with autoimmune diseases (Rouzaut et al., 1999) and in the PBMC fraction of patients with active pulmonary tuberculosis (Zea et al., 2006). Among peripheral circulating human leukocytes of normal blood donors only PMN express arginase (Munder M et al., 2005). By biochemical fractionation and immunoelectron microscopy they had demonstrated that the enzyme is constitutively present in azurophil granules of human PMN, where it constitutes a novel oxygen-independent anti-microbial defense mechanism (Munder M et al., 2005). After fusion of azurophil granules with a phagosome, arginase is present in the phagosome and likely depletes the intraphagosomal microenvironment of L-arginine during phagocytosis of pathogenic microorganisms which enhances the fungicidal activity of human PMN. Interestingly, *Saccharomyces cerevisiae* and *Candida albicans* up-regulate genes of their endogenous L-arginine biosynthetic pathways upon phagocytosis by human neutrophils (Rubin-Bejerano et al., 2003). This transcriptional response likely reflects the L-arginine-deprived intraphagosomalmicro-milieu of PMN and is not detectable upon phagocytosis by human monocytes (Rubin-Bejerano et al., 2003) which do not express arginase (Munder M et al., 2005). Another study confirmed the expression of arginase I in human PMN but localized the enzyme to the gelatinase granules (Jacobsen et al., 2007). The discrepancy in results is still unclear at the moment. In vitro, constitutive human PMN arginase activity was not modulated by a variety of pro- and anti-inflammatory stimuli, including cytokines that typically lead to arginase induction in

murine myeloid cells (Munder M et al., 2005). In contrast, arginase is inducible in a variety of other human cell types like, for example, endothelium, epithelial cells and smooth muscle. Arginase I shares this feature with other important constitutive PMN proteins or peptides involved in inflammation and microbial defense like human cationic anti-microbial protein of 18 kDa (hCAP18), neutrophil gelatinase-associated lipocalin (NGAL), bactericidal/permeability-increasing protein (BPI) and the defensins (Borregaard et al., 2007). The fundamental discrepancies of arginase expression and regulation between murine and human immune cells fit into a growing list of differences in the immune systems of both species (Mestas and Hughes, 2004). This must be kept in mind when data from animal models are extrapolated to the human situation. Also, data on arginase expression in the human PBMC fraction without further purification need to be interpreted with caution. It remains to be analysed if arginase protein and activity is really induced in monocytes within the PBMC fraction. Alternatively, activated PMN are known to aberrantly co-purify within the PBMC fraction of patients with tumours or inflammation (Schmielau and Finn, 2001), so that de novo arginase activity in the PBMC population under conditions of inflammation might actually be confined to the neutrophil subset (Munder, 2009)

### 1.2.5 Immunosuppressive mechanism of Arginase in the human immune system

Arginase expression and L-arginine depletion have emerged as a powerful immunosuppressive pathway of the mammalian immune system (Bronte and Zanovello, 2005). Ochoa et al. analysed the influence of L-arginine deficiency on the function of human T lymphocytes and found a down-regulation of the T cell receptor (TCR) ζ chain, a critical signaling element of the TCR, as a possible mechanism for the impaired T cell function under conditions of L-arginine depletion (Rodriguez Pc et al., 2002). Also, an arrest of T cells in the G0-G1 phase of the cell cycle, associated with the absence of up-regulated cyclin D3 and cyclin-dependent kinase 4 (cdk4) was seen upon L-arginine depletion (Rodriguez Pc et al., 2007). Murine macrophages express arginase after Th2 stimulation (Munder et al., 1998) and this also leads to depletion of extracellular L-arginine and consecutive down-regulation of the TCR ζ chain in activated T cells (Rodriguez et al., 2003). T cell hyporesponsiveness associated with down regulated TCR ζ chain can be the consequence of various circumstances (Baniyash, 2004) and transcriptional (Tsokos et al., 2003), posttranscriptional (Rodriguez Pc et al., 2002) or posttranslational (Bronstein-Sitton et al., 2003)

mechanisms of TCR ζ down-regulation have been described. In humans, immunosuppression in association with T cells that have partially down-regulated their TCR ζ chain is a recurrent finding in patients with cancer, autoimmunity or chronic infections (Baniyash, 2004). The mechanism(s) and cells that induce the observed T cell phenotype and/or the associated immunosuppression in vivo are largely unknown.

As intracellular constituents are liberated from dying PMN and accumulate in the micromilieu human T cell activation should be blunted in the case of arginase liberation from human PMN. In fact, of all naturally occuring amino acids only L-arginine is depleted (and L-ornithine and urea synthesized) within the extracellular micromilieu of dying PMN (Munder et al., 2006). Within such an L-arginine-depleted milieu human T lymphocytes remained viable, but stopped proliferation and secretion of cytokines, while TCR activation-induced transcription of cytokine genes remained intact. Interestingly, human purulent exudates contains extraordinarily high arginase activities and liberated PMN arginase I fully accounts for the profound T cell suppressive properties of human pus by L-arginine depletion (Munder et al., 2006). Arginase-mediated T cell hyporesponsiveness is also involved in the suppression of the maternal anti-fetal immune response. A successful pregnancy depends on largely unknown mechanisms by which the immune system of the mother is made tolerant to the semi-allogeneic fetus. Myometrial arginase activity in the vicinity of the placental implantation site is >25 higher than in myometrium from non-pregnant guinea pigs (Weiner et al., 1996). In humans, arginase activity is also highly up-regulated in term placenta and increased in the peripheral blood of pregnant women (Kropf et al., 2007). While placental arginase might be important in the supply of polyamines, the local depletion of L-arginine via PMN-expressed arginase clearly dampens invading T lymphocytes (Kropf et al., 2007), possibly by down-regulating their TCR ζ chain. Arginase I is also expressed constitutively in human erythrocytes (Kim et al., 2002) and is liberated into the extracellular milieu upon hemolysis. It was also shown that the immune suppression associated with transfusion of packed red blood cells might be due to the liberation of arginase from erythrocytes during storage and consecutive systemic L-arginine hydrolysis upon transfusion (Bernard et al., 2008).

## 1.3 Statement of the problem

According to World Health Organization (WHO), there were an estimated 8.7 million incident cases of TB (range, 8.3 million-9.0 million) globally (WHO., 2012). Africa, more specifically Sub-Saharan Africa, faces the worst TB epidemic, where TB associated morbidity and mortality occurs mainly in the economically productive age group (15-60 years) (WHO., 2000). Available data indicated, Ethiopia has been one of the high TB burden countries (Prasad, 2010) with an incidence and prevalence rate of 300 and 470 cases per 100,000 populations respectively. Among all new TB cases notified to federal ministry of health, 30% were smear positive (WHO., 2010, MOH., 2008). Moreover, Moreover, Acquired immunodeficiency syndrome has become one of the world's most serious health and development challenges (UNAIDS., 2013). The HIV/AIDS pandemic marks a severe development crisis in Africa, which remains by far the worst affected region in the world, 70.0% of the people living with HIV are from sub-Saharan Africa (UNAIDS., 2002). The national adult human immunodeficiency virus (HIV) prevalence infection in Ethiopia was 1.5% (Demissie and Solomon, 2011). Ethiopia is among the countries most heavily affected by the HIV and TB. The World Health Organization has classified Ethiopia 11$^{th}$ among the 22 high burden countries with TB and HIV infection in the world (WHO., 2013).

TB and HIV co-infection has also been well documented in Sub-Saharan African set up (WHO., 2004 ). Worldwide, 13% of TB patients have HIV co-infection, and as many as 37% have HIV co-infection in parts of African Region, which accounted for 75% of TB cases among people living with HIV worldwide (WHO., 2013).

Tuberculosis is the most prevalent disease in HIV positive people and the majority of the people at risk of HIV and TB are living in Sub Sahara Africa. The risk of developing active TB in HIV positive individuals is increased many fold despite antiretroviral chemotherapy (Lawn et al., 2005). Tuberculosis may occur at any stage of HIV disease and is frequently the first recognized presentation of underlying HIV infection (Sonnenberg et al., 2007). As compared to people without HIV, PLHIV have a 20-fold higher risk of developing TB (WHO., 2011) and the risk continues to increase as CD4 T cell counts progressively decline (Sonnenberg et al., 2007).

Both TB and HIV have profound effects on the immune system, as they are capable of disarming the host's immune responses through mechanisms that are not fully understood. HIV infection progressively impairs the immune system and makes the individual more vulnerable for opportunistic infection like by *Mycobacterium tuberculosis*. The progressive quantitative and qualitative impairment of CD4+ T cells is well established, and the loss of activated CD4+ T cells starts in the early phases of HIV infection and is accompanied by reduced proliferative responses and altered cytokine secretion (Boasso et al., 2009). Although depletion of CD4+ T cells explains much of the immune suppression in HIV infections, the precise mechanisms involved in the onset of immune pathology during the infection have not yet been resolved (Appay and Sauce, 2008). The metabolism of L-arginine by arginase is emerging as a crucial mechanism for the regulation of immune responses against infectious diseases. Depletion of L-arginine by arginase enzyme has emerged as a powerful immunosuppressive pathway of the immune system (Bronte and Zanovello, 2005).

The influence of L-arginine deficiency on the function of human T lymphocytes was analyzed and found a down-regulation of the T cell receptor (TCR) ζ-chain, a critical signaling element of the TCR, as a possible mechanism for the impaired T cell function under conditions of L-arginine depletion (Rodriguez and Ochoa, 2008). Arginase enzyme reduces the bioavailability of L-arginine and impairs T cell responses: high arginase activity results in reduced availability of extracellular L-arginine in the microenvironment. In turn, this decrease in L-arginine results in T cell hypo responsiveness (Bernard et al., 2001, Bronte and Zanovello, 2005, Muller et al., 2009). And this major arginine metabolizing enzyme, arginase 1 is expressed by myeloid cell in the presence of immune stimulation beyond the normal level (Luiking et al., 2004). High arginase enzyme expressions have been reported in a variety of infectious diseases, including tuberculosis (Zea et al., 2006), and HIV (Cloke et al., 2010a).

### 1.4 Significance of the study

Increased arginase activity has been shown to impair T cell responses by reducing the bioavailability of immunologically important amino acid, L-arginine. Previously higher arginase activity has been described in various infectious diseases including in AIDS/HIV and TB, so this study is important to validate the consistency of the result and also so as to test for the first time in the blood of HIV/TB co-infection. Our study also will have important implications for the

advancement of knowledge about the pathogenesis of HIV, TB and HIV/TB co-infection. This study may also open up new and novel therapeutic avenues to target dysregulated T cells responses. Moreover, this study will stimulate further study on arginine metabolism and arginase function in other pathological condition and supports the growing body of literature regarding the potential role of arginase on host's immune response and on modulation of T cell responses by the catabolic pathway of arginase.

## 1.5 Objective

### 1.5.1General objective

- To determine the level of arginase activity in the blood of patient with HIV, TB and HIV/TB Co infection.

### 1.5.2 Specific objectives

- To compare arginase activity in PBMC of HIV, TB and HIV/TB co-infected patient with healthy controls.
- To compare Arginase activity in PBMCs and plasma of HIV patients with HIV/TB co-infected patients.
- To compare Arginase activity in PBMCs and plasma of TB patients with HIV/TB co-infected patients.
- To determine correlation of arginase activity with BMI of HIV, TB and HIV/TB co-infection and CD4 T cell counts of HIV and HIV/TB co-infection.

# 2. Material and methods

## 2.1 Study area and

The study area, Addis Ababa, administrative region, is the capital city of Ethiopia covering an area of 540 sq. km. The total population of the city is about 3.3 million with 5046 peoples per square kilometer, more of slum and overcrowded. The Administrative region has 10 sub cities and 106 woredas (districts). According to Addis Ababa health bureau report of 2010, there were 49 hospitals of which 13 were government owned, 5 NGOs and 31 are private, 36 public health centers, and 130 public health stations, 700 different levels private clinics are found in Addis Ababa city Administrative region (Hhi., 2007). This study was conducted at St. Peter's and Zewditu hospital, Addis Ababa, Ethiopia. These hospitals are conveniently selected which provide voluntary HIV counseling and testing, well known in their ART services, tuberculosis diagnosis and treatment. These hospitals provide services to the population in the surrounding area of the city and also patient from every corner of the country.

## 2.2 Study subject

For this cross-sectional study, 17 apparently healthy HIV seropositive patients, 17 AFB positive pulmonary TB patients (HIV seronegative) and 17 patients who are co-infected by the two pathogen (HIV/TB) were recruited from St. Peter's and Zewditu hospital; informed written consent was obtained from each patient. Moreover, 9 apparently Healthy Volunteer individuals were also used as control.

The diagnosis of TB was based on positive smear microscope for AFB whereas HIV seropositivity was based on the standard HIV test algorithm.

## 2.3 Study design and period

A cross-sectional study design with convenient sampling method was employed in order to determine the level of arginase in the blood of HIV, TB and HIV/TB co-infected patients from April 2014 to September 2014.

## 2.4 Sampling Technique

After briefly describing of the purpose, advantage, and over all nature of the study to the study participants, the consent of every individual was taken. The consent form was translated into simple (non-medical), local languages, and read for those who were illiterate. The blood sample was taken from those who gave their consent. 20 milliliter blood sample was collected to EDTA tube (anti- coagulated) and isolation of PBMC and plasma was done within 2 hours of sample collection. The arginase enzyme assay as well as isolation of PBMC and plasma was done at Black lion Hospital. Basic information on the socio demographic characteristics and clinical data like CD4 T cell count, BMI and TB constitutional symptom of the patient was obtained from the patient's hospital records (cards).

## 2.5 Selection Criteria

**Inclusion criteria:** Exclusive volunteers and patients whose age is eighteen and above.

**Exclusion criteria:** Extra pulmonary tuberculosis patients, pregnant women, unconscious and patients on anti-TB therapy and ART are excluded.

## 2.6 Study variables

The level of arginase activity is the only dependent variable.

The independent variables are:-

- CD4
- BMI
- HIV infection
- TB infection
- HIV/TB co-infection

## 2.7 Laboratory procedures

### 2.7.1 Peripheral Blood

Twenty ml of blood was collected from patients before initiation of treatment and controls in BD Vacutainer EDTA tubes. Plasma was obtained after centrifugation of 1ml of blood at 1800 rpm for

10 minutes and stored in 1.8ml tube at -20$^0$C until use. Peripheral blood mononuclear cells (PBMC) were isolated without delay using a large bore pipette tip after density gradient centrifugation on Histopaque®-1077(Sigma) following manufacturers instruction. Briefly, 18ml of Histopaque-1077 was added to a labeled 50ml conical centrifuge tube and kept at room temperature and almost equal volume of blood from EDTA tube was carefully overlaid onto the Histopaque-1077. After centrifuged at 1900rpm for 20minutes the PBMC layer was aspirated with a Pasteur pipette to within 0.5 cm of the plasma/Histopaque-1077 (opaque) interface carefully without/with minimum plasma and Histopaque-1077. Aspirated cells were transferred into new conical centrifuge tube and mixed with 10ml phosphate buffered saline (PBS) and washed by centrifuging at 1800rpm for 10 minutes and supernatant decanted. This procedure was repeated twice and the cells were resuspended in lysis buffer (0.1% Triton X-100, 25mM Tris-HCl and 10mM $MnCl_2$, Sigma) and stored at -20$^0$C until further use.

### 2.7.2 Determination of arginase activity

The activity of arginase in plasma and PBMC of HIV, TB, HIV/TB co-infected patients and healthy controls was determined using colorimetric assay based on color formed when urea produced is heated in acid with α-isonitrosopropiophenone as described elsewhere (Kropf et al., 2007). The urea that forms color comes from the hydrolysis of a known concentration of L-arginine added in the procedure following the activation of the enzyme arginase in the samples tested. The method was described to be sensitive and unaffected by other metabolites (Munder et al., 2006).

The procedure briefly is as follows, 50µl of plasma or PBMC were heated at 56$^0$C for 10 min using thermal cycler to activate the enzyme. Fifty µl of 0.5M L-arginine (pH 9.7) was added to the activated enzyme and incubated at 37$^0$C for 60 min to facilitate the hydrolysis of L-arginine. During the activation and hydrolysis steps a standard urea solution of different concentration (50, 25, 12.5, 6.25, 3.125, 1.625mg/dl) was prepared using distilled water. The reaction was stopped by adding 400µl of Acid mix ($H_2SO_4$ (96%)/$H_3PO_4$ (85%)/H2O (1:3:7, v/v/v)). Twenty µl α-isonitrosopropiophenone (ISPF, dissolved in 100% ethanol, Sigma) was added and mixed thoroughly and incubated using thermal cycler at 98$^0$C for 45 min followed by incubation at 4$^0$C for 30 min for the development of characteristic color. The urea standard was treated similarly

with samples. After incubation at $4^0$C for 30 min thoroughly mixed 200µl of the mixture was used for measuring optical density at 550nm. For the determination of arginase activity in the plasma, in addition to the above step arginase activity was also measured without activation and hydrolysis steps and the value obtained was subtracted from the value obtained from the step with activation and hydrolysis. The standard curve developed from the standard urea is used to calculate the amount of the enzyme or its activity. The enzyme activity is described as mU/ml. One unit of enzyme activity is defined as the amount of enzyme which catalyzes the formation of 1 µmol urea/min.

Calculation of arginase activity

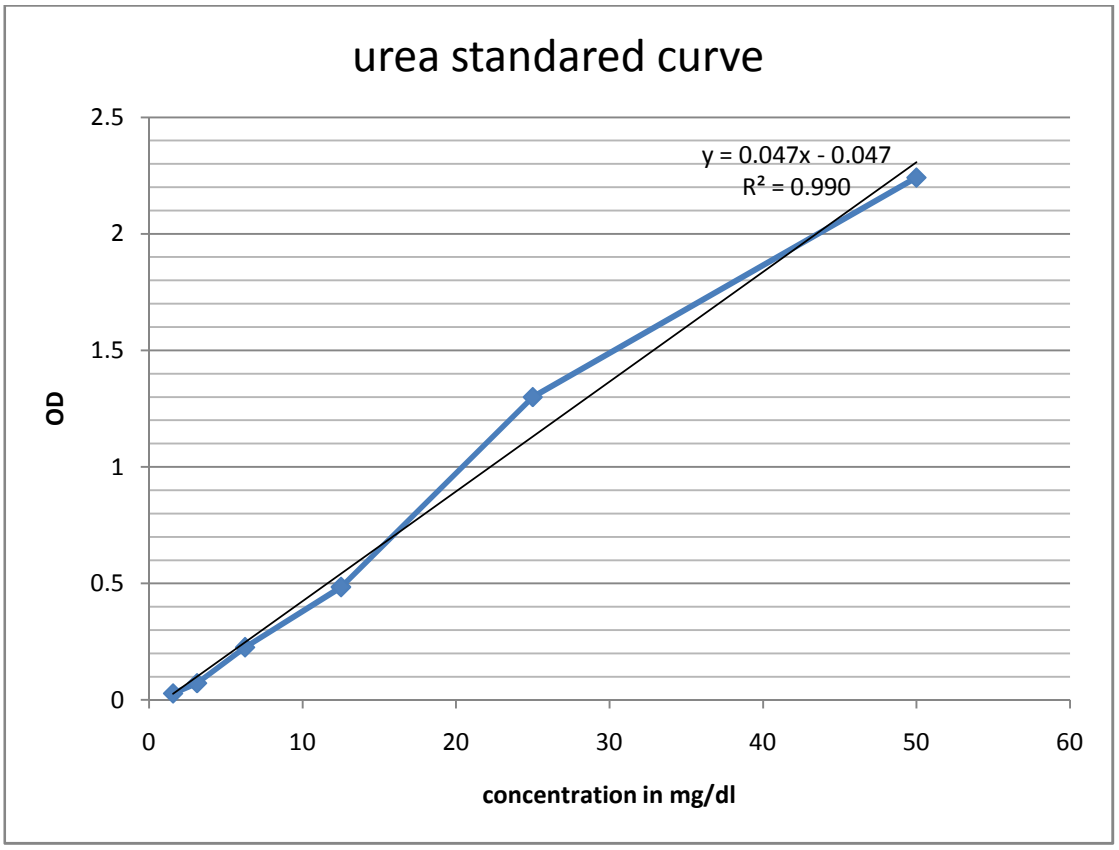
$$m\ p\ sc = (\text{«}g\ u \times m\ ) \div (\text{«}M\ u \times ti\ )$$

| **Note** | |
|---|---|
| µg urea | = Optical density measured in the arginase enzymatic assay |
| µM urea | = 60 |
| mU | = 1000 |

### 2.7.3 Preparation of urea standard and development of standard curve

1. 5ml of 1000mg/dl urea standard solution was prepared by dissolving 50mg urea powder () in 5 ml of distilled water.
2. Then 1ml, 100mg/dl urea standard solution was prepared by diluting the 1000mg/dl stock solution 1:10 by taking 100µl of stock and 900 µl of distilled water.
3. Urea standard solution of different concentration (50, 25, 12.5, 6.25, 3.125, 1.625mg/dl) was then prepared by serially diluting the 100mg/dl solution 1:2.

| Urea concentration | Amount of stock transferred | Distilled water |
|---|---|---|
| 100mg/dl | 100 µl from 1000mg/dl | 900 µl |
| 50mg/dl | 1ml from 100mg/dl | 1 ml |
| 25mg/dl | 1ml from 50 mg/dl | 1ml |
| 12.5mg/dl | 1ml from 25mg/dl | 1ml |
| 6.25mg/dl | 1ml from 12.5mg/dl | 1ml |
| 3.125mg/dl | 1ml from 6.25mg/dl | 1ml |
| 1.625mg/dl | 1ml from 3.125mg/dl | 1ml |



### 2.7.4 Preparation of lysis Buffer

Lysis buffer was prepared by adding 20ml of 0.1% triton, 20ml 50mM tris-HCl of 7.5 ph and 2ml 100mM manganese chloride (previously in cold room, now in fridge) into labelled centrifuge tube and vortex and Stored in fridge.

### 2.7.5 Preparation of 0.5M Acid Mix

0.5M Acid Mix was prepared by mixing 320ml $dH_2O$, 135ml phosphoric acid and 45ml sulphuric acid slowly in fume cupboard on ice.

## Overall Laboratory procedures

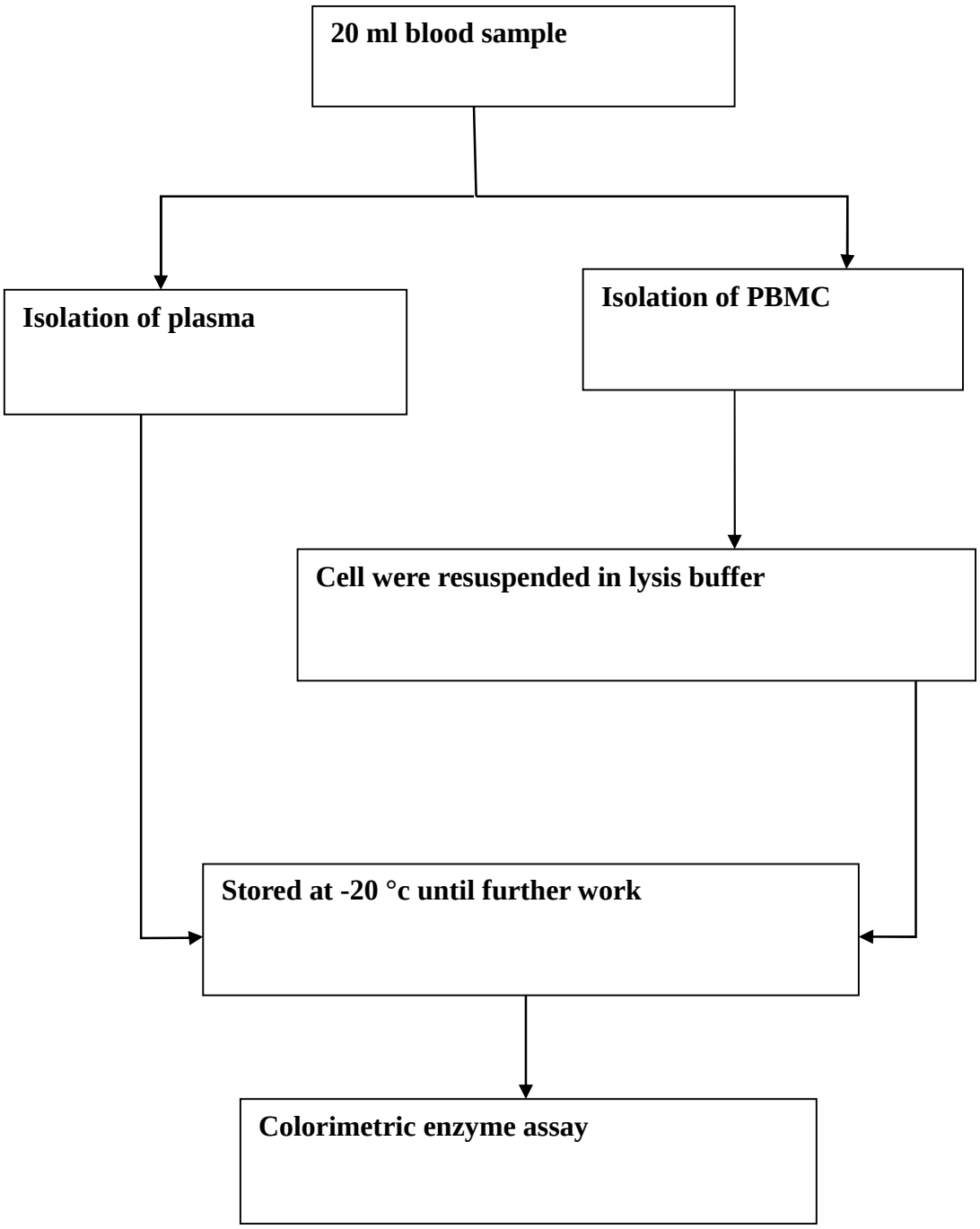



Figure 2.7 Chart showing overall procedures of the lab

## 2.8 Quality assurance

The blood sample was collected by using aseptic techniques. The functionality and the sterility of the equipment for transportation and material for sample taking laboratory work have been carefully monitored. The urea standard was run daily while running the sample.

## 2.9 Data Analysis

Data were evaluated for statistical differences by use of a 2-tailed Mann-Whitney U test and for correlation by use of a Spearman correlation, with Prism software (version 6.0; GraphPad) and differences considered as statistically significant at $p < 0.05$. Unless otherwise specified, results are expressed as mean±SEM.

## 2.10 Ethical clearance

Ethical clearance was secured from Departmental Ethical Review and Research Committee (DERC) of department of Medical Microbiology, Immunology and Parasitology (DMIP), College of Health Science, Addis Ababa University before starting the field work. Official letter of co-operation was written to each selected Hospital by department of Medical Microbiology, Immunology and Parasitology, College of Health Science, Addis Ababa University. The proposal also reviewed by the Ethical and Research Committee of St. Peters Hospital and Consent was obtained from each hospital. There was a high degree of confidentiality during data collection and informed consent has been obtained from each study subject, no name and other identifier are not written. The instrument and procedures used in this study did not cause any harm to the study subjects, data collectors and supervisor who were involved in the study.

## 2.11 Dissemination of results

The findings of this study will be presented to department of microbiology, parasitology and immunolog. The findings will also be disseminated to different organizations (governmental and non-governmental) that will have a contribution to improve and preventing the wide spread of Tuberculosis and HIV. Findings will also be presented in different seminars and workshops to disseminate and it may also be submitted for possible publication and scientific journals as well.

# 3. RESULT

## 3.1 Clinical data

A total of 51 patients (a group of 17 patients for each group) and 9 healthy controls were recruited for this cross sectional study. Of all patients, 30 are female and 21 are male where as 7 of the control are females and had a median age of 24 (IQR, 25-28). HIV/TB patient had a median age of 39 (IQR, 32-44) and the median age of HIV and TB group was 30 (IQR, 25-39.5) and 32 (IQR, 25- 38.5) respectively. All HIV/TB and TB patients' had a complaints of cough (> 2 weeks duration), fever, night sweat and weight loss (TB constitutional symptom).

The nutritional status of the patients and healthy control was assessed by calculating their body mass index (BMI). As shown in fig.3.1 (A) The BMI of healthy control was statistically significantly higher as compared to the BMI of HIV/TB co-infected patient (HIV/TB: 17.61±0.5115 $kg/m^2$, control: 22.12±0.7816 $kg/m^2$, p = 0.0002), TB patient (19.20±0.4108 $kg/m^2$, P = 0.0045) and HIV patient (18.63±0.5057 $kg/m^2$, p = 0.0015). The difference between the mean BMI of HIV/TB patients and the mean BMI of TB patient was statistically significant (p = 0.0326) but we didn't found statistically significant difference between BMI of HIV/TB and HIV patients (p = 0.1903). Seven out of 17 HIV patients (41.1 %), 6 out of 17 TB patients (35.3 %) and 10 out of 17 HIV/TB co-infected patient (59%) had a BMI below 18.5 which were malnourished (Who., 1999). None of the TB patient , 5 out of 17 HIV/TB co-infected group (29.4 %) and 2 out of 17 HIV group (11.7 %) had a BMI of less than 16 which were severely malnourished (Who., 1999). Therefore, HIV/TB co-infected patients were the most malnourished and severely malnourished group than both HIV and TB groups.

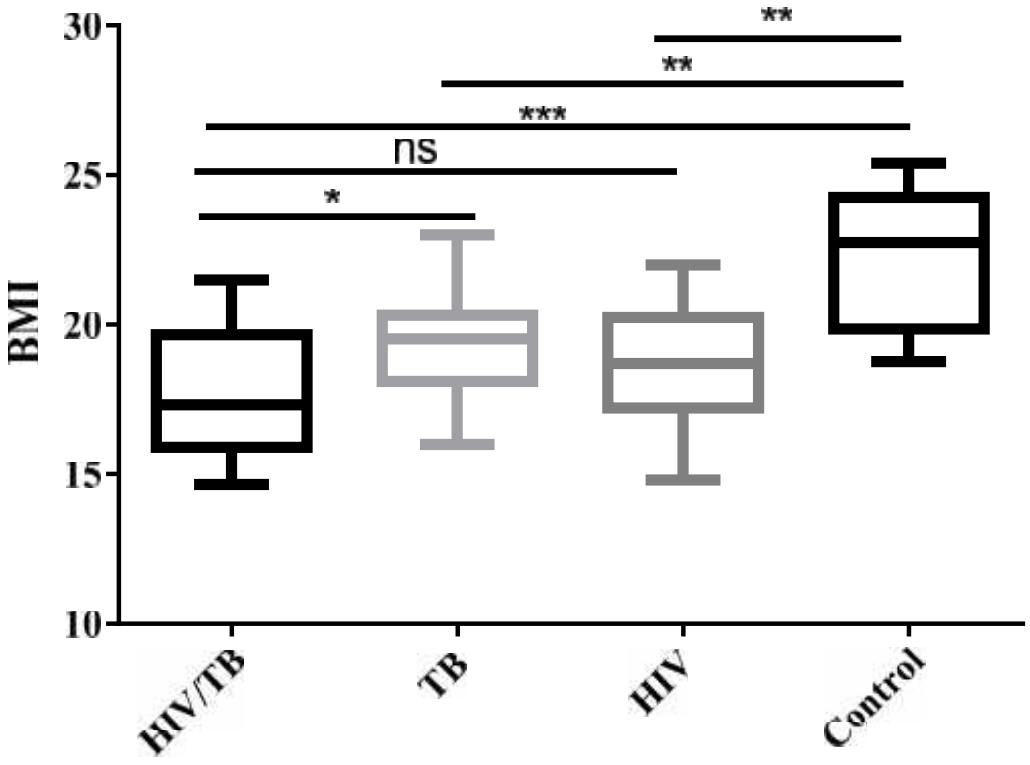



Figure 3.1(A) Measurements of BMI (HIV/TB group: n = 17, TB group: n = 17, HIV group: n = 17 and control group: n = 9). The Box represents the median and IQR and the whisker represent the range. NS = not significant and * represent level of significance.

Both HIV and HIV/TB co-infected patients had remarkably reduced CD4+ T cell counts. Moreover, the result in (figure 3.1 (B)) shows that CD4+ T cell counts were significantly lower in the blood of HIV/TB co-infected patient than in the blood of HIV patients (HIV/TB: 192.1±17.12 and HIV: 267.6±30.48, p = 0.0476).

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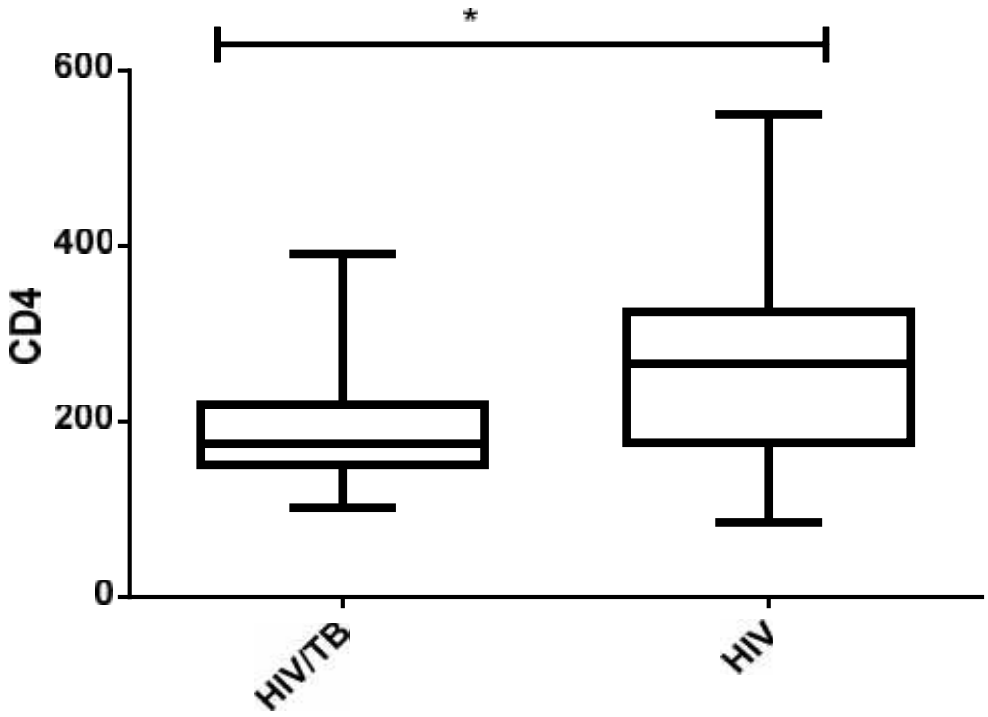


Figure 3.1 (B) CD4+ T cell counts (cells/ml of blood) of HIV/TB patients vs. HIV patients (HIV/TB group: n = 17 and HIV group: n = 17). The Box represents the median and IQR and the whisker represent the range. * represent level of significance.

### 3.2 Arginase activity in PBMC

Arginase activity in PBMC of healthy controls was measured and compared with PBMC arginase activity of HIV, TB and HIV/TB patients. As shown in Figure 3.2 statistically significantly lower level of arginase activity were measured in PBMC of healthy controls as compared with PBMCs arginase activity of HIV/TB co-infected patients (HIV/TB: 157.5±1.298mU/ml blood, controls: 28.26±4.663 mU/ml blood, p = < 0.0001), TB patients (76.73±10.76 mU/ml blood, p = 0.0023) and HIV patients (60.67±9.927 mU/ml blood, p = 0.0380). Arginase activity in PBMC of HIV/TB co-infected patient was also compared with arginase activity in PBMC of TB and HIV patients. Interestingly, the difference was statistically significant in PBMCs arginase activity of HIV/TB co-infected patients with TB patients (P = < 0.0001) and HIV patients (P = < 0.0001).

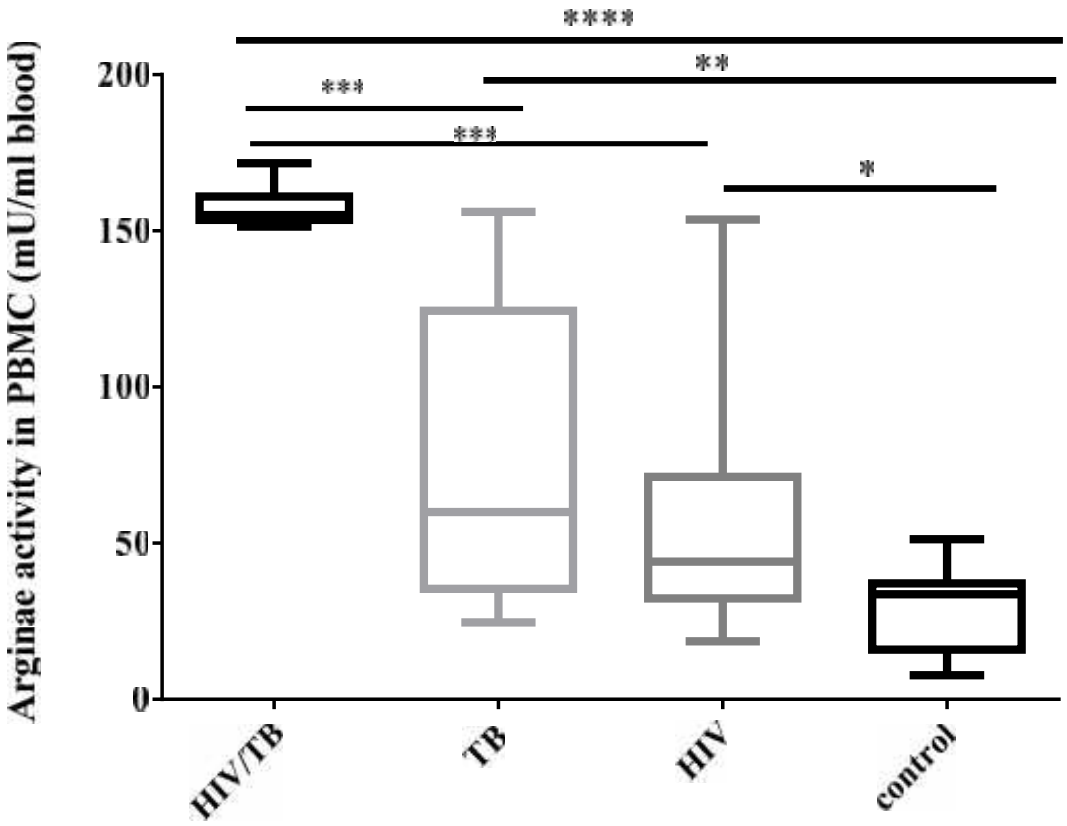


Figure 3.2 Arginase activity in PBMC of patients and healthy controls (HIV/TB: n = 17, TB: n = 17, HIV n= 17 and control: n = 9). The Box represents the median and IQR and the whisker represent the range. The activity level of arginase was measured by enzyme assay. * represent level of significance.

### 3.3 Arginase activity in plasma

Similar to PBMC arginase level, the level of arginase enzyme in we plasma of HIV/TB, TB, HIV patients and healthy controls and compared with each other. As shown in fig. 3.3 statistically significantly lower level of arginase activity was detected in plasma of healthy controls as compared to arginase activity in plasma of HIV/TB co-infected patients (HIV/TB: 14.94±1.329 mU/ml blood and control: 6.219±0.6087, p = < 0.0001) and TB patients (8.116±0.7410 mU/ml blood, P =0.0429). The mean arginase activity in plasma of health control was almost similar with the mean arginase activity in plasma of HIV patients (6.297±0.9163, p =0.4994). Furthermore, arginase activity in plasma of HIV/TB co-infected patients was compared with arginase activity in

plasma of HIV and TB patients and the result demonstrated that the higher mean level of arginase activity in the plasma of HIV/TB co-infected than the mean arginase activity in the plasma of TB and HIV patient was statistically significant (P =0.0003 and p = < 0.0001 respectively).

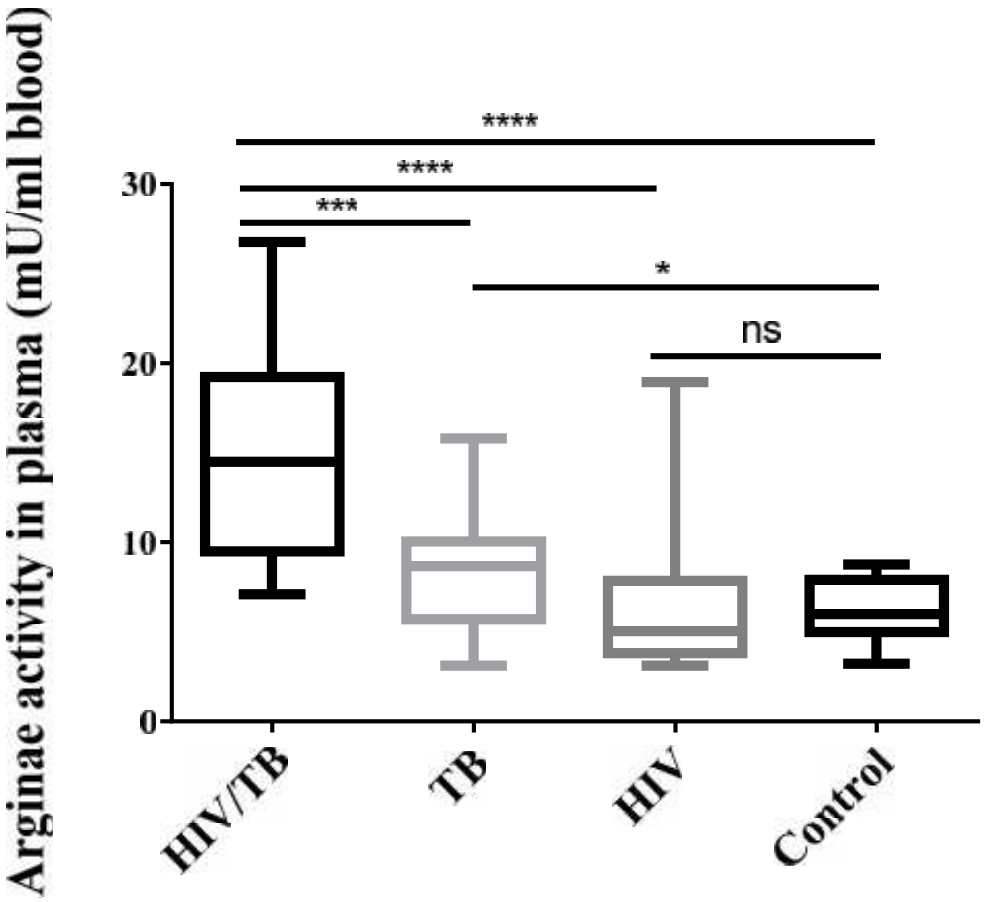


Figure 3.3 Arginase activity in plasma of patients and healthy control (HIV/TB: n = 17, TB: n = 17, HIV: n = 17 and control: n = 9). The Box represents the median and IQR and the whisker represent the range. The activity level of arginase was measured by enzyme assay. NS = not significant and * represent level of significance.

### 3.4 Arginase activity with BMI and CD4

BMI is used to assess the nutritional status of adults and a CD4 count measures how well the immune system is working, next we assessed the correlation of arginase activity with BMI and CD4 count. Spearman's rho correlation was used to correlate arginase activity with CD4 T cell count of HIV and HIV/TB patients and with BMI of HIV, TB and HIV/TB patients. As shown in fig 3.4(A & B) Interestingly a statistically significant negative association was found between arginase activities in PBMC of HIV/TB and HIV patient and their CD4 T cell count (HIV/TB: $r = -0.8694$, $p < 0.0001$ and HIV: $r = -0.8554$, $p < 0.0001$). Moreover Spearman's rho correlation also shows strong negative association between arginase activity in PBMC of HIV, TB and HIV/TB patients and their body mass index, however the association between arginase activity in PBMC of HIV/TB patients and BMI is not statistically significant (HIV/TB: $r = -0.4353$, $p = 0.0807$, HIV: $r = -0.6838$, $p = 0.0032$ and TB: $r = -0.8750$, $p< 0.0001$) (Fig.3.4 C-E).

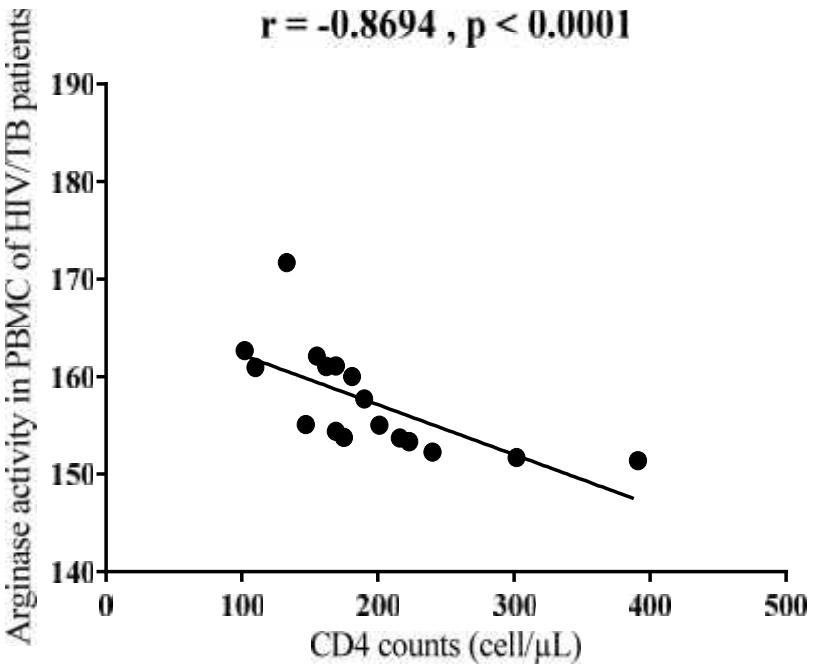


Figure 3.4 (A) Correlation between the level of arginase activity in peripheral blood mononuclear cells and CD4+T cell counts, among HIV/TB co-infected patients ( n = 17)

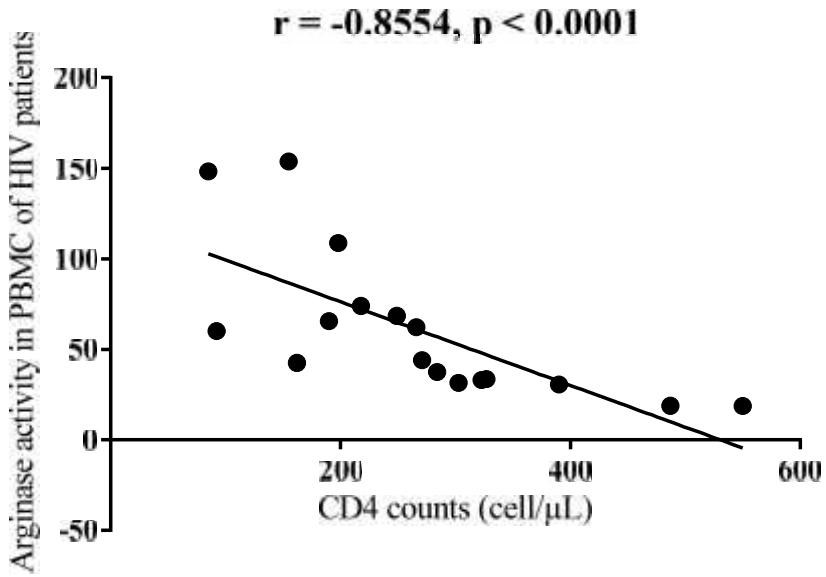


Figure 3.4 (B) Correlation between the level of arginase activity in peripheral blood mononuclear cells and CD4+T cell counts, among HIV patients (n = 17)

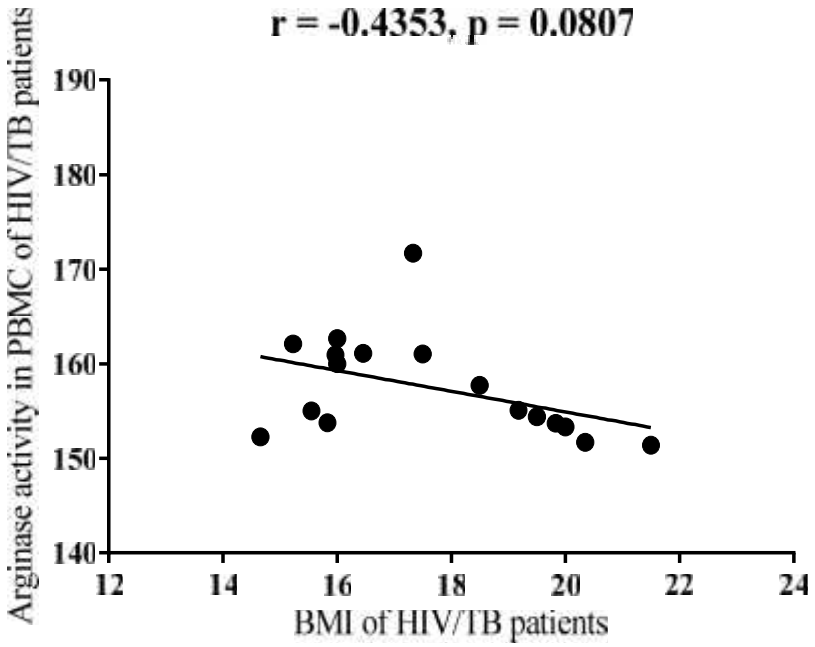


Figure 3.4 (C) Correlation between the level of arginase activity in peripheral blood mononuclear cells and Body mass index, among HIV/TB patients (n = 17).

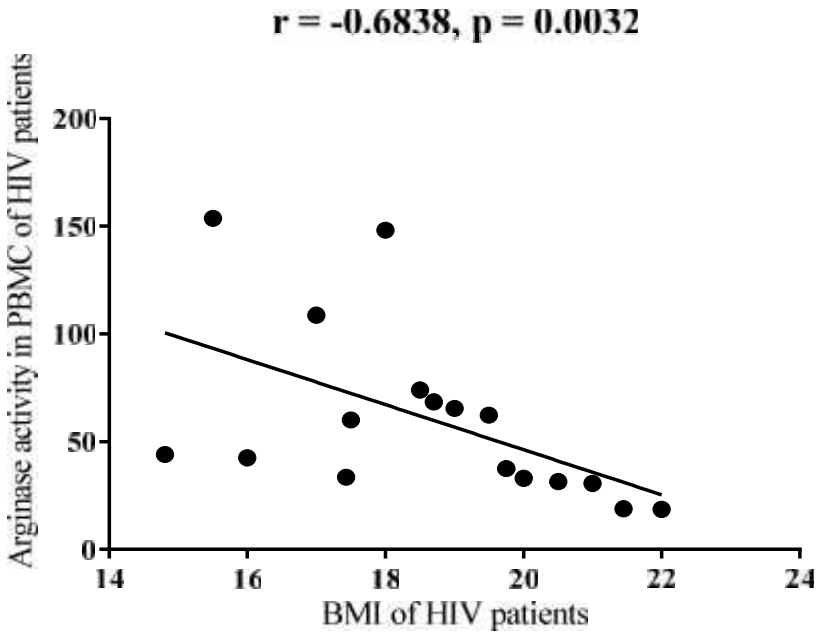


Figure 3.4 (D) Correlation between the level of arginase activity in peripheral blood mononuclear cells and Body mass index, among HIV patients (n = 17).

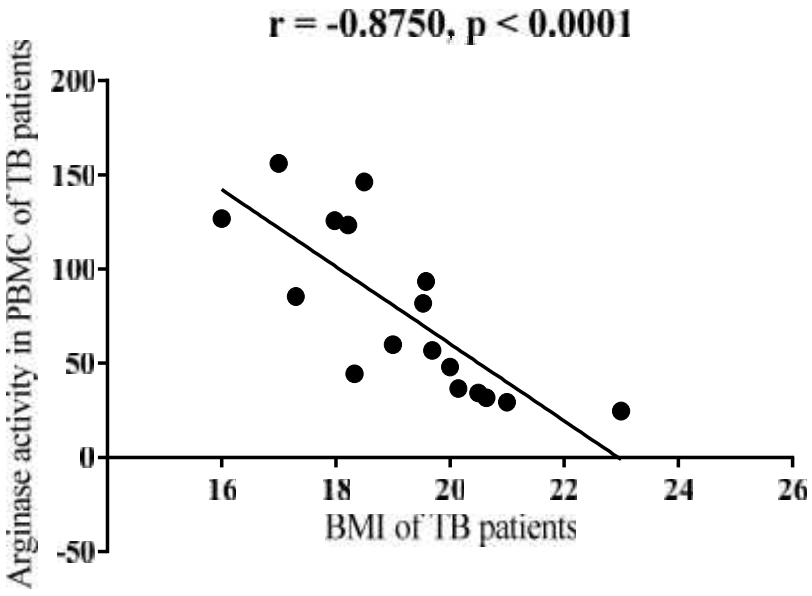


Figure 3.4 (E) Correlation between the level of arginase activity in peripheral blood mononuclear cells and Body mass index, among TB patients (n = 17).

# 4. Discussions

Amino acid starvation is increasingly recognized as a potent immune regulatory mechanism. For example, cysteine depletion leads to cell cycle arrest in human T cells, due to intracellular glutathione reduction and altered redox regulation (Sido et al., 2000). Reduction of the levels of another amino acid, tryptophan, by the enzymatic activity of indoleamine 2 3-dioxygenase (IDO) in the kynurenine pathway suppresses maternal T cell responses during pregnancy and thereby contributes to the tolerance of allogeneic fetuses (Munn et al., 1998). The catabolism of the semi-essential amino acid L-arginine by arginase is also emerging as a potent mechanism for the regulation of T cell responses (Munder et al., 2006, Kropf et al., 2005, Makarenkova et al., 2006) because availability of these amino acid is essential for normal T-cell proliferation and function (Bronte and Zanovello, 2005, Rodriguez et al., 2003, Ochoa and Makarenkova, 2005). Several investigators have further investigated the importance of L-arginine on specific cellular and molecular functions in the T lymphocyte (Rodriguez et al., 2005, Rodriguez et al., 2003, Bronte et al., 2003a). Upon arginine deprivation, there is a progressive reduction (to 25% of basal levels) in the number of T-cell receptors on the cell membrane. This is principally due to the translational regulation of the expression of the ζ-chain peptide (Rodriguez et al., 2005, Rodriguez et al., 2003, Taheri et al., 2001), an essential component of the T-cell receptor complex. Importantly, CD3 ζ chain expression and T cell proliferation have been restored by addition of both a competitive arginase inhibitor and by the addition of exogenous L-arginine (Kropf et al., 2007). Interestingly, loss of the CD3 ζ chain is observed in certain cancers (Kusmartsev and Gabrilovich, 2006) and in HIV infection (Cloke et al., 2010b), both disease processes associated with decreased T-cell function and increased arginase expression.

In this study, we measured the level of arginase, an enzyme with potent immunosuppressive properties in PBMC and plasma of healthy control as well as in PBMC and plasma of HIV, TB and HIV/TB co-infected patient and compare with each other. The level of arginase is statistically significantly increased in PBMCs of HIV-seropositive patients as compared to healthy control. Consistently, the level of arginase activity in PBMC were statistically significantly higher in HIV-seropositive patients with a low CD4 T cell count (< 350 cells/μL) than those in control participants (Cloke et al., 2010b), and our patients also had a mean CD4 T cell count of less than 350 cells/μL too. Moreover, arginase activity in PBMCs and plasmas of pulmonary tuberculosis

patient were also still notably higher than arginase activity in PBMC and plasmas of healthy controls. In other study conducted by Zea et al. showed that high levels of arginase activity were measured in patients who have active tuberculosis and it coincide with reduced levels of L-arginine in the plasma (Zea et al., 2006). Finally, we report for the first time, to our knowledge, that the level of arginase, an enzyme that catabolizes L-arginine is statistically significantly increased in PBMCs and plasmas of HIV/TB co-infected patients as compared to healthy controls.

The level of arginase activity in PBMC and plasma of HIV/TB co-infected patients was also compared with HIV and TB patients in order to test whether arginase activity was even further increased in co-infected patients. Interestingly, statistically significant increased levels of arginase were measured in both PBMC and plasma of HIV/TB co-infected patients as compared to plasmas and PBMCs arginase activity of HIV and TB patients. Similar study conducted on VL and VL/HIV co-infected patient, statistically significantly higher level of arginase was measured in PBMC and plasma of VL/HIV co-infected patients as compared to VL patients (Takele et al., 2010).

High arginase levels imply a high rate of substrate (L-arginine) consumptions and decrease level of extracellular L-arginine, immunologically essential amino acid. It has been shown that higher arginase activity coincides with lower levels of L-arginine and lower expression levels of CD3 ζ in T cells (Cloke et al., 2010b). In this study, we have not measured the level of L-arginine in the plasma of our patients. Arginase-induced L-arginine depletion has been associated with profound T cell dysfunction, and the expression level of CD3 ζ in T cells is used as a marker of T cell hyporesponsiveness (Bronte and Zanovello, 2005, Kropf et al., 2007, Ochoa et al., 2007). The molecular basis for this T cell dysfunction induced by L-arginine deprivation has recently been identified: the absence of L-arginine arrests T cells in the G0–G1 phase of the cell cycle (Rodriguez Pc et al., 2007).

In addition to impairing T cell functions, arginase activity has also been shown to favor parasite growth in macrophages: arginase catabolises L-arginine into polyamines, which are the main intracellular source of the polyamines necessary for the growth of the parasites (Kropf et al., 2005). Similarly, polyamines also appear be important for HIV replication, since blockage of a key enzyme in polyamine synthesis efficiently suppresses HIV-1 replication

(Schafer et al., 2006). Besides, it is also shown that *Mycobacterium* enhances arginine transport in infected macrophage and acquires the metabolites necessary for bacterial growth (Peteroy-Kelly et al., 2003). So it is possible that the degradation product of L- arginine resulted from the hydrolysis of L- arginine by arginase could benefit both TB and HIV replication. This might also account for exacerbated disease outcome associated with TB, HIV, and HIV/TB co-infection.

Malnutrition is associated with immune dysfunctions and increased susceptibility to infections (Morley, 2012), and our result indicate that the highest arginase activity was measured in PBMC and plasma of the most malnourished HIV/TB group. Moreover, there was an inverse correlation between arginase activities in PBMC of HIV, TB and HIV/TB patients and their BMI though the association between PBMC arginase activity of HIV/TB patients and their BMI is not statistically significant: increase arginase in PBMC of HIV, TB and HIV/TB patients was correlated with reduced body mass index. Malnutrition is remarkably common in HIV/TB co-infected patient as 59 % of HIV/TB patient had BMI below 18.5. Macrophages and monocytes from malnourished mice express significantly more arginase both in vitro and in vivo (Corware et al., 2014). Similarly our results also show that relatively higher arginase activity was measured in peripheral blood mononuclear cells of the most malnourished HIV/TB patients.

Furthermore, we have also associated CD4+ T cell count of HIV and HIV/TB co-infected patients and arginase activity. Interestingly, a highly significant inverse correlation was found between increased arginase activity in PBMC of HIV/TB and HIV patient and their CD4 + T cell count. Similar association between CD4+T cell count and arginase activity was observed in HIV seropositive patients (Cloke et al., 2010b). Our study also revealed that PBMCs from HIV/TB co-infected patients with a lower CD4 T cell count (as compared to HIV patients) express statistically significantly higher levels of arginase activity than PBMCs arginase activity from HIV-seropositive patients with a higher CD4 T cell count (as compared to HIV/TB co-infected patients). *Mycobacterium 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 CD4+ T cells are most important in the protective response against *Mycobacterium tuberculosis* (Raja, 2004). Likewise, immune dysfunction leading to AIDS is characterized by a profound decline in the number and effector functions of CD4+ T cells (Boasso et al., 2009). Here, we propose that the higher levels of

arginase activity observed in PBMC and plasma from HIV/TB patients with a lower CD4 T cell count result in lower levels of L-arginine, thereby causing dysregulation of CD4+ T cell responses. In other word, the CD4+ T cell counts, which are closely associated with disease severity and immune suppression (Clerici et al., 1989, Shearer and Clerici, 1991, Appay and Sauce, 2008) were significantly lower in HIV/TB patients as compared to HIV patients. The results presented in our study could explain this weakened immune response as we had shown that arginase, an enzyme with potent immunosuppressive properties, is even further increased in the PBMCs from HIV/TB patients.

# 5. Conclusion and recommendation

## 5.1 Conclusion

In conclusion, we found a significant increased arginase activity in PBMC of HIV, TB and HIV/TB co-infected patients than in PBMC of healthy controls. Similar result was also observed in the plasma of TB and HIV/TB co-infected. The level of Arginase activity was also found higher in the PBMC and plasma of HIV/TB co-infected patients than HIV and TB patients. Moreover, arginase activity was found inversely correlated with BMI of HIV, TB and HIV/TB patients and with CD4+ T cell count of HIV and HIV/TB patients.

## 5.2 Recommendations

In accordance with our study we recommend:

- ✓ A better understanding of the events that result in the up-regulation of arginase and the subsequent down-regulation of T cell responsiveness will improve our understanding of the complex mechanisms that lead to progressive immune dysfunctions in patients with HIV, TB and HIV/TB co-infection and provide new targets to improve the efficacy of therapy.
- ✓ Further work on the impact of arginase-mediated L-arginine catabolism on the pathogenesis of other infectious disease, as this may result in the identification of novel therapeutic interventions that might interfere with this arginase enzyme pathway and therefore improve immune responses and finally resistance to infectious diseases.
- ✓ Increased arginase activity is known to cause depletion of L-arginine (immunologically essential amino acid), so the following mechanism are proposed in order to treat L-arginine starvation:-
    - ➢ prevention of arginase up regulation and
    - ➢ Dietary strategies in order to restoring arginine concentrations.

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# ANNEXES

## Annex 1: Participant Information Sheet

Date ----------

**Title:** Arginase activity in the blood of patient with HIV, TB and HIV/TB co infection at Addis Ababa, Ethiopia**.**

**Objective of the study:** To measure arginase activity in the blood of HIV, TB and HIV/TB co infection at Addis Ababa, Ethiopia**.**

**Organizations:** The study will be conducted by Addis Ababa University, school of Graduate studies. Laboratory procedure is to be carried out at Black lion Hospital.

**Procedure:** Expert (in the area) will collect blood sample from vein in the left arm other than for routine diagnosis, but only for this research purpose. Laboratory method to be used is arginase enzyme assay.

**Participation:** The procedure is to be carried out after getting your willingness to participate. Those patients who are exclusive volunteers and fulfilling inclusion criteria will be included.

**Risks associated with sample collection:** No risk during blood specimen collection except some sort of discomfort during vein puncture.

**Benefi**t**:** there is no direct benefit in involving in this study but the result of this work can contribute something in the advancement of knowledge towards restoring immune dysfunction caused by these arginase enzymes and for the better understanding of pathogenesis of HIV and TB.

**Compensation:** No compensation will be given for your participation in this study except for elapsed time and transportation.

**Confidentiality:** From medical ethics point of view and research ethics, every part of your personal information will be kept confidentially. Information to be collected from your hospital record and variables expressing your identity will be coded secretly. The only responsible person to link your variables (important for your follow up and treatment) with the code number is the principal investigator. However, other researchers can see your clinical information, which is without your identity

**Dissemination of the result:** Eventually, the result devoid of your identity will be reported through publication or by other means. Have no suspicion on the confidentiality of your information, even at this time.

**Right to refuse or withdraw:** Thus, it is your right to agree or to refuse to participate in the study. Refusing to participate will not have any impact on your medical follow up.

You can address your problem or question through one of the addresses given bellow.

**Contact Addresses:**

1. **Mohammedmensur Abdu,** Address: Addis Ababa University, College of Health Science, department of Medical Microbiology, Immunology and Parasitology, Mobile: +251911519684, E-mail – mohammedmensur@gmail.com
2. **Dr. Tamirat Abebe,** Address: Addis Ababa University, College of Health Science, department of Medical Microbiology, Immunology and Parasitology, Mobile: +251911447227, E-mail tabebezeleke@yahoo.com
3. **Addis Ababa University (College of Health Science),** address: Office of Associate Dean, Postgraduate Programs and Research. Tel. + 251-011-551-28-765, P.O. Box 9086, Addis Ababa, Ethiopia

Thank you for your patience and kindness

## Annex 2: Informed Consent Form

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

Identification No ----------

I read and/or well informed about the nature of the study, entitled **"arginase activity in the blood of patient with HIV, TB and HIV/TB co infection at Addis Ababa, Ethiopia. "**. Then, she/he asked my willingness to give the blood sample to be collected for this research purpose. Finally, she/he told me that this will be certain if I agree on the following points and signed bellow.

a) I understood the objective of the study

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

c) I understood that clinical data about me which is important for this study will be extracted from my hospital record (card)

d) I am aware of any information describing my identity which is taken from my hospital record, won't be disclosed and also will be kept confidential

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

f) I clearly informed as I have the right to refuse to participate.

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

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

The information sheet was given/ explained to me by: ----------, signature --------, Phone ----------

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

Name of laboratory technician: ----------, signature --------, phone, ----------

**ለተሳታፉዎች የጥናቱ መረጃ ቅፅ**

ቀን ----------

**ርዕስ፡** የአርጂኔዝ ኢንዛየም ልካት በቲቢ፣ኤች አየ ቪ እና ቲቢ/ኤች አየ ቪ ህሙማኖች ደም ዉስጠ

**የጥናቱ ዓላማ፡** የዚህ ጥናት አላማ በቲቢ፣ኤች አይ ቪ እና ቲቢ/ኤች አይ ቪ ህሙማኖች እና ከችግር ነፃ በሆኑ ሠዎች ደም ዉስጥ አርጂኔዝ ኢንዛይምን መለካት ነው፡፡

**ጥናቱን የምያካሂደው ድርጅት፡** ኣድስ አበባ ዩንቨርሲቲ ፣ የድህረ ምረቃ ትምህረት ቤት ነዉ፡፡

የላቦራቶሪዉም ስራ የሚካሄደዉ በኣድስ አበባ ዩንቨርሲቲ ህክምና ፋካልቲ ጥቁር አንበሳ ሆስፒታል ዉስጥ ነዉ፡፡

**የናሙና አወሳሰድ ህደት፡** የደም ናሙና ከክንድ ላይ በባለሙያ አማካኝነት ይወሠዳል፡፡

**ተሳትፎ፡** በጥናቱ ዉስጥ ያለመሳተፍ መብትዎ የተጠበቀ ነዉ ፡፡ ናሙናዉን የምንወስደዉ የእርስዎን ሙሉ ፍቃድ ካገኘን ብቻ ነዉ፡፡ መስፈርቱን የምያሟሉና ፍቃዶኛ የሆኑ በጥናቱ ዉሰጥ ይገባሉ፡፡

**በናሙና አወሳሰድ ጊዜ ሊገጥሞት የሚችሉ ችግሮች፡** በዚህ ጥናት ላይ በመሣተፈዎ ሊደርስበዎት የሚችል አደጋ የለም ፡፡

**ከጥናቱ ሊያገኙት የሚችሉት ጥቅም፡** በዚህ ጥናት ላይ በመሣተፈዎ ምንም አይነት ቀጥተኛ ጥቅም የለውም ነገር ግን ይህ ጥናት ወደፊት ከቲቢ እና ኤች አየ ቪ ጋ የተየየዙ ችግሮች ላይ ለሚሰሩ ጥናቶች ኣወንታዊ ጥቅም ኣለው ፡፡

**ማካካሻ፡** በዚህ ጥናት ላይ ተሳታፊ ስለሆኑ ምንም አይነት ጥቅም አያገገኙም ግን በጥናቱ ምክንያት ያወቱት ወጭ (ለትራንሰፖርትና ላባከኑት ጊዜ) ካለ ተመጣጣኝ ክፍያ የከፈላል፡፡

**ምስጥር ስለ መጠበቅ፡** በህክምና ሙያ ስራት መሰረት፣ ማንኛዉም ዓይነት የግል መረጃዎ በምስጥር ይጠበቃል፡፡ ከሆስፒታል መዝገብ የተሰበሰበዉ መረጃዎ ማንነትዎን በማይገልጽ በምስጥር ኮድ ወደ ኮምፒተር እንዲገባ ይደረጋል፡፡ የላቦራቶሪ ዉጤትዎም ምስጥሩ የተጠበቀ ከመሆኑም በላይ የእርስዎን ስም አይዝም፡፡ ይሁን እንጂ፣ ሌሎች ተመራማሪዎች የርስዎን ማንነት የለለበትን ዉጤትዎን ሌያዩ ይችላሉ፡፡ መረጃዎና ዉጤትዎም ለተጠቀሰዉ አላማ ብቻ ይዉላል፡፡

**የዉጤት አገላለፅ ሂደትና መንገድ፡** ጥናቱ ከተጠናቀቀ በኃላ ፣ ዉጤቱ በተለያየ መንገድ ይገለፃል፡፡ የእርስዎን ማንነት እንደማይዝና ምስጥርዎ እንደተጠበቀ ይሆናል፡፡ ለዚህም የእርስዎን በጎ ፍቃድ እንጠይቃለን፡፡

**በጥናቱ ያለመሳተፍ መብት፡** በጥናቱ ዉስጥ የመሳተፍም ሆነ ያለ መሳተፍ ሙሉ መብት አለዎት፡፡ በጥናቱ ዉስጥ አለመሳተፍም በሚሰጠዎት ህክምና ላይ ምንም አይነት ችግር አያመጣም፡፡

**ተመራማሪዎችን ሊያገኙበት የሚችሉባቸዉ አድራሻዎች፡** ስለ ጥናቱ ማንኛውንም አይነት ጥያቄ ካልዎት ከሚከተሉት ዉስጥ የአንዱን አድራሻ ይጠቀሙ፡፡

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**የስምምነት መጠየቅ ቅፅ**

ስም---------- ፣ ዕድሜ ---------፣ ፆታ ----------

መለያ ቁጥር ----------

ካነበብኩት ወይም ከሰማሁት “አርጂኔዝ ኢንዛይም በ ቲቢ፣ኤች አይ ቪ እና ቲቢ/ኤች አይ ቪ ህሙማኖች ደም ዉስጥ በዘዉዲቱ እና ቾዱስ ፒጥሮስ ሆስፒታል” ስለ ተባለዉ ጥናት በቂ ግንዛቤ አግንቸአለሁ፡፡ የምሰጠዉ ናሙና ከታዘዘልኝ ምርመራ ዉጭ ለጠናቱ አላማ ብቻ መሆኑን ተገንዝበአለሁ፡፡ በመጨራሻም ናሙናዉን መፍቀዴን የማረጋገጠዉ ፤ በሚከተለት ነጥቦች ከተሰማማሁ ብቻ መሆኑ ተነግሮኛል፡፡

ሀ. የጥናቱ ዓላማ ገብቶኛል ፡፡

ለ. ናሙናዬም ሆነ መረጃዬ ከተገለጸዉ ዓላማ ወጪ እንደማይዉልና የተረፈ ናሙና ካለም በጥንቃቄ እንደሚወገድ ተረድቻለሁ፡፡

ሐ. ለጥናቱ የሚጠቅሙ እኔን የሚገልጡ መረጃዎች ከሆስፒታል መዝገብ እንደሚዎሰድ አዉቄአለሁ፡፡

መ. መረጃዎቹም ማንነቴን እንደማይገልፅ እንዲሁም ሚስጥራዊ እንደሚሆን ተረድቻለሁ፡፡

ሠ. በጥናቱ ተሳታፉ ስለሆንኩ ሳይሆን የትራንሰፖርትና ላባከንኩት ጊዜ የምሆን ተመጣጣኝ ገንዘብ እንደሚሰጠኝ አዉቄአለሁ (ካለ)፡፡

ረ. በጥናቱ ዉስጥ ያለመሳተፍ መብት እንዳለኝ ተረድቻለሁ፡፡

ሰ. በጥናቱ ዉስጥ ባለመሳተፊ በሚሰጠኝ ህከምና ላይ ምንም ችግር እነደማያመጣ አዉቀአለሁ፡፡

ስለ ጥናቱ ካነበብኩት ወይም ከሰማሁት ፣ በቂ ጊዜ ወስጀ ካሰብኩ በኋሊ ፣ በቂ ግንዛቤ አገንቻለሁ፡፡ ስለሆነም ፣ በጥናቱ ዉስጥ መሳተፌ ጠቃሚ ሆኖ ስላገኘሁት ፤ በራሴ ወስኘ ናሙናዉን ለመስጠት ፍቃደገኛ መሆኔን በፊርማዬ አረጋግጣለሁ፡፡

መረጃውን የሰጠው ሰው ስም---------- ፉርማ ----- ስልክ --------

የተሳታፉ ስም ---------- ፉርማ ----- ስልክ --------

ናሙናዉን የወሰደዉ ሰው ---------- ፉርማ ---- ስልክ ---------

## DECLARATION

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

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