A COMPARISON OF ARGINASE ACTIVITY IN THE SALIVA, PLASMA AND PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) OF PULMONARY TUBERCULOSIS PATIENTS IN ADDIS ABABA, ETHIOPIA

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LIST OF ABBREVIATIONS

ARG: Arginase

CAT: Cationic amino acid transporter

DOT: Directly observed short course therapy

EPT: Extra pulmonary Tuberculosis

HIV: Human Immuno deficiency Virus

ISPF: α-isonitrosopropiophenone

LPS: Lipopolysaccharide

LTA: Lipo techoic acid

MDR: Multidrug resistant

MSC: Myeloid suppressor cell

MTB: Mycobacterium tuberculosis

NOS: Nitric oxide synthase

ODC: Ornithine decarboxylase

PBMC: Peripheral blood mononuclear cells

PMN: poly morpho nuclear cells

PTB: Pulmonary TB

TB: Tuberculosis

TCR: T cell receptor

TGF: Transforming growth factor

TLR: Toll like receptor

SEM: Standard Error of Mean

WHO: World Health Organization

XDR: Extensively drug-resistant

Abstract

Background: *Mycobacterium tuberculosis (MTB)* is the causative organism of the chronic bacterial infection, tuberculosis (TB). TB is one of the leading causes of death due to infectious diseases worldwide. Recently it has been observed that arginase (ARG)-is no longer a mere enzyme responsible only for urea cycle but is a key enzyme that plays a great role in modulation of the immune response and thus, pathogenesis. Infection with MTB and other intracellular pathogens induces the activation of ARG and inducible nitric oxide synthase (iNOS) which competitively metabolize L-arginine as a common substrate to produce L-ornithine and nitric oxide (NO), respectively. ARG induced deprivation of L-arginine is important for pathogens to limit the intracellular production and killing action of NO and T cell responses. Therefore, induction of ARG might be an important escape mechanism for the survival of MTB**.**

Materials and Methods: A cross-sectional study was carried out to compare the level of ARG activity in the saliva, plasma and peripheral blood mononuclear cells (PBMCs) of newly diagnosed HIV sero -negative smear positive TB patients (n=18) and healthy controls (n=8). The level of arginase activity in all type of samples was measured by enzymatic assay and a two-tailed Mann-Whitney U test was performed to assess statistical differences.

Results: There was a statistically significantly higher level of arginase activity in the PBMCs ($p=0.015$) and saliva ($p=0.008$) of TB patients than those of healthy controls. Furthermore, an increased level of arginase activity was detected in the plasma of TB patients as compared to healthy controls, however it was not statistically significant ($p=0.211$). A significantly higher ($p=0.024$) frequency of arginase expressing cells were detected in the PBMCs of TB patients as compared to healthy controls. The phenotype of these cells was identified as $CD15^{+}$ neutrophile.

Conclusions and recommendation**:** Infection with MTB can result in an increased frequency of ARG-expressing cells as well as in increased level of arginase activity in the PBMCs, saliva and plasma of the TB patients. Therefore, the measurement of ARG activity in saliva might be used as a biomarker of TB infection.

Key words: Arginase, TB, PBMC and saliva.

1.0. INTRODUCTION

Tuberculosis (TB), also called the "white plague" and "consumption," is a disease that has plagued the citizens of nearly every nation in the world for centuries. It is one of the most prevalent bacterial infections worldwide and constitutes a leading global health threat (Schreiber et *al*., 2009). According to the 2008 WHO latest estimates of the global burden of TB, there were an estimated 8.9–9.9 million incident cases of TB, 9.6–13.3 million prevalent cases of TB, 1.1–1.7 million deaths from TB among HIV-negative people and an additional 0.45–0.62 million TB deaths among HIV-positive people (WHO,2009). The disease is caused by a group of similar bacilli, most commonly *Mycobacterium tuberculosis* complex, (Brook et al., 2007), which *is* usually transmitted through the airborne spread of droplet nuclei produced by patients with infectious pulmonary tuberculosis (Braumwald *et al*., 2008).

Since TB produces acute, chronic and latent diseases involving every organ of the body, although the lungs remain the primary site of infection, it is classified as pulmonary, extra pulmonary, or both. Extrapulmonary Tuberculosis (EPT) is infection of the extra pulmonary sites, such as, lymph nodes, pleura, genitourinary tract, bones and joints, meninges, peritoneum, and pericardium. However, virtually all organ systems may be affected (Braumwald *et al*., 2008). Before the advent of HIV infection, ~80% of all new cases of tuberculosis were limited to the lungs. However, up to two-thirds of HIV-infected patients with tuberculosis may have both pulmonary and extrapulmonary disease and extra pulmonary disease alone (Braumwald *et al*., 2008). Active TB of the lungs classically present different signs and symptoms, such as fatigue, weakness, weight loss, fever, chronic cough and spitting of blood usually is associated with far-advanced lesions ,but meningitis or urinary tract involvement can also occur in the absence of other signs of tuberculosis (Brook et al., 2007).Therefore, the diagnosis of tuberculosis should be made on the basis of a history of exposure, physical examination, chest radiograph, tuberculin test, and sputum examinations with culture and acid fast staining procedures (Brook et al., 2007).

Among the antimycobacterial strategies of the host, the free radical nitric oxide (NO) is an important innate response which is directly toxic to MTB and other diverse intracellular pathogens. Its production from the amino acid, L-arginine in inflammatory cells like

macrophages is catalyzed by the inducible nitric oxide synthase (iNOS). As one means of survival MBT limits this intracellular production and killing action of NO by inducing the production of another macrophage enzyme called arginase (ARG), which compete with iNOS and deplete L-arginine (Chul et al., 2009). In human ARG is mainly expressed by neutrophils and their phenotype in the PBMCs of HIV sero positive persons found to be $CD15^{+}CD14^{-}$ (Cloke et al., 2010).

Although, all cases of tuberculosis caused by drug-susceptible strains are curable with proper treatment and possible to reduce its global incidence, the emergence of HIV/AIDS pandemic and multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis during the past decade has created a serious, and potentially deadly, healthcare problem for developing countries particularly for Africa (WHO, 2009 and Wright *et al*., 2009). Because, if untreated; the disease may be fatal within 5 years in 50–65% of cases (Braumwald *et al*., 2008). Furthermore, in addition to its high rate of mortality the disease also leaves a lifetime sequelae on people who are cured from the disease by substantially reducing their quality of life (Miller *et al*., 2009).The direct and indirect costs of tuberculosis, and the social consequences, are often catastrophic for the individual patient, the family, and the wider community (Hanson *et al*., 2006).

1.1. Literature review

1.1.1. Immunopathology

According to classic animal model studies, there are different stages of mycobacterial infection (Lurie, 1964). The infection starts, when *Mycobacterium tuberculosis* contacts with macrophage in which some of the organisms are destroyed and some are able to continue to multiply. Pathogenic mycobacteria are able to survive and multiply within macrophages by inhibiting the fusion of phagosomes with lysosomes (Russell, 2001) as well as by hindering the acidification of the phagolysosome (Kaufmann, 2001). During the second stage, blood monocytes and other immune cells are attracted to the site of infection. The monocytes differentiate into macrophages which are unable to kill the mycobacteria (Qualls *et al*., 2010). It is only a few weeks later, the third stage, when the influx of antigen-specific T cells, that mycobacterial multiplication is controlled. This happens because of

secretion of IFN-γ which activates the macrophages and releases the block on phagosomal maturation (Russell, 2001, Qualls *et al*., 2010). Efficient killing of mycobacteria depends on nitric oxide produced by nitric oxide synthase and by other related radicals (Kaufmann, 2001 and Qualls *et al*., 2010). This process is enhanced by acidification of the mycobacteria-containing vacuole which can now occur because the block is now lifted. The final stage infection may become dormant or, alternatively, if the immune cells fail to control the multiplication of the bacteria dissemination occurs and active disease develops. Infected macrophages produce large amounts of proinflammatory mediators such as TNF-α, IFN-γ, IL-6, IL-1β and IL-12. A range of anti-inflammatory cytokines is produced including IL-4, TGF-β and IL-10 (van Crevel *et al*., 2002, Popovic et *al*., 2007 and Lahiri *et al*, .2010). The balance between proinflammatory and anti-inflammatory cytokines defines the expression of tuberculosis (van Creval *et al*., 2001, Braumwald *et al*., 2008). Uncontrolled cytokine release is responsible for many of the symptoms and signs of tuberculosis such as fever and wasting.

Pulmonary tuberculosis can be broadly categorized as primary and post primary (secondary) tuberculosis. Primary PTB occurs soon after the initial infection with tubercle bacilli. The middle and lower lung zones are most commonly involved in the primary TB. The lesion forming after infection is usually peripheral and accompanied in more than half of cases by hilar or paratracheal lymphadenopathy, which may not be detectable on chest radiography. Unless in children and in persons with impaired immunity (primary PTB may progress rapidly to clinical illness) in the majority of cases, the lesion heals spontaneously and may later be evident as a small calcified nodule (Braumwald *et al*., 2008).

Secondary tuberculosis occurs in 10% to 15% of the patients who have tubercle bacilli that have survived in the primary lesion. Reactivation tuberculosis is characterized by chronic tissue lesions, the formation of tubercles, caseation, and fibrosis. Regional lymph nodes are only slightly involved, and they do not caseate. The risk that latent *MTB* infection will proceed to active disease depend on the patients's Age (late adolescence and elderly) and degree of immunosuppression (CDC, 2007 and Braumwald *et al*., 2008). In a study of HIV-infected, tuberculin skin test–positive persons, this risk varied from 2.6 to 13.3 cases per 100

person-years and increased as the CD4+ T cell count decreased. (CDC,2007 and Braumwald *et al*., 2008).

Age (late adolescence and elderly) and depleted immunity plays important role for the reactivation (CDC, 2007).Generally primary TB is thought to depend on a combination of genetic susceptibility of the host and virulence of the infecting MTB strain, whereas post primary TB is due to a temporal dysregulation of otherwise intact immune defenses (Schreiber *et al*., 2009).

Both innate (Tsolaki, 2009) and adaptive immunities are essential to eradicate MTB from the body, particularly cell-mediated immune (CMI) response is known to be critical in the host defense against infection with intracellular pathogens such as mycobacteria(Schreiber *et al*.,2009).Recent findings clearly indicated that Toll-like receptors (TLRs) play an essential role in the recognition of MTB components by macrophages and dendrite cells, resulting in not only activation of innate immunity but also development of antigen-specific adaptive immunity (Kawamura, 2006). Alveolar macrophages, monocytes, and dendrite cells are critical for directly phagocytizing, processing and presenting the bacilli's antigens to T lymphocytes (Braumwald *et al*., 2008). Clearance of bacteria by macrophages is in part dependent on macrophage activation by the cytokine interferon gamma (IFN-γ) secreted by CD4 T cells, CD8 T cells, and NK cells (Flynn *et al*., 1993). The primary defense mechanism of monocyte and macrophage is probably related to production of nitric oxide (Popovic *et al*.,2007), which has anti mycobacterial activity (Kaufmann, 2001) ,And increases synthesis of cytokines such as TNF-α and IL-1, which in turn regulate release of reactive nitrogen intermediates (Chan *et al.,*1995). It has been also reported that induction of apoptosis of the infected cells may be one of the strategy of host defense against MTB because, it can result suppression of the intracellular replication of the bacilli (Kawamura, 2006 and Stenger *et al*., 1997). Up on exposure to the Mycobacterial antigens T lymphocytes will be activated and proliferated. The activated CD4+ T lymphocytes can differentiate into cytokine-producing Th1 or Th2 cells (Braumwald *et al*., 2008). Th1 lymphocytes play an important role in granuloma formation by secreting type 1 cytokines, primarily IFN-γ (an activator of macrophages and monocytes) (Schreiber *et al*., 2009) and

TNF (Cooper *et al*., 1993). T_H2 cells produce IL-4, IL-6, IL-10, and IL-13 and also may promote humoral immunity. The interplay of these various cytokines and their cross-regulation determine the host's response (van Creval *et al*., 2001, and Braumwald *et al*.,2008).CD8+ T cells have been associated with protective activities via cytotoxic responses and lysis of infected cells as well as with production of IFN-γ and TNF-α (Sud *et al*.,2006).

The cytokine that is produced from NK cells and dendritic cells at the early period of infection strongly induces not only macrophage activation but also development of antigen-specific IFN-gamma-producing CD4+T cells (Kawamura, 2006). During the course of infection with MTB, innate immune responses control the spread of the bacteria, but T lymphocyte recruitment to the lung is required for containment of MTB in granulomas and production of IFN-γ (Giacomini *et al*., 2001).

The successful replication and survival of MTB in the presence of different antibacterial agents inside host macrophages depends on the intelligent strategies employed by the bacteria. Among the antimycobacterial agents, nitric oxide (NO) is one of the most important, which is directly toxic to MTB, (Bogdan, 2001 and Pieters, 2008). During acute bacterial infection, NO is produced as one of host resistance factor through the enzymatic activity of inducible nitric oxide synthase (iNOS) on the amino acid L-arginine (Bronte *et al*., 2005).

1.1.2. Role of arginine metabolism in immunity

Arginine, first discovered over 100 years ago, is a basic amino acid naturally ingested in our diets at a rate of 3–5 g/d (Wu *et al*., 1998). It is classified as a semi-essential or conditionally essential amino acid, depending on the age and health of an individual. Adults can synthesize enough L-arginine so that it is not a nutritionally essential amino acid whereas newborns are unable to produce arginine efficiently. However, in cases of physical stress (surgery or trauma) and infection, since demands cannot be met via endogenous routes L-arginine needs to be supplemented (Popovic *et al*., 2007). Regulation of L-arginine homeostasis is dependent on dietary intake, whole body protein turnover and arginine synthesis and catabolism (Castillo *et al*., 1993). Arginine is an important amino acid that

serves to modulate the cellular immune response during infection and also a common substrate for the two inducible enzymes, such as arginase (ARG) and nitric oxide synthase (NOS) (Wu *et al*., 1998 and Bogdan,2001).These enzymes are competitively regulated by Th1 and Th2 cytokines and other factors (Bronte *et al*.,2005 and Popovic *et al*.,2007).

The inducible enzyme NOS is not constitutively expressed but highly induced by proinflammatory cytokines such as interferon gamma (IFNγ), tumour necrosis factor alpha (TNF-α), interleukin 1 (IL-1), IL-2 and other membrane associated substances such as lipopolysaccharide (LPS), lipoteichoic acid (LTA)(Munder *et al.,*1998 and Bernard *et al*.,2000), and oxidizes L-arginine in a two-step process into NO. Therefore, the availability of L-arginine in the extra-cellular environment is one of the main factors that regulate the expression of inducible nitric oxide synthase (Lee *et al*., 2003 and Chaturvedi et al., 2007).The generation of nitric oxide from arginine contributes to the innumerable physiological processes and efficient immune response and cytotoxicity of host cells to kill MBT and other invading intracellular pathogens (Wu *et al*.,1998 , Bogdan,2001 and Alderton *et al*.,2001).This is also supported by a study showing the loss of iNOS from the host by genetic ablation or pharmacologic inhibition leads to increased susceptibility to mycobacteria and early death in infected mice (MacMicking *et al*., 1997 and Flynn *et al*.,1998).

There are two isoforms of arginase (Arginase I and Arginase II), which differ with regard to tissue distribution and subcellular localization (Das *et al.,* 2010). Arginase I , is a trimeric cytosolic protein located in the erythrocytes and cytosole of hepatocytes (Mori, 2007), is an important component of the urea cycle. Salivary arginase also has been demonstrated in the parotid gland of man with up to 400 times higher activity than its activity in serum (Konarska *et al*., 1985). Furthermore, the kinetic, chromatographie and immunological similarities of liver, red blood cell and salivary arginase suggest their genetic identity ((Konarska *et al*., 1985). Arginase II is also a trimeric mitochondrial protein, which is expressed in extrahepatic tissues like the small intestine, kidney, brain, monocytes, and macrophages (Mori, 2007).

In higher organisms, ARG hydrolyses L-arginine to L-ornithine and urea. L-Ornithine is a precursor for the synthesis of polyamines by the ornithine decarboxylase (ODC) pathway and for the synthesis of L-proline by the enzyme ornithine aminotransferase: polyamines are involved in cell growth and differentiation, whereas L-proline affects collagen production (Wu *et al*., 1998 and Bogdan, 2001).The expression of ARG induced in mouse myeloid cells by exposure to the Th2 cytokines, such as IL-4, IL-13 or IL-4, and IL-10 (Barksdale *et al*.,2004 *and* Munder *et al.,*1998), and also by IL-6 and TGF-β (Munder *et al*.,1999), prostaglandins (PGE2) (Bansal *et al*.,2005), and catecholamines (CA)(Bernard *et al*.,2000).

Human ARG 1 has been recently found to be constitutively expressed by granulocytes, activated macrophage and myeloid derived suppressor cells (Kropf *et al*.,2007 and Ochoa *et al* .,2007). Activation of both iNOS and arginase reflects inflammatory responses in specific disease process (Holan *et al*., 2006). And one can regulate the expression of the other through different mechanisms: iNOS can control arginase by the generation of the NO intermediate, hydroxy-L-arginine, which is a competitive inhibitor of arginase activity; in turn, arginase can regulate NO production via the depletion of L-arginine from the extracellular milieu (Modolell *et al*., 1995).

The metabolism of L-arginine by iNOS and arginase 1 in myeloid cells is emerging as an important mechanism of T cell regulation (Iyer *et al*., 2002). Myeloid suppressor cells (MSCs) use ARG and NOS, either separately or in combination to inhibit T-cell responses to antigen. The choice of which of these enzymes to use is regulated by a network of signals, cytokines and membrane-bound receptor–ligand interactions that underlie the crosstalk between MSCs and activated T cells (Bronte *et al*., 2005). Regulating the availability of arginine is the potential mechanism for controlling the production of NO (Efron and Barbul, 1998), as well the proliferation and function of T-cell (Bronte *et al*., 2005). 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 z-chain peptide (Rodriguez *et al*., 2003 and Taheri *et al*., 2001), an essential component of the T-cell receptor complex. Interestingly, loss of the z-chain is observed in certain cancers (Zea *et al*., 1995) and after surgery or trauma (Taheri *et al*.,

2001), both disease processes associated with decreased T-cell function and increased ARG 1 expression. High level of arginase expression observed during infection with different infectious organisms, such as *Trypanosoma cruzi* (Bronte *et al*., 2005), *Salmonella typhimurium* (Lahiri *et al*., 2008), Leshmania (Gaur *et al*., 2007), HIV (Colke *et al*., 2010),*schistosoma mansoni* (Hesse *et al*.,2001) and *Mycobacterium tuberculosis*(Zea *et al*.,2006). Therefore, induction of arginase expression through regulation of NO production and T-cell function can be taken as one means of survival for different intracellular organisms from the killing action of NO and T-cell response, For example, infection of macrophages by mycobacteria drove the production of IL-6, IL-10, and granulocyte colony–stimulating factor (G-CSF), which subsequently induced the expression of Arg1 in uninfected cells by the STAT3 signaling pathway (Qualls *et al*., 2010). Thus, macrophage Arg1 limits the production of NO in vitro and in vivo, thereby inhibiting efficient clearance of the mycobacteria (Qualls *et al*., 2010).The molecular mechanisms by which L-arginine depletion leads to a hyporesponsive state in T cells have been shown to be due to cell cycle arrest in the G0-G1 phase whereas in the presence of sufficient L-arginine, cells progressed into S and G2-M phases (Rodriguez *et al*., 2007).

Though little is known about the role of arginase activity in immune response against TB infection, some studies indicated that T cells from TB patients display reduced CD3ζ expression, which is correlated with increased level of ARG activity and ARG-induced L-arginine deficiency (Zea *et al*., 2006). These expression levels were normalized with successful TB treatment (Zea *et al*., 2006). Other study conducted on HIV sero-positive patients also suggests that, increased level of arginase activity might contribute to T cell dysfunction, as well as correlated with disease severity (Colke *et al*., 2010). In addition, the suppression of T cell responses might be mediated by the increased production of IL–10, which is one of the anti-inflammatory cytokine produced during mycobacterial infection and responsible for increased level of arginase activity in the PBMCs of TB patients (Zea *et al*., 2006 and Qualls *et al*., 2010).

Our hypothesis was that following TB infection increased level of arginase would be induced in the patients PBMC s and saliva. We therefore measured and compared the

activity of arginase in the PBMCs, and saliva of HIV sero-negative, smear positive TB patients and healthy controls. Therefore, the present study investigate whether infection with *Mycobacterium tuberculosis* induce an increased level of arginase activity in the peripheral blood mononuclear cells and saliva of tuberculosis patients. And, also the result of this study may indicate the correlation between mycobacterial infection and level of salivary arginase activity, so as to use salivary arginase as an alternative diagnostic marker of active pulmonary tuberculosis disease.

1.2. Objective of the study

1.2.1. General objective

- To measure the level of arginase activity in the PBMCs, plasma and saliva of HIV sero-negative and smear positive TB patients attending Health Centers in Addis Ababa, Ethiopia.

1.2.2. Specific objective

- To compare arginase activity in the PBMC, plasma and saliva of TB patients and controls
- To correlate salivary arginase activity and active pulmonary tuberculosis infection
- To identify the phenotype of arginase expressing cells in the PBMC
- To provide a base line information about the activity of arginase in the PBMC, plasma and saliva of TB patients.

2.0. MATERIALS AND METHODS

2.1. Study area, period and design

A cross sectional study was conducted from November, 2010 through April, 2011 at four Health centers in Addis Ababa, Ethiopia. Addis Ababa is the capital city of Ethiopia. Its altitude is 2300 meters above sea level with annual temperature and rainfall of 10.6 ^{o}C to 22.8 ^{o}C and 1,180.4mm respectively. Based on the preliminary 2007 census results, Addis Ababa has a total population of 2,738,248, with a male to female ratio of 0.9:1.

2.2. Patient recruitment

A convenient sampling technique was used to recruit eighteen HIV- seronegative smear positive pulmonary tuberculosis (PTB) patients, who were newly diagnosed (based on both clinical examination and sputum smear results) and had not received anti-TB treatment from all TB suspected patients attending the TB clinic of the health centers during the study period. Eight apparently healthy (individuals have no sign and symptoms of TB) volunteer controls were selected from first year medical microbiology postgraduate students. Then, after the informed consent was obtained, all the necessary demographic and other clinical datas were collected by attending nurses using a pre- structured questionnaire prepared for this study (appendix IV).

Inclusion criteria: Patients with HIV negative sero status and smear positive TB, whose age was above 5 years.

Exclusion criteria: Patients with Leishmaniasis, Schistosomiasis, HIV positive sero status, Pregnancy and TB patients who has already begun the treatment.

2.3. Sample collection, transportation and processing

2.3.1. Sample collection and transportation

After signed informed consent (appendix III) had been obtained, 20 ml peripheral blood was drawn into two 10 ml EDTA Vacutainer tubes from each of the 18 patients and 8 negative controls. In addition, 500-1000µl of saliva was collected from both patients and controls by spitting in a clean 50ml tube after thorough rinsing of their mouth with water. Immediately after collection, all samples were transported to and processed in Tikur Anbessa Hospital.

2.3.2. Sample processing

Blood plasma isolation

Plasma was collected by the centrifuging of EDTA anti-coagulated blood at 1800rpm for 10 minutes. Then 1-2 ml of plasma was transferred with a clean Pasteur pipette to a tube and frozen until required (appendix VI).

PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with Ficoll: after separation of the plasma the remaining blood was adjusted to 20 ml by adding phosphate buffer saline (PBS), then carefully layered onto 20 ml of Ficoll (Histopaque®-1077, Sigma) and centrifuged at 1900 rpm for 20 min without brake. Using a sterile pipette the interface layer containing the PBMC was collected and transferred into a 50ml centrifuge tube and washed with PBS. PBMCs were counted by mixing 10µl of PBMCs with 90 µl of trypan blue solution to make a 1:10 dilution. The stained PBMCs were counted using 10X objective and eye piece of light microscope after charging it in to the hemocytometer chamber (VII). The remaining cells were suspended in 300µl lysis buffer (0.1% Triton x-100, 50 mmol/L Tris-hydrogen chloride at pH7.5, and 10 mmol/L manganese chloride (sigma)) and then the cell lysate frozen at -20°C until further use (appendix VI).

Arginase activity assay

The enzymatic activity of arginase in PBMCs, plasma and saliva was measured as described elsewhere (Cloke *et al*., 2010). Briefly, arginase was activated by incubating 50µl of defrosted sample (cell lysates, plasma or saliva) at 56^{o}Cfor 7 minutes; then 50 µl of L-arginine (0.5M at pH 9.7) aliquot was added, and the solution was incubated at 37^{o}C for 60 min (for PBMCs) and 120 minutes (for plasma and saliva). Simultaneously, several standards of urea were prepared using milliQ water and urea volume, and treated the same as the samples. The reaction was stopped by adding 400µl acid mixture, and then 20µl 6% ISPF was added to all tubes for the development of color following the production of urea as a result of the hydrolysis of L-arginine by the enzyme in the PBMC. The color product from the reaction was measured using plate reader, at 550nm, after the reaction mixture was

incubated at 98^0c for 30 minutes, followed by 45 minutes incubation at 4^oC (AppendixVIII).

Note: ISPF is a coloring agent which can react with urea and produce color. The intensity of the color product is directly proportional to the amount of urea produced by arginase activity.

Flow cytometric analysis of the phenotype of arginase- expressing cells

Flow cytometric analysis of the phenotype of arginase-expressing cells was done using the following antibodies: anti-CD14 and anti-CD15 (BD Pharmingen: cloneM5E2, Beckman Coulter: RMO52). For the intracellular detection of arginase I, 1 x 10^6 PBMCs were incubated with 20µL FcR blocking reagent for 20 min at room temperature. Anti-CD14 and anti-CD15 mono-clonal antibodies were added directly to the tubes so as to label the cells. After 20 min of incubation at 4^0C, cells were washed and fixed with 4% formaldehyde, then permeabilized with 1% saponine in order to allow anti-arginase enter in to the cells and bound with arginase. Then, the cells were labled with intracellular antibodies (0.2 µg FITC-conjugated anti-arginase I) and 15 min later, cells were washed again with PBS at 1900 rpm for 5 minutes and resuspended with staining buffer and identified by flow cytometry using a FACS Calibur (Becton Dickinson) (Appendix IX).

2.4. Statistical analysis

The arginase activity in the PBMCs, plasma and saliva of both TB patients and controls compared and evaluated for statistical difference with a 2-tailed Mann-Whitney U test. Statistical significance denoted when $p < 0.05$.

2.5. Ethical consideration

This M.Sc. research project was approved and ethically cleared by the research and ethical review Committee of Department of Microbiology ,Immunology and Parasitology (DMIP) , Faculty of Medicine; Addis Ababa University. Official permission from the study sites and written informed consent from study participants were obtained (Appendix III).

3.0. RESULTS

3.1. The age and sex distribution of TB patients and healthy controls

In this study a total of 18 smear positive HIV negative TB patients from four health centers (Teklehaymanot, Cazanchis, Arada and Addis ketema) and 8 healthy controls enrolled. Of the total 18 patients, 10 (55.6%) were males and 8 (44 .4%) were females with the mean (median) age of 26.3(24.5) years, range being 15–48 years.

Table 1: The age and sex distribution of TB patients and healthy controls.

Group	No	Mean age (range)	Sex	
			Male	Female
TB patients	18	26 (15-48)	10	8
Controls	8	27 (24-33)	6	2

3.2. PBMC counts in smear positive TB patients

The PBMC count of TB patients and healthy controls was performed by trypan blue exclusion and as shown in Figure 1, the counts of PBMCs in smear positive TB patients were found to be significantly higher (median concentration ± SEM, 17.0 ±3.18 cells/5 ml of blood, $p<0.05$) than the counts in the PBMCs of controls (median concentration ± SEM, 11.78 ±1.35 cells/5 ml of blood).

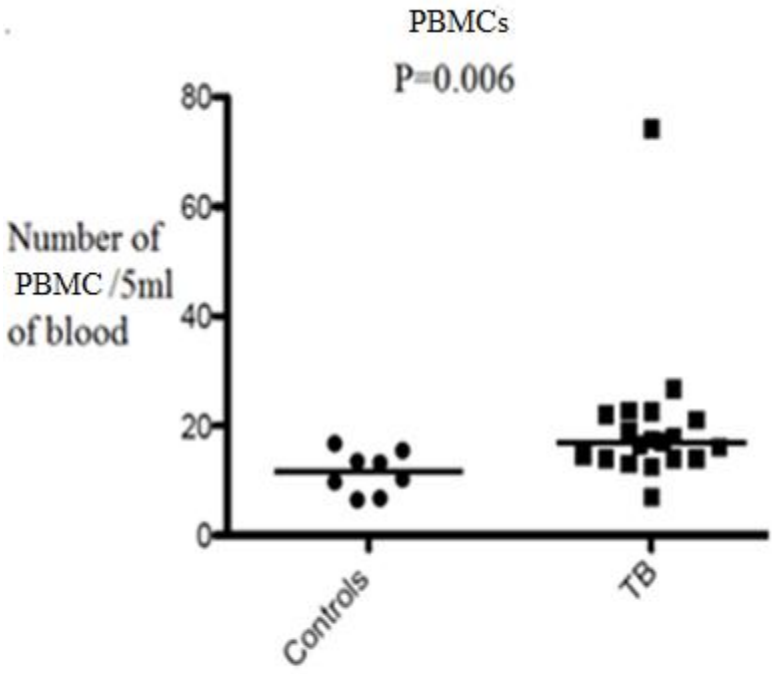

Figure 1.PBMC count of TB patients (n=18) and healthy controls (n=8)

3.3. Arginase assays

Arginase activities in PBMCs isolated from HIV sero-negative smear positive Tuberculosis patients.

To assess the level of arginase activity in treatment naïve smear positive TB patients, 18 HIV sero-negative smear positive TB patients were recruited, and arginase activities were measured by enzymatic assay in PBMCs and compared to those of 8 healthy controls. As shown in the (Table 2 and figure 2), the arginase activity in the PBMCs isolated from smear positive TB patients was significantly higher (median concentration ± SEM, 12.15 ±1.45 mU / 10^6 cells, p= 0.015) as compared to controls (median concentration ± SEM, 5.22 ±1.14 mU/10^6 cells).

Levels of arginase activity in plasma isolated from HIV sero-negative smear positive TB patients

To determine whether arginase activities can be measured in the plasma of smear positive TB patients, plasma samples from 18 HIV sero-negative smear positive TB patients were collected and arginase activities were measured and compared to those of 8 healthy controls. As shown in (Table 2 and figure 2), the arginase activity in the plasma of TB patients was higher than the activity seen in the plasma from smear negative control , but the difference was not statistically significant(median concentration ± SEM, 19.89 ±4.12 vs 13.59 ±8.52 mu/ml of plasma; p=0.211).

Activity of arginase in the saliva of HIV sero-negative smear positive TB patients

To evaluate the enzyme activity in the saliva of HIV sero-negative smear positive TB patients and healthy controls, arginase was assayed in saliva taken from both 18TB patients and 8 healthy controls. And we have found a statistically significant higher arginase activity in the saliva of TB patients than the activity of arginase seen in the control group (Table 2and figure 2), (median concentration ± SEM, 198.8 ±32.19 vs 127.3 ±30.16 mu/ml of saliva; p=0.008).

Table 2: Arginase activity in PBMCs, plasma and saliva of HIV sero-negative smear positive TP patients and healthy controls

Study groups	NO	**Concentration of arginase (mean ± SD, range values in brackets) in**		
		PBMCs ($mu/10^6$ cells)	Plasma (mu/ml of plasma)	Saliva (mu /ml of saliva)
Controls	8	6.4±3.2 (2.3 – 11.6)	22.6±24.1 (3.7– 78.1)	141.1 ± 85.3 (63.6 – 303.1)
TB Patients	18	12.6±6.2 (3.6–24.1)	27.2±17.5 (6.3– 71.1)	227.8 ± 136.6 (74.2 – 564.8)

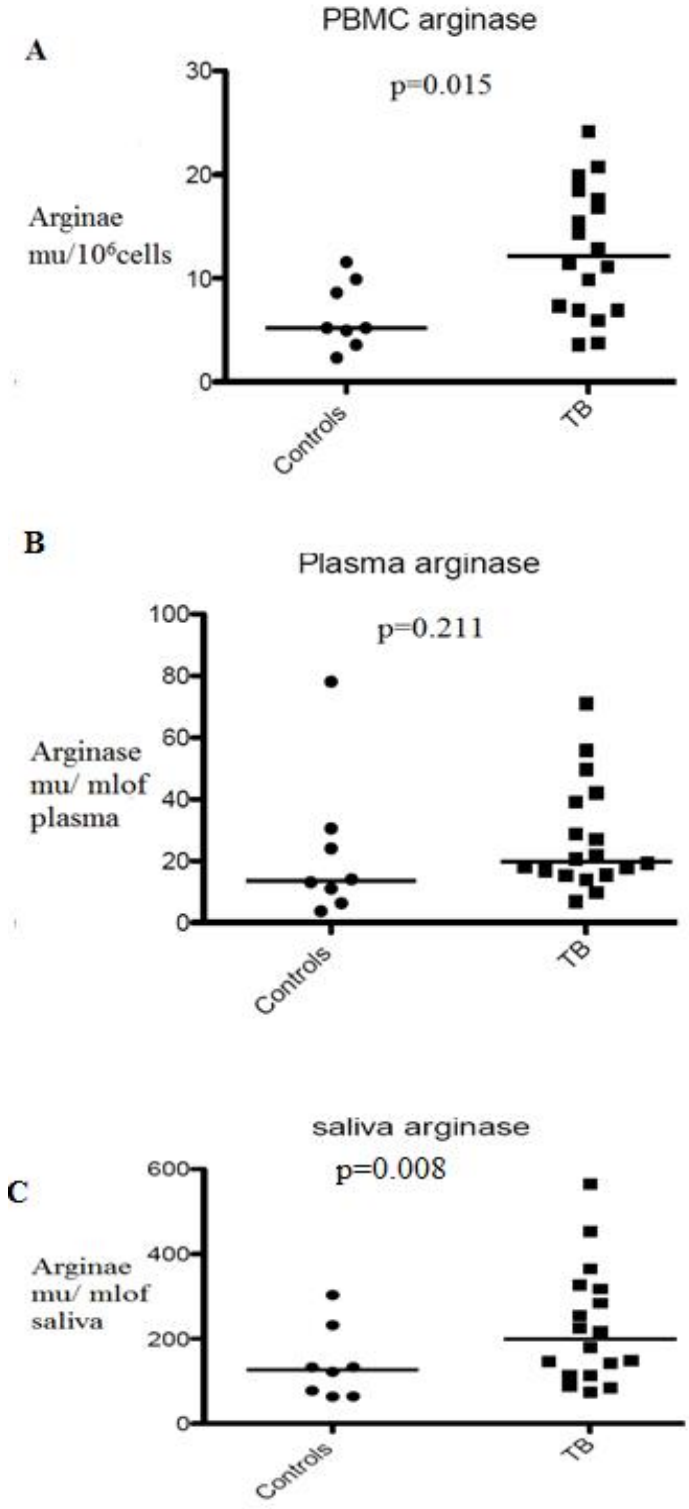

Figure 2. Level of arginase activity in the PBMC (A),plasma(B) and saliva (C) of TB patients(18) and healthy controls(8).

3.4. Phenotype of arginase-expressing cells

The phenotype of arginase-expressing cells was identified by flow cytometry (FACS) as $CD15^{+}CD14^{-}$ in the PBMCs of HIV sero-negative and smear positive TB patients and controls subjects using extracellular and intracellular markers. PBMCs from 6 smear positive TB patients and 8 smear negative controls were tested. The frequency of CD15+ arginase+ cells in the PBMCs of smear positive TB patients was found to be significantly higher as compared to healthy controls (mean ± SD, 18.8 ± 9.9 vs 6.03 ±1.83 respectively, p=0.024) (Table 3).The result shown in (figure 3) is one representative experiment of six independent experiments which indicates a higher frequency of occurrence of $CD15^{+}CD14^{-}$ arginase expressing cells in the PBMCs from HIV sero-negative smear positive TB patients than healthy controls, that is 22.5% and 4.6% of the gated cells were $CD15^{+}CD14^{-}$ arginase expressing cells respectively.

Table 3: The phenotype of arginase-expressing cells in the PBMCs from patients and healthy controls.

Controls & patients identification number	**Percentage of $CD15^{+}$ cells**	
	Controls	**TB patients**
00 1	4.6	21.1
002	6.9	9.0
00 3	6.4	9.0
00 4	2.4	35.2
00 5	5.6	15.8
00 6	8.3	22.5
00 7	7.0	
00 8	7.0	
Mean	6.03	18.8
Std.Deviation	1.83	9.90
Std.Error	0.65	4.04
P-value	0.024	

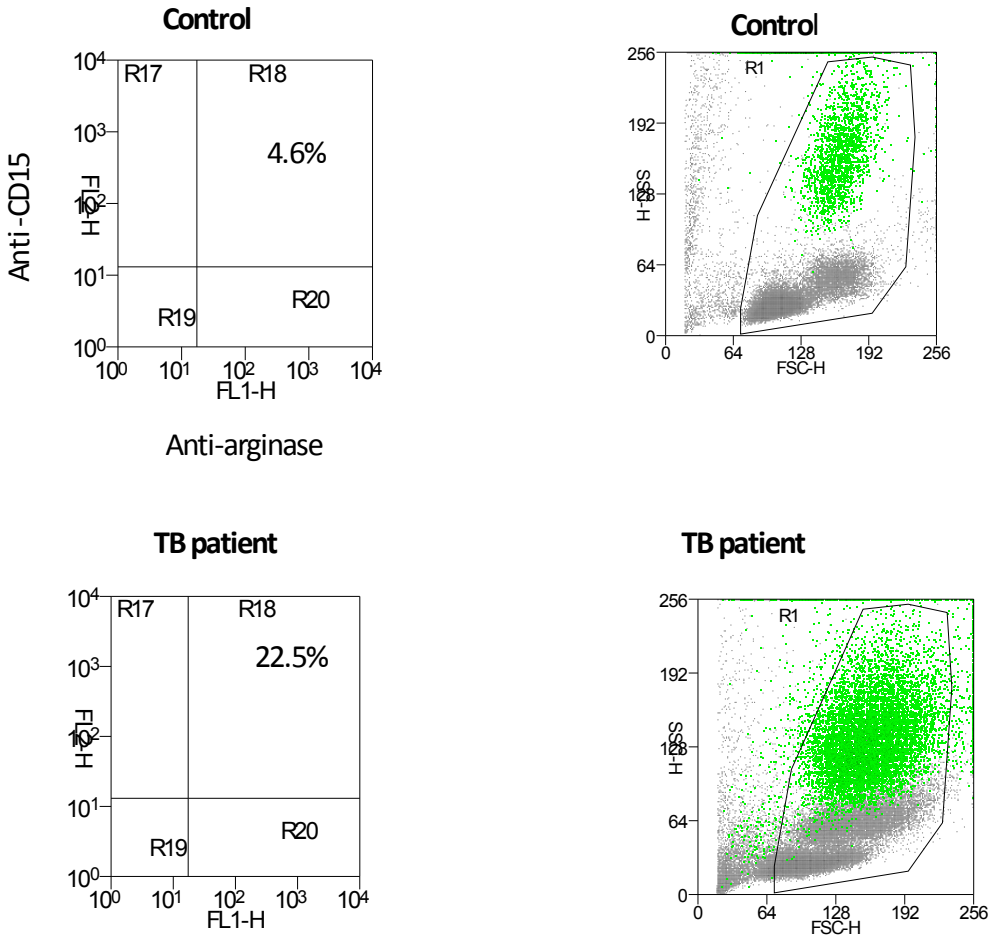

Figure 3. Characterization of the phenotype of arginase expressing cells done in peripheral blood mononuclear cells isolated by density gradient from the blood of seronegative and smear positive TB patients and controls using extracellular and intracellular markers by flow cytometry (FACS).Data are the result of one representative experiment of six independent experiments.

4.0. DISCUSSION

Tuberculosis (TB) is a disease with diverse clinical manifestations which appear to be associated with the level of alteration of the immune response against the TB bacilli. Therefore, understanding the interaction of *Mycobacterium tuberculosis* and its host during infection is very vital in order to design effective therapeutic as well as immune mediated clearance of the bacilli.

The result of this study reveals a statistically significantly higher (p=0.006) PBMCs count in HIV- sero negative smear positive TB patients than healthy controls. The reason behind for this high count of PBMCs in TB patients observed here is probably due to the activation and proliferation of mononuclear cells as a means of body defensive response against mycobacterial infection. Likewise, it is indicated that during *Mycobacterium tuberculosis* infection both innate and cell mediate immune responses are activated which involve the a ctivation and proliferation of different mononuclear cells, such as monocytes, macrophages, dendriticcells, Tcells (CD4 and CD8) and natural killer cells (Braumwald *et al*., 2008).

The finding presented here also shows a higher level of arginase activity in the PBMCs from HIV sero-negative smear positive TB patients than in the PBMCs from health controls. In line with our finding, other studies also indicated the same increased level of arginase activity in the PBMCs from persons infected with *Mycobacterium tuberculosis* (Zea *et al*., 2006) and HIV (Cloke *et al*., 2010). Furthermore, different studies conducted on laboratory mouse infected with *Trypanosoma cruzi* (Bronte *et al*., 2005), *Salmonella typhimurium* (Lahiri *et al*., 2008), Leshmania (Gaur *et al*., 2007) and *Schistosoma mansoni* (Hesse *et al*., 2001), have also shown an increased level of arginase activity which serves as a means of host defense evading mechanisms. That is, arginase is involved in the regulation of immune responses through metabolism and depletion L-arginine, because the presence of sufficient amount of L-arginine in the macrophage allow the inducible nitric oxide synthase (iNOS) to produce more nitric oxide and peroxynitrite molecules, which are the most important effecter molecules during the killing of *Mycobacterium tuberculosis* and other intracellular organisms in the infected host cells (Chul *et al.,* 2009).Furthermore, mycobacterial induced arginase can also control the proliferation and function of T-cells

(Zea *et al*.,2006). That is, deprivation of L-arginine by increased arginase activity is one determinant factor for the hypo responsiveness of T cell during TB infection, because T lymphocytes depend on arginine for proliferation, z-chain peptide and T-cell receptor complex expression, and the development of memory (Popovic *et al*., 2007). In this study we propose that the higher (statistically significant, p=0.015) level of arginase activity seen in the PBMCs of TB patients is probably due to the increased induction of the expression of arginase activity following infection with *Mycobacterium tuberculosis*, because TB bacilli use this increased expression of arginase activity as a means of survival strategy from T cells response by depleting arginine which is important for T cell function. This increased expression of arginase can also be important for *Mycobacterium tuberculosis* to survive from the bactericidal action of NO through depletion of L-arginine by competing with iNOS.

An increased level of arginase activity in pulmonary tuberculosis patients play a central role for the depletion of L-arginine and T cell dysfunction (Zea *et al*., 2006). In the present study an increased level of arginase activity in the plasma of TB patients than healthy controls was observed; however the difference was not statistically significant (p=0.211). This high level of plasma arginase is not unique to TB. The same increased plasma arginase level was also observed in patients with other infectious disease (Munder *et al*., 2005) and renal carcinoma (Paulo *et al.,* 2009) which is contributed by degranulated PMN cells. Thus, the possible reason for increased plasma level of arginase observed in our study might be due to the release of the intracellular arginase from the intracellular compartments of activated and degranulated granulocytes during TB infection. In addition, during microbial infection those arginase expressing granulocytes undergo apoptosis, which is critical for the resolution of the inflammation (Munder *et al*., 2005). A similar mechanism could also probably contribute for the increased level of plasma arginase observed in our study. Furthermore, it has been shown by various studies that both mammalian and pathogenic arginase localized in the intracellular compartments (Das *et al.,* 2010), and are not secreted out. Therefore, in order to metabolize L-arginine, PMN cells of murine do not release their ARG1 rather they will express CAT-2B and increase uptake of L-arginine. Whereas, human PMN cells do not express CAT-2B, instead they release arginase I from intracellular granules into the

microenvironment, where it depletes L-arginine and induces T-cell dysfunction (Paulo *et al.,* 2009).This might also be one of the reasons for increased plasma arginase level in this study.

Here we report for the first time to our knowledge, the presence of a statistically significantly (p=0.008) increased level of arginase activity in the saliva of TB patients than in the healthy controls. Similarly, an increased level of salivary arginase activity has been reported in the saliva of patients with periodontal diseases, which have resulted less NO production and susceptibility to bacterial infection and it came down after antibiotic treatment (Ozer *et al*., 2011). The increased level of salivary arginase activity observed in TB patients here might also be a good strategy to limit the production of NO and survive from the host immune responses. However, because of the limited scope of the present study it might be difficult to draw a conclusion beyond the presence of enhanced salivary arginase activity following TB infection, since the type of cells producing salivary arginase, their relationship with TB infection and how they are stimulated during TB infection and the role of salivary arginase activity for pathogenesis of *Mycobacterium tuberculosis*, requires further work. Furthermore, since the liver, blood cells and salivary arginase is coded by the same gene (Konarska *et al.,* 1985) and saliva is simpler to collect than blood, the increased salivary arginase activity in TB patients perhaps might be used as an important biomarker for the diagnosis of TB infection.

We have identified the relatively higher (p=0.024) proportion of arginase expressing cells observed in the PBMCs of TB patients than in healthy controls as CD15+ neutrophils. In agreement with our finding, other studies conducted on patients with HIV (Cloke *et al*., 2010) and renal cell carcinoma (Paulo *et al.,* 2009) reported the presence of high proportion of $CD15^{+}$ neutrophils in their PBMCs and the correlation of these cells with the increased level of arginase activity. In addition, it is indicated that human peripheral blood neutrophils are the predominant arginase expressing cells, which constitutively express high amount of arginase-I within their intracellular granules (Cloke *et al*., 2010 and Munder *et al*., 2005) and when they are activated they have a tendency to co purify with the PBMC fraction in the peripheral blood samples, therefore are called low density granulocytes (LDGs) (Cloke *et al*., 2010). Likewise, the higher proportion of arginase expressing cells observed in this

study probably due to the activation of these cells as a consequence of TB infection and thereby they might contribute for the enhanced arginase activity seen in the PBMC and plasma of TB patients and consequently suppresses T cell activation, that is the degranulation or death of these cells results the release of their arginase into the micromilieu where it binds to L-arginine and reducing its bioavailability for transport into other cells. The impairment of T cell response by enhanced arginase activity of TB patients has already been demonstrated (Zea *et al*., 2006).

5. CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

The present study concluded that infection with *Mycobacterium tuberculosis* results in higher PBMC count and higher frequency of $CD15^{+}CD14^{-}$ arginase expressing cells along with increased level of arginase activity in the PBMCs, saliva and plasma of pulmonary TB patients compared to healthy controls. Therefore, *Mycobacterium tuberculosis* might induce the production of high arginase expressing cells as well as high arginase activity in the saliva, PBMC and plasma of TB patients as one means of survival strategy from host immune defense by limiting the production of NO and impairing of T-cell function. That is, arginase-induced deprivation of L-arginine might impact negatively on both the production of NO and T cell responses:

- In the absence of L-arginine, the levels of NO are reduced, thereby resulting in less efficient killing of the bacteria
- T cells unconditionally require L-arginine for effective activation, therefore, in the absence of L-arginine, T cells responses will be hampered

5.2. RECOMMENDATION

The following recommendations are forwarded based on the finding of this study:

- A better understanding of the relationship between the stages of TB infection and level of salivary arginase activity might enable us to use saliva instead of blood to measure arginase activity as one diagnostic and severity marker for TB infection. Therefore, further work should be considered so as to find out:
  - the type of cells producing salivary arginase during TB infection
  - the molecular mechanism of arginase production by these cells
  - the relationship between the activation of these cells and TB infection
  - Whether these cells are affected by other oral infectious and non infectious organism and other pathological conditions.
  - the role of salivary arginase activity for pathogenesis of MTB
- Furthermore, large scale study should also be considered in order to set the cut value for normal level of salivary arginase.

5.3. Limitations of the study

During the study few limitations were faced, such as:

- Unable to measure plasma L-arginine level
- Unable to compare the expression of CD3 zeta between TB patients and health controls and with their level of arginase in saliva, PBMCs and plasma.
- Lack of PPD test (Tuberculin skin test) which limit the criterion for health control selection

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7. APPENDIXES

I. DECLARATION

The work provided in this thesis is the researcher's own original research work and has not been submitted elsewhere for any other degree or qualification.

M.Sc candidate: Yayehyirad Tassachew (B.Sc.)

Signature:…….

Date of submission……..

The work provided in this thesis is the researcher's own work, and we confirm that the research has been conducted under supervision and completed as per the condition of the technical and ethical requirements needed.

1. Supervisor: Mr. Tamrat Abebe
   Signature: _____
   Date and place _____
   Addis Ababa, Ethiopia
2. Supervisor: Dr. Adane Mihret
   Signature: _____
   Date and place _____
   Addis Ababa, Ethiopia
3. Supervisor: Dr. Pascale kropf
   Signature _____
   Date and place _____
   London, UK

II. INFORMATION SHEET

You are kindly invited to participate in this study, which involves about 18 smear positive PTB patients and 8 healthy controls in Addis Ababa. The title of the study is "A comparison of arginase activity in the PBMCs, saliva and plasma of HIV sero negative smear positive TB patients in Addis Ababa, Ethiopia. The aim of this study is to compare the level of ARG activity in the PBMC, saliva and plasma of smear positive TB patients and healthy controls. Our hypothesis is that Patients with active PTB has increased level of arginase activity. There for this study will compare the arginase activity in the blood and saliva of smear positive TB patients so as to use arginase activity in the saliva as a diagnostic marker of TB infection.

Purpose: the purpose of this study is to compare the level of ARG activity in the PBMC, saliva and plasma of smear positive TB patients in Addis Ababa, Ethiopia.

Duration: the duration of this study depend upon the availability of study subjects. it can probably take about three months or more.

Procedures to be carried on: the procedure of sample collection is easy and straight forward; 20 ml of blood will be collected using butter fly needle and vacutainer tube and also 500-1000µl of saliva by spiting in a tube by attending physician/health officer/nurse/lab. Technologist and then it will be analyzed in the research laboratory at Tikur Anbesa Hospital.

Risk and discomfort: There will not be any risk associated during sample collection without little discomfort.

Expected benefits: From this study you may not be directly benefited however; the result (increased salivary arginase activity) can be used for diagnosis and treatment follow up of other TB patients in the future.

Confidentiality: All your personal information collected for the purpose of the present study will be kept confidential.

Compensation: No compensation will be provided for participating in this study.

Termination of the study: Participation in the study is voluntary, and refusal to participate involves no penalty or loss of benefits to which you are otherwise entitled. The study

participants have a right to Keep hold information; decline to cooperate in the study or to refuse provision of specimens

I would also like to inform you that this study will be approved by Department Ethical and Review Committee and ethically cleared by Institutional Review Board (IRB), Faculty of Medicine Addis Ababa University. If you have any question about the right of the study participant the address is:

Faculty of Medicine Addis Ababa University

Office of Associate Dean, Postgraduate Programs and Research

P.O. Box 9086. Addis Ababa, Ethiopia

Tel. 251-011-551-28-765

If you have question about the study the address of the principal investigator is:

Yayehyirad Tassachew

Department of Microbiology, Immunology and Parasitology

Faculty of Medicine, Addis Ababa University

P.O. Box. 9086, Addis Ababa, Ethiopia

Tel: 0916829464

II. መረጃ መስጫ

የጥናቱ አላማ ፡- በሳንባ ነቀርሳ በሽተኞች ምራቅና ደም ውስጥ የሚገኘውን አርጅኔዝ የሚባለውን ኢንዛይም መጠን ለመለካትና ለማወዳደር ነው

የሚፈጀው ጊዜ፡- እንደሚገኙት የጥናቱ ተሳታፊዎች መጠን ጥናቱ 3 ወር ወይም ከዚያ በላይ ሊፈጅ ይችላል፡፡

አጠቃቀም፡- ለጥናቱ የሚሆን 20 ml ደምና ከ500 – 1000 µl ምራቅ በወቅቱ የጥናቱን ተሳታፊ የሚከታተለውን ሃኪም /ጤና መኮንን/ ነርስ /ላብራቶሪ ቴክኖሎጂስት አነስተኛ መርፌና ቢልቃጥ በመጠቀም ይሰበስባል፡፡

ሊደርስ የሚችል አደጋ፡- በናሙና አወሳሰድ ወቅት ከጥቂት ምቾት መቀነስ በስተቀር ምንም አይነት አደጋ አይኖረውም፡፡

የሚያገኙት ጥቅም፡- ከዚህ ጥናት እርስዎ በቀጥታ ላይጠቀሙ ይችላሉ፡፡ ነገር ግን በሳንባ ነቀርሳ በሽተኞች ምራቅ ውስጥ የሚገኘው አርጅኔዝ ኢንዛይም መጠን ከፍ ያለ እና በደማቸው ውስጥ ከሚገኘው አርጅኔዝ መጠን እኩል ከሆነ ወደፊት ለሌሎች የሳንባ ነቀርሳ በሽተኞች እንደበሽታ አመላካችነትና የሚሰጠው የሳንባ ነቀርሳ ህክምና በበሽታው ላይ ያመጣውን ሰውጥ ለመከታተል ይጠቅማል፡፡

ሚስጢራዊነት፡- ለጥናቱ ሲባል የሰጧቸው መረጃዎች ሁሉ ሚስጢራዊነታቸው የተጠበቀ ይሆናል፡፡

ማካካሻ፡- ጥናት ውስጥ በመሳተፍዎ የሚያገኙት ምንም አይነት ማካካሻ የለም፡፡

ፍቃደኝነትዎን ስለማቋረጥ፡- ጥናቱ ውስጥ የሚሳተፉት ፍቃደኛ ሲሆኑ ብቻ ነው፡፡ ጥናቱ ውስጥ ለመሳተፍ ፍቃደኛ ባይሆኑ የሚደርስብዎት ቅጣትም ሆነ ሊያገኙ ሲገባ የሚያጡት ጥቅም የለም፡፡ ማንኛውም የጥናቱ ተሳታፊ መረጃ ያለመስጠት፤ ከጥናቱ የማፈግፈግ እና ናሙና ያለመስጠት መብቱ የተጠበቀ ነው፡፡ በተጨማሪም ጥናቱ ከትምህርት ክፍሉ ኤቲካል ሪቪው ኮሚቴ እና ከአዲስ አበባ ዩኒቨርስቲ ህክምና ፋኩሊቲ ማረጋገጫ የተሰጠው ሲሆን ማንኛውም አይነት የጥናቱን ተሳታፊዎች መብት በተመለከተ ጥያቄ ቢኖርዎ በሚቀጥለው አድራሻ መጠየቅ ይችላሉ፡-

ለ አዲስ አበባ ዩኒቨርስቲ

ህክምና ፋኩሊቲ ተባባሪ ዲን፤ ድህረ ምረቃትና ምርምር ክፍል

የፖስታ ሳጥን ቁ. 9086

አ.አ ኢትዮጵያ

ስልክ፡ 251-011-551-28 765

ጥናቱን በተመለከተ ማንኛውም አይነት ጥያቄ ሲኖር የዋናውን ተመራማሪ አድራሻ በመጠቀም ይጠይቁ፡-

ለ ያየህይራድ ጣሳቸው፤

አዲስ አበባ ዩኒቨርስቲ

ህክምና ፋኩሊቲ

ማይክሮ ባዮሎጂ፤ ኢሚኖሎጂ እና ፓራሳይቶሎጂ ትምህርት ክፍል

የፖስታ ሳጥን ቁ. 9086

ስልክ፡ 0916-82 94 64

III. CONSENT FORM Serial no…….

Card no…..……

Name of study participant: _____

I have been requested to participate in this study which involves collecting of 20 ml of blood and 500-1000 µl of saliva. During collection of the specimen I have told that there is no harm without little discomfort I have also read the information sheet (or it has been read to me); I have understood that this study is about arginase activity in the saliva and blood of smear positive pulmonary tuberculosis patients at Health centers, Addis Ababa, Ethiopia in which TB victims like me will be benefited from the findings. I have asked some questions and clarification has been given to me. I have given my consent freely to participate in the study, and I herby to approve my agreement with my signature.

Participants signature _____Date_____

Investigateurs signature _____ Date_____

Witness signature 1. _____Date _____

III. የመረጃ ቅጽ

የጥናቱ ተሳታፊ ስም …… ተ.ቁ ……

የካርድ ቁ. ……

በጥናቱ ውስጥ እንድሳተፍ ጥያቄ ቀርቦልኝ ለጥናቱም የሚሆን 20ml ደምና ከ500 – 1000 µl የምራቅ ናሙና እንደሚያስፈልግ ተገልፆልኛል፡፡ የናሙና አወሳሰዱ ሂደት ከትንሽ ምቾት መቀነስ በስተቀር ምንም አይነት ጉዳት እንደማያስከትልብኝ ተነግሮኛል፡፡ በተጨማሪም መረጃ ለመስጫ የተዘጋጀውን ወረቀት በተገቢው መንገድ አንብቤያለሁ (ተነቦልናል)፡፡ ጥናቱ በጤና ጣቢያ ህክምና ለመውሰድ በሚመጡ የሳንባ ነቀርሳ በሽተኞች ደምና ምራቅ ውስጥ ያለውን አርጅኒየስ ኢንዛይም መጠን ለመለካትና ለማወዳደር እንደሆነና እንደእኔ ያሉ የሳንባ ነቀርሳ ተጠቂዎች ከጥናቱ ግኝት ሊጠቀሙ እንደሚችሉ ተረድቻለሁ፡፡ በተጨማሪም አንዳንድ ያልገቡኝን ነገሮች ጠይቄ ተገቢ ማብራሪያ ተሰጥቶኛል፡፡ ስለሆነም በጥናቱ ውስጥ በነፃ ለመሳተፍ ፍቃደኛ መሆኔን በፊርማዬ አረጋግጣለሁ፡፡

የጥናቱ ተሳታፊ ፊርማ …….. ቀን ……..

የተመራማሪው ፊርማ ……. ቀን ……..

የምስክሮች ፊርማ 1ኛ. …… ቀን ……..

IV. Questionnaire

This questionnaire is for the investigation of arginase activity in the PBMC, saliva and plasma from active pulmonary tuberculosis patients in Addis Ababa, Ethiopia

Participant Identification

1. Serial No……... 2. Card no…….
3. Address …….. 4. Participant name……
5. Age……sex……. 6. Ethnicity……....
7. Marital status…….. 8. Occupation……

V. Reagent preparation

Lysis Buffer

1. 20ml 0.1% triton
2. 20ml 50mM tris-HCl at pH7.5
3. 2ml 100mM manganese chloride (previously in cold room, now in fridge)
4. into labelled centrifuge tube
5. Vortex
6. Store in fridge

6% ISPF

1. 3g ISPF 2
2. 50ml absolute ethanol
3. Vortex
4. Store in fridge with foil

0.5M Acid Mix

1. 320ml dH_2O
2. 135ml phosphoric acid
3. 45ml sulphuric acid
4. Mix slowly in fume cupboard on iced dH_2O – mix on ice
5. Keep an eye on warmth of bottle – stop if mixture warms too much

VI. Sample preparetion

Blood Plasma Isolation

1. Collect 20 ml of Venus blood in two EDTA vacutainer tubes
2. Centrifuge EDTA anti-coagulated blood at 1800rpm for 10 min.
3. Then, harvest 1-2 ml of plasma by transferring with a clean pasture pipette to a labeled tube and frozen until required .

Blood Ficoll

1. Pipette 20ml ficoll into a 50ml centrifuge tube
2. Slowly pipette 20ml blood on top of ficoll (slant tube when dispensing blood)
3. Centrifuge at 1900rpm with no brake for 20 minutes at room temp.
4. using a sterile pipette collect the interphase (collecting as little ficoll as possible)
5. Wash the interphase with PBS (fill centrifuge tube (up o 50ml))
6. Centrifuge 1800rpm for 10 minutes at room temp.
7. Pour away the supernatant
8. Repeat the PBS wash
9. Pour away the supernatant
10. And re-suspend in 300µl lysis buffer
11. Then the cell lysate frozen at -20°C until further use

VII. PBMC count

1. Stain the washed cells by mixing 90 µl of PBMCs with 10 µl of trypan blue solution by making making a 1:10 dilution.
2. Then charge the stained PBMCs in to hemocytometer chamber
3. And count the PBMCs with a 10 X objective and eye piece of light microscope.
4. Then the number of PBMCs counted are calculated using the following formula

   PBMC count = Number of cells counted x 10^4 (the volume of the chamber) x DF

VIII. Arginase Assay

1. Defrost sample, e.g. saliva
2. Vortex defrosted sample and allow settling
3. Pipette 25µl footpad supernatant into a 0.5ml eppendorf
4. Pipette and add 25µl lysis buffer to each sample.
5. Vortex (can leave at 4°C if necessary)
6. using a thermal cycler, incubate at 56°C for 7 minutes – this activates the arginase present in the footpad samples
7. Take an aliquot of L-arginine (0.5M at pH9.7) from the freezer
8. Pipette 50µl L-arginine into the incubated footpads
9. Vortex
10. using the same thermal cycler incubate at 37°C for 60 minutes. Incubation can range from 15-120 minutes depending on amount of arginase present
11. Pipette and add 400µl acid mixture to stop the reaction (lab coat, gloves and GOGGLES!!!!) (Can leave at 4°C if necessary).
12. In 5 new 0.5ml eppendorfs, pipette MilliQ water and urea standard:

Tube number	**MilliQ Water (µl)**	**Urea Volume (µl)**	**Urea Conc. (µg)**
1.	96.875	3.125	3.75
2.	93.75	6.25	7.5
3	87.5	12.5	15
4	75.0	25.0	30.0
5	50.0	50.0	60.0
6	25.0	75.0	90.0
7	0.0	100.0	120.0

13. Pipette and add 400µl acid mixture to the urea standards (GOGGLES!!!!) (Can leave at 4°C if necessary) .
14. Pipette 20µl 6% ISPF to ALL tubes.

15. VORTEX TUBES EXTREMELY WELL until the phases are mixed – this is EXTREMELY IMPORTANT!

16. using the same thermal cycler, incubate all samples and standards at 100°C for 30 minutes using a weight on top of the tubes

17. Pipette 200µl of urea standard and experiment onto a 96 flat bottom well plate.

18. Read the 96 well plate at 550nm

19. Rinse plate under running water and put into non-contaminated waste bin

20. Discard all tubes into acid bin.

IX. FACS Protocol for phenotyping of arginase expressing cells

1. Label FACS tubes and adds 150 µl of FCS / staining buffer to each tube.
2. Distribute 150 µl of washed PBMC to each tube.
3. Label the cells with anti-CD15 and anti-CD14 monoclonal antibodies (surface markers), vortex and incubate at 4^0C for 20 minutes.
4. Wash the cells with PBS at 1800 rpm for 5 minutes.
5. Then, fix the cells with 500 µl of cold PBS and then 500µl of 4% formaldehyde and vortex and incubate at 4^0C for 20 minutes.
6. Wash the cells with PBS or appropriate buffer at 1900 rpm for 5 minutes
7. Permeabilize cells with 1ml of appropriate buffer at 1900 rpm for 5 minutes.
8. Wash the cells with PBS at 1900 rpm for 5 minutes.
9. Label the cells with intra cellular antibodies (FITC-conjugated anti-arginase monoclonal antibodies), vortex well and incubate in dark for 15 minutes.
10. Wash the cells with PBS or appropriate buffer at 1900 rpm for 15 minutes.
11. Resuspend cells with 250µl of staining buffer.
12. Run FACS.