Effect of Diabetes Mellitus on Effector T-Cell Immune Response among Active Pulmonary Tuberculosis Patients in Addis Ababa, Ethiopia

By

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Abstract

Background: -*The merging epidemics of tuberculosis (TB) and diabetes mellitus (DM) become major causes of morbidity and mortality throughout the world. Despite the clinical and public health significance posed by the dual burden of the two disease worldwide, there is little evidence on the prevalence of DM among pulmonary tuberculosis patients in Ethiopia, and very little is known about the immunological relationship of DM and TB. Accordingly, the objective is devised to determine the prevalence and the effect of DM on T-cell immune response among active pulmonary tuberculosis (PTB) patients in Addis Ababa, Ethiopia.*

***Methods**: - A comparative cross sectional study was conducted and consecutive screening of 205 active PTB cases for DM was done from June 2014-February 2015. Socio demographic and clinical data were collected using structured questionnaire. Blood was collected from 17 PTB with diabetic co-incidence, and 20 PTB patients for T cell phenotyping. Flow cytometry was used to determine the frequency of memory and T regulatory cells. Cross tab, logistic regression and Mann–Whitney test were done using SPSS V22 and Graph pad prism V5.03. P value < 0.05 was taken as statistically significant.*

***Results**: - The overall prevalence of DM and Impaired Fasting Glucose was 8.3 % and 26%, respectively. BMI with > 25 kg/m^2 (p=0. 000), alcohol drinking (* OR 2.942 and 95% CI [1.077-8.035]*) and smear positive PTB (*OR 3.036 and 95% CI [1.029-8.961]) *had a statistical association with increased occurrence of DM. PTB coincident with DM is characterized by the increased percentage of activated T cell (Median, 3.360; IQR, 2.515-3.910 vs 2.605 IQR 2.130-3.078, (p=0. 0373)) and T regulatory cells* (*Median, 3.660; IQR, 2.445 -4.010 vs 2.165; IQR, 1.943-2.858, (p=0. 0174)). Poor glycemic control (95 % CI; 0.0793 to 0.8205, p=0. 0221) was also correlated with an increased activated and T regulatory cell percentage. There was no significant difference in CD4$^+$, CD8$^+$ and CD45RO + memory cells.*

***Conclusion and recommendation**: - The present study has shown a higher prevalence of DM than the estimated prevalence in the general population. PTB with coincident DM was characterized by elevated T activated and T regulatory cell indicating that immune alteration is associated with expansion of these cells in favor pathogen survival. Further studies with a comprehensive study design are warranted to get a representative figure and functionality assay of pathogen specific Tregulatory cells in TB-DM patients should be done.*

***Key word**: Diabetes mellitus. Pulmonary Tuberculosis, T regulatory cells, Memory T cells.*

Table of Contents

Contents **Page**

List of Tables

List of Figures

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Abbreviations and Acronyms

AFB: Acid Fast Bacilli

AHRI: Armauer Hansen Research Institute

ALERT: All African Leprosy Rehabilitation and Training center

CD: Cluster of Differentiation

CFP-10: Culture Filtrate Protein-10

DM: Diabetes mellitus

ESAT-6: Early Secreted Antigen Target -6

FBG: Fasting Blood Glucose

FBS: Fetal Bovine Serum

FoxP3: Forked head box P3

HbA1C: Hemoglobin A1C

HDL: High density lipoprotein

HIV: Human Immunodeficiency Virus

IDF: International Diabetes Federation

IFN: Interferon

IL: Interleukin

LDL: Low density lipoprotien

M. tuberculosis : Mycobacterium tuberculosis

PBMC: Peripheral Blood Mononuclear Cells

PBS: Phosphate Buffered Saline

PPD: Purified Protein Derivatives

RPMI: Roswell Park Memorial Institute

TB: Tuberculosis

TGF: Transforming growth Factor

Th: T-helper cells

TNF: Tumor Necrosis Factor

Treg: T regulatory cell

STZ: Streptozotocine

WHO: World Health Organization

Chapter One

1. Introduction

1.1. Background

Tuberculosis (TB) is the leading cause of death in the world from a bacterial infectious disease. It is caused by a genus Mycobacterium and member of mycobacterium complex especially *Mycobacterium tuberculosis* is the main etiological agent of disease in human (1). It is a fairly large, non-motile rod-shaped, acid fast, obligate aerobe, facultative intracellular parasite usually in macrophages, and has a slow generation time in the ranges between 15 to 20 hours. It typically affects the lungs (pulmonary TB) but can affect other sites as well (extra pulmonary TB) (2, 3).

M. tuberculosis usually enters the alveolar passages of exposed humans in an aerosol droplet, where its first contact is with resident macrophages, but it is also possible that bacteria can be initially ingested by alveolar epithelial type II pneumocytes (4, 5). Alveolar epithelial cells may contribute to the local inflammatory response in human tuberculosis by producing chemokines monocyte chemotactic protein-1 and interleukin-8 (IL-8) and attract monocytes, lymphocytes, and polymorphonuclear cells (6, 7). *M. tuberculosis* can persist for decades within the granuloma structures and, due to some intervening medical case (e.g., HIV infection, diabetes mellitus (DM), cancer, malnutrition, aging, etc.), genetic and environmental factors, bacteria can reactivate (8)

The relationship between DM and tuberculosis posited to be bidirectional; people with DM are more susceptible to tuberculosis and tuberculosis also affects DM by causing hyperglycemia, causing impaired glucose tolerance; impaired glucose tolerance is the major risk factors for developing DM (9). People with DM are more susceptible to infections and suffer from relatively severe illness due to their immunosuppressed status, with reactivation of older foci of TB rather than through fresh contact (10).

1.2. Statement of the problem

Tuberculosis remains the major public health concern, with an estimated one third of the world's population infected with *M. tuberculosis*, intensified due to DM and HIV and other pandemic providing a large reservoir of highly susceptible individuals (11). According to 2014 WHO report, in the world there were 9 million new cases of TB: Ethiopia ranks 10^{th} among 22 tuberculosis high burden countries (12) ,and tuberculosis have been also reported as most frequent co-morbidity in Ethiopian increasing DM population (13- 15)

DM is recognized to increase the risk of person developing tuberculosis by a threefold, fourfold higher risk of death and relapse during TB treatment, and slow smear conversion (13,16). Moreover, frequent treatment failure and multi drug resistance tuberculosis are being more associated with devastating phenomenon of the increasing epidemic of DM (16-18). This link may become even more meaningful in coming years, as the prevalence of DM is expected to rise dramatically in the resource-poor areas where TB thrives, including Ethiopia.

It could be one reason why those efforts to reduce the global incidence of tuberculosis are having little effects despite high case detection rates and cure rate (17,19). There are many challenges in screening and diagnosis of tuberculosis in DM patient and vice versa; as the two disease managed in two different clinics. According to current estimate; failure to address the DM epidemics could offset tuberculosis rate by 8% (20). Although there have been few studies conducted on the prevalence of DM in different part of Ethiopia, they mainly focus either on general population or tuberculosis other than active pulmonary tuberculosis (PTB),so this study was planned to assess the prevalence of DM among HIV negative active PTB patients in Addis Ababa.

Despite the clinical and the global public health significance posed by the dual burden of tuberculosis and DM, very little is known about the immunological mechanisms of susceptibility. Different literatures with different model suggested inconsistent conclusion on immunological profile in relation with DM. Some suggested that type 1 DM pathogenesis is related with imbalanced expansion between effector T cell especially T 17 and low non-defective T regulatory cells (Tregs) (21). In the other study it's been showed there is functional defect and

turn off FoxP3 expression in certain Tregs and lose the control of Th17 expansion (21).To the contrary, *M. tuberculosis* also shown in to induce Myeloid-derived suppressor cells (22) and pathogen specific Tregs that antagonize the inflammatory Th1 cell differentiation and recruitment which could induce disease progression (24).

Conflicting findings in profile of T cell cytokines are published with; impaired host resistance against *M. tuberculosis* infection in diabetic mice is associated with reduced or delayed production of Th1-related cytokines (IL-12 and IFN- γ) and impaired development of Th1 cells (25). In humans, conflicting reports have appeared which suggest no difference and lower ratio of IFN γ/IL-4, Treg production, (26) or higher production of IFN- γ by diabetic than non-diabetic TB patients (27).

We hypothesized that the significant differences in clinical symptoms as well as effects of treatment, in patients with DM, particularly when they develop TB, might be related to an imbalance b/n T reg and effector T cell response in these patients. There is still inadequate information concerning the relationships between Effector T cell/Treg cells in DM and tuberculosis co-morbidity. So this study was designed to explore further the effect of DM on T cell immunological response in pulmonary tuberculosis (PTB) patients in the setting with highly endemic tuberculosis and DM such as Ethiopia.

1.3. Significance of the study

TB is an indiscriminate disease affecting people all over the world. Globally a third of the world's population is thought to be infected. DM has received increasing attention as a risk factor for TB. It weakens the immune system making it easier for people to succumb to infection by TB, increasing risk by two to three times. But what does this actually mean in countries such as Ethiopia where the incidence of TB is high? This becomes a real challenging issue. People with DM are also more likely to progress from latent to active TB. But the immunological mechanism of this susceptibility is still unknown.

It is therefore important to know the prevalence of DM in persons with tuberculosis and understand the relationship between host responses and disease to identify which T cell populations are involved in disease processes. So the study findings will be important for vaccine development, design of therapeutic and for policy makers in design of diagnosis approach in the control of tuberculosis particularly in DM. The study will also provide a platform for longitudinal studies examining the role of immunological biomarkers in the development of tuberculosis in DM patients and data on prevalence of DM among PTB patient in TB endemic areas like Ethiopia.

Chapter Two

2. Literature review

1.2. Epidemiology of diabetes and tuberculosis co-morbidity

In the setting of the increasing overlap of population at risk for DM and TB, the combination of these diseases represents a worldwide health threat. Worldwide there were 9 million new cases of TB: 5.7 million were individuals newly diagnosed in 2013 and 0.4 million were previously diagnosed TB patients (28). DM currently affects at least 5% of the world's population, especially people of working age group, which is a serious risk to the economic potential of the countries. The number of people with DM in the world was 366 million people in 2011; by 2030 this will climb to 552 million, according to the International Diabetes Federation (29). Eight of the ten countries with the highest incidence of DM worldwide are also classified as high burden countries for TB by the World Health Organization (30).

The duo relationships between TB and DM was recognized long time ago, but recently abundant evidence of high rates of DM in people with TB and vice versa have appeared. Several studies have looked at the association between DM and tuberculosis both in developed and low and middle income countries (16, 30). According to one modeling study on data from 13 high burden countries, by 2035, the cumulative reduction in tuberculosis incidence would be instead offset by 8.8% and mortality would be 34·0% . Lowering the prevalence of DM by an absolute level of 6·6–13·8% could accelerate the decline of tuberculosis incidence by an absolute level of 11·5–25·2% and tuberculosis mortality by 8·7–19·4% (31). It was also shown by a systematic review and a meta-analysis of 13 observational studies assessing the association of DM and TB, that DM was associated with an increased risk of TB regardless of study design and population (16).

In across sectional assessment of DM in TB patient from Mexico-Texas border, a higher prevalence of 36% in Mexico and 39 % in Texas were reported (32).Similarly from a study in this region on the binomial occurrence of type 2 DM with TB, age ≥35 years previous contact, person infected with tuberculosis, Body Mass Index ≥25 kg/m^2 and inherited family history of DM was associated with the development of drug and multidrug resistance (33). Highest DM

among individuals with tuberculosis foreign-born populations in San Francisco, USA as compared to USA born was reported and patients with co-morbidity were more likely to be male, older than 45 years and born in the Philippines. As indicated, the preexisting exposure to TB and high DM in the migrant is more likely associated with higher rate among this group (34).

In low and middle income countries where DM is sky rocketing on the existing high burden of TB; it is stipulated that five of the top ten countries projected to have the greatest numbers of people living with DM by 2035 are countries with high burdens of TB (29).

In a community based cohort study on the prevalence of type 2 DM among newly diagnosed PTB patients in China 6.3% of DM was reported (35). PTB patients had a higher odds of DM than non-TB controls (adjusted OR 3.17, 95% CI 1.14–8.84) DM. Increased age (> 30 years), (BMI 18.5–23.9), family history of DM, positive sputum smear, and cavity on chest X-ray were significantly associated with DM (35,36). From a cohort study on 'Diabetic Control and Risk of Tuberculosis; DM was associated with a modest increase in the risk of active, culture-confirmed, and pulmonary (with or without extra pulmonary involvement) tuberculosis (35). Correspondingly from state-wide representative sample of TB patients in Kerala, India, 44% had DM which is fairly large prevalence and 23% had previously known DM and 21% were newly diagnosed. Age >50years and male gender were found to be associated with higher prevalence (38).

With the advent of demographic and economic transition in African countries the overwhelming diabetic epidemics poses a serious concern on the existed thrives of TB burden. High prevalence (29.8%) of *M. tuberculosis* infection and disease diagnosed with tuberculin skin test chest x-ray and culture in children and adolescents with type 1 DM was reported in South Africa (39). In a study from Conakry, Guinea; TB patients diagnosed for DM using capillary blood glycaemia test: a prevalence rate of 3.35% (95% CI 1.35–5.35) was reported. The diagnosis of DM preceded that of TB by an average of 5 years (range 1–9 years) (40). A study conducted on newly diagnosed Ugandan adult TB patient 8.5% of patient were having DM and only 1.9% patients with TB had a known diagnosis of DM at enrolment with the majority had type 2 DM (41) and 16.7% prevalence of DM was found in a study in relatively young urban Tanzanian

adults (42).This could give an indication for a bidirectional screening of tuberculosis patients for DM and vice versa.

Despite higher dual disease burden of TB and DM, few studies have been conducted in in different geographic regions of Ethiopia. In a retrospective data in Tikur Anbesa hospital from data analysis of 1352 diabetic patients, 5.8% of cases were diagnosed for tuberculosis using clinical evaluation, chest x-ray, acid fast bacilli in sputum or measures tissue and histopathological characteristic of biopsy specimens. Fever, sweating and cough were the most common symptoms reported (13).

A study from Gondar referral hospital reported 8.5 % DM and 29.6% of impaired fasting glucose on active TB patients and it was higher (11.1%) among male than female participants (4.9%). The proportion of newly diagnosed diabetic cases was 52.9%, and all of them were between 25-44 years of age. Moreover, 28.6% of diagnosed DM patients had concomitant HIV infection. The proportion of DM was 9.6% among smear positive and 8.2% among smear negative cases (14).

In a screening of DM population for PTB using AFB smear microscopy at the Dessie referral hospital, Northeast Ethiopia 8.5% prevalence were reported. History of TB (AOR: 13.4; 95% CI: 2.74–65.73), contact history with known TB patients (AOR: 9.4; 95% CI: 1.822–48.50), and long duration of DM (AOR: 8.89; 95% CI: 1.88–58.12) were independently associated with the development of active TB in people living with DM (44).

In a recent study conducted at St Peter's TB specialized hospital in Addis Ababa which was done with a reverse screening of PTB population for DM, there was 15.8% prevalence of DM and 26.7% impaired fasting glucose. DM was higher among higher age and 25.4% among male than female 3.8% TB patients. In most of the PTB patients diagnosed for DM, HIV was also common concomitant occurrence with 15.9% than HIV negative TB patients 15.8% which could also be attributable to HIV. Higher proportion DM (25%) were observed among the smear positive than smear negative patients (6.7%) (41).

In the past 20 years, the debate over whether DM causes increased susceptibility to tuberculosis, as well as differences in presentation, severity, and response to therapy, has been rekindled. DM

and tuberculosis are found to be dangerous line up in which DM preliminary role with immune impairment and becoming a risk factor to reactivation of latent tuberculosis.

2.2. Immunity in *Mycobacterium tuberculosis* and diabetes mellitus coincidence

Natural infection with *M. tuberculosis* occurs by inhalation of a small number of bacilli that invade and replicate in resident alveolar macrophages, which are the predominant myeloid cell type in the airspace of the healthy lung (46). An enhanced innate immune response is believed to abrogate *M. tuberculosis* infection (negative tuberculin skin test or interferon gamma release assay) 70% of the exposed contacts, but the mechanisms comprising this protective immunity remain a major knowledge gap in the TB field. Among those infected, the most frequent outcome is the progression to a latent TB infection where most mycobacteria are killed and the few that remain viable are in a latent state characterized by altered metabolism and persistence. To reach the equilibrium between infection and immunity the innate cell particularly residential macrophages orchestrate the priming of adaptive immune response with activation of other inflammatory cells, monocytes, myeloid dendritic cells, neutrophils and B cells for the formation of granuloma, hallmark of TB immune response (47). But equilibrium birched by underlying factors, such as DM, HIV, and malnutrition.

Despite the clinical and public health significance posed by the dual burden of tuberculosis and DM, very little is known about the immunological and biochemical mechanisms of susceptibility. A growing body of research suggested that a defective immune response, metabolic effect of fat tissue in DM may contribute to an increased susceptibility (25).This review summarizes studies conducted on both animal models and human studies.

Increased susceptibility to TB in animal models combining TB and DM by assimilating the physiological conditions to DM has been done. Sugawara *et al*. used the Komeda DM-prone rat, as a model of type 1 DM (48) and Goto Kakizaki Rats as type 2 DM (49). The infected Komeda DM Prone rats developed large granulomas without central necrosis in their lungs, liver or spleen significant increase in the number of colony-forming units of *M. tuberculosis.* Alveolar macrophages from DM rats were not fully activated by *M. tuberculosis* infection and did not

secrete nitric oxide that can kill *M. tuberculosis* but no significant difference in phagocytosis of tubercle bacilli by alveolar macrophages was observed between DM and wild type rats. But pulmonary levels of interferon-γ, tumor necrosis factor-α and interleukin-1β mRNAs were higher in the infected KDP DM rats than in wild type rats (48) but the opposite were recorded for Goto Kakizaki Rats (49).

Most, not all animal studies used streptozotocin (STZ) to deplete insulin producing cell to cause hyperglycemia which is the main feature of DM. In one study on (STZ)-induced diabetic mice, resistance to live tubercle bacillus (Schacht strain) challenge were used to look the T cell and monocyte response. At 3 months post infection,>90% of hyperglycemic mice had died versus <10% of the euglycemic controls (50). Yamashiro *et al*. challenged STZ-treated mice with Mtb H37Rv (10^5 CFU i.v.) and found a 0.5 log higher lung bacterial burden at 14 days compared with that in untreated controls, rising to 1.5 logs higher at day 35. The IFN-γ, IL-12 and Nitric Oxide content in lung tissues homogenates was significantly lower in the diabetic group at 14 days, as was IFN-γ production by Mtb antigen Purified Protein Derivative (PPD) and *M. bovis* BCG stimulated splenocytes and peritoneal exudate cells tested at 8 days. Addition of high glucose levels (33 mM) to the cultures of PPD-restimulated spleen cells reduced the synthesis of IFN- γ in diabetic mice, but Insulin treatment starting 2 days after STZ administration resulted in a lower bacterial burden at day 35 (51) .

Similar study was extended by Martens *et al*. (52) on TB susceptibility in STZ-treated C57BL/6. They applied relatively long time to induce the chronic hyperglycemia <4 weeks (acute) or ≥12 weeks (chronic) before low-dose aerosol challenge with *M. tuberculosis* Erdman strain (compared to Yamashiro *et al*. (51). Chronic diabetic mice had peak bacillary burden, pulmonary inflammation, greater absolute numbers of CD4+ and CD8+ T cells, macrophages, neutrophils ,and levels of IFN-γ,IL-1β,and TNF-α in the lung tissue were observed in later 8-16 weeks compared with euglycemic mice. The expression of adaptive immunity was delayed in chronic diabetic mice, shown by reduced early production of IFN-γ in the lung and by the presence of fewer antigens (ESAT-6) responsive T-cells compared with euglycemic mice within the first month of infection. But there was no difference in the expression of inducible nitric oxide synthase between groups (52).

TB susceptibility, like other diabetic complications, results from the cumulative effect of chronic hyper rather than from an immediate impact of elevated blood glucose. As it is observed in 40% sucrose diet-induced hyperglycemic guinea pig simultaneously exposed to H37Rv strain of *M. tuberculosis* continued for 30 or 60 days of infection had more severe and more numerous extra pulmonary lesions in sucrose feed pig (49). Preserved TB defense in acutely hyperglycemic mice indicates that susceptibility is not a consequence of insulin deficiency or confounding effect of STZ (52,53).

The author extended the experiment on guinea pig model by taking into account a variety of other metabolic defects associated with impaired glucose tolerance and type 2 DM through dietary manipulation. The guinea pigs were fed a diet consisted of 18% protein, 30% fat, and 52% carbohydrate, with the carbohydrate portion consisting of 45% sucrose and 55% fructose. M. tuberculosis infection of diabetic guinea pigs resulted in severe and rapidly progressive tuberculosis with a shortened survival interval, more severe pulmonary and extra pulmonary pathology, and a higher bacterial burden compared with glucose-intolerant and non-DM controls. Compared with non-DM, DM guinea pigs with TB had an exacerbated proinflammatory response with more severe granulocytic inflammation and higher gene expression for the cytokines/chemokines interferon-g, IL-17A, IL-8, and IL-10 in the lung and for interferon- γ, tumor necrosis factor-a, IL-8, and monocyte chemoattractant protein-1 in the spleen. TB disease progression in guinea pigs with impaired glucose tolerance was similar to that of non-DM controls in the early stages of infection but was more severe by day 90 (54).

Most of the animal model studies cited above give rise to the hypothesis that impaired innate and delayed hyperactive adaptive immune is related with the extent of chronic hyperglycemia. It is also shown in a controlled intervention studies, glucose lowering treatment of TB patients warranted to justify screening for and tight control of DM (55).

Vallerskog *et al*. showed that *M. tuberculosis* specific IFN- γ producing T cells arise later in the lymph nodes of DM mice than controls, with a proportionate delay in recruitment of these cells to the lung and stimulation of IFN- γ dependent responses. Dissemination of *M. tuberculosis* from lung to lymph nodes was also delayed in diabetic mice, although they showed no defect in dendritic cell trafficking from lung to lymph nodes after LPS stimulation. Lung leukocyte

aggregates at the initial sites of *M. tuberculosis* infection developed later in diabetic than in non-diabetic mice, possibly related to reduced levels of leukocyte chemo attractant factors including CCL2 and CCL5 at early time points post infection (56).

The other hypothesis, barrier hypothesis of delayed innate inflammation is the chronic glucose induces advanced glycated end products. Lung matrix proteins have slow turnover and accumulative glycation over time with DM (57). The chronic up-regulation of glucose can lead to the abnormal accumulation of advanced glycation end products that are highly reactive and can bind and modify immune response molecules (e.g. antibodies, complement) (58, 59). Hyperglycemia mediated by *M. tuberculosis* infection also shown to cause an accumulation of advanced glycation end products in lung granulomas, tissue and serum which are shown to interfere attraction of cell to lung (53). Plasma levels of heme oxygenase-1 and tissue inhibitors of metalloproteinase were higher in patients with TB and T2DM than in patients without DM, which is positively correlated with random plasma glucose, circulating glycosylated hemoglobin, and low-density lipoprotein levels (27).

From human studies, most of the results are inconsistent with animal model studies and indicate paradoxical hyper inflammatory response associated with chronic hyperglycemia. A study on human blood monocyte from healthy non DM and DM were stimulated *in vitro* to *M. tuberculosis H37Rv* strain to perform Monocyte-*M. tuberculosis* association assays. The percentage of monocytes with at least one associated (attached or ingested) mycobacteria were lower in DM group. Altered route of entry of the pathogen in DM patients may influence the downstream activation of signaling pathways in the monocyte and the survival of mycobacteria (60). In Genome wide association studies two genes, *HK2 and CD 28,* emerged as potential culprits in DM-increased TB susceptibility (61).The gene *HK2* encodes hexokinase 2 which is a critical mediator of aerobic glycolysis which is a unique energy source for macrophages. Roles of *HK2* for in the development of insulin resistance and DM have been demonstrated in experimental studies (62). Decreased expression of *HK2* may impair macrophage function, and decreased T-cell activation and the Th1 response thus increasing of the risk of tuberculosis (61). This leads to investigation of adaptive immune response.

2.3. Adaptive immune response in tuberculosis and diabetes co-morbidity

Adaptive immune responses are mediated by B and T lymphocytes. Adaptive immune responses mediated by T cells play a vital role in the elimination of *M. tuberculosis*. Cytotoxicity and cytokine production are the two major effector mechanisms utilized by T cells against intracellular pathogens (63). T helper cells are activated in the lymph nodes when APCs present peptides bound onto their HLA class II molecules. After the activation, naive CD4 T cells may differentiate into helper cells (Th1, Th2, Th17 Th9, and Th22), T follicular helper cells and inducible T regulatory cells, according to their cytokine profiles (64, 65). Production of $CD4^+$ effector subsets depends on the type of antigen, dose of antigen, mode of entry and cytokine milieu at the microenvironment (65). Among various cytokines, the indispensable role of IFN-γ in *M. tuberculosis* infection was demonstrated in various experimental systems. A timely Th1-biased adaptive immune response is the major determinant of outcomes in human TB and in animal models of TB (52).With the recognition that DM increases susceptibility to TB, it was anticipated that impaired expression of adaptive immunity would be identified as the culprit mechanism; this hypothesis was suggested by early data from a mouse model (54, 56).

2.3.1. Pro-inflammatory immune response

The interaction of T helper cells (Th) with infected macrophages is the central protective immunity against *M tuberculosis*. *M tuberculosis* resides and replicates within phagocytes and persists in susceptible hosts by modulating protective immune responses or altering host protective Th cell responses (9). There is a considerable body of literature describing adverse effect of DM on the inflammatory, protective milieu, of TB immune response. Inconsistent findings have been reported; delayed but hyper active adaptive response in both human and animal human model studies (52, 54) ,lower expression of inflammatory Th 1 response, IFN-γ release(51, 66) no significant differences in the qualitative or quantitative T-cell IFN- γ, Th 17 responses (67, 68).

A study on DM-TB patients from Southern Texas and northern Mexico with PPD restimulation of whole blood, higher production of IL-12, IL-2, and IFN- γ in especially type 2 DM involving chronic hyperglycemia were reported (27). This finding was extended and confirmed by Kumar

et.al that compared Th1 and Th17 cells in TB- DM with non-DM TB patients, on mycobacteria-specific immune responses (PPD, ESAT-6, and CFP-10) in the whole blood. Diabetic TB patients had increased frequencies of antigen specific mono and dual functional CD4+ Th1 cells and) cytokine levels of both Th1 and Th17 s both from plasma and antigen stimulated supernatant. DM is not associated with significant alterations in the frequencies of naive, central memory, and effector memory CD4+ T cells, but lower Treg cell frequencies compared with no DM (69, 70). The decreased frequencies of natural Tregs could be associated with enhanced Th1 and Th 17 cells and hyper inflammatory response.

Diminished inflammatory response were also reported in different settings; in a study in India persons with DM or pre-DM, latent infection diminished circulating levels of type 1 (interferon γ, interleukin 2, and tumor necrosis factor α) and type 17 (interleukin 17F) cytokines as well as a decreases *M. tuberculosis* antigen-specific levels of type 1 and type 17 cytokines from antigen-stimulated whole blood analysis (68). These result were repeated in Tanzania, African setting where interferon gamma release assay measurement were found to be lower in TB –DM than the control. DM patients without tuberculosis, however, showed strongly reduced non-specific IFN-γ production as compared TB-DM, (26) which is essential for inhibition of the initial growth of M. tuberculosis that could be inherited impaired response of DM patients. These variations merit further investigation such as geographic host and pathogen difference in TB-DM interaction.

Type two DM were also shown to impair the CD8+ and NK cell function in tuberculosis patients with elevated frequencies of mycobacterial - antigen stimulated CD8+ T cells expressing Type 1 (IFN-γ and IL-2) and Type 17 (IL-17F) cytokines .In contrast significantly diminished expression of the cytotoxic markers - Perforin, Granzyme B and CD107a both at baseline and following antigen or anti-CD3 stimulation, while NK cells were associated with significantly decreased antigen - stimulated expression of CD107a only (71). This possibly contributing to the pathology of this comorbid disease, as well this aggravated hyper inflammatory response can be associated with problem of angiogenesis, common manifestation in DM host.

2.3.2. Anti-inflammatory immune response

T regulatory constitutes 5–10% of CD4+ T cells in normal mice and humans, and is essential for maintenance of peripheral tolerance and homeostasis. These cells express CD25on the cell surface and a transcriptional regulator, Foxp3. The discovery of the forkhead/winged helix transcription factor FOXP3 as a marker for these cells allows a more accurate characterization of Treg (72). Some studies suggested that human FOXP3+ cells (in contrast to mouse FoxP3+Treg) are functionally heterogeneous (73) and that transient expression of FOXP3 on activated T cells does not correlate with suppressive function (74). However FOXP3 is still considered the most accurate marker for naturally occurring Treg. Recent studies have further characterized the phenotype of Treg by the expression of low levels of CD127. $CD4^+$ $CD25^+$ FOXP3+$CD127^{low}$ Tregs play a role in in auto immune disease and recent advances suggest their role in infectious diseases. Most Tregs arise during normal maturation in the thymus and survive in the periphery as "natural Tregs" (75). Some Tregs develop from conventional CD4+ T cells in response to infectious challenge, and are called inducible Tregs or antigen-specific Tregs (76).

Recent studies found that Tregs proliferate and accumulate at sites of infection, and prevent efficient clearance of infection in mice infected with *M. tuberculosis*. During tuberculosis, Treg cells proliferate in the pulmonary lymph nodes, change their cell surface phenotype, and accumulate in the pulmonary lymph nodes and lung at a rate parallel to the accumulation of effector T cells. The role of Tregs in maintaining tuberculosis were supported by controlled experiment on Treg depleted mice which showed ~1 log less of colony-forming units of M. tuberculosis in the lungs as compared to the wild type (77).

Lower response of the anti-inflammatory milieu of the immune response has been shown in TB-DM, than healthy subjects. Freshly isolated PBMC from tuberculosis patients had increased percentages of Tregs, compared to healthy tuberculin reactors (78). But decreased frequencies of Treg but increased anti-inflammatory IL-10, IL-4 were reported in TB_DM patients (69, 70, 79). Naturally in DM, particularly in type 1, reactive T cell and antibodies outweigh the suppressive activities of T reg and are cause of immune pathogenesis. This scenario is remained to be investigated in TB-DM subjects.

2.3.3. The Balance between pro inflammatory and anti-inflammatory response

Maintaining the equilibrium between infection and immunity to tuberculosis is hallmark of TB immune response by protecting the host tissue damage by the inflammatory response. Shifting the balance towards protective immune response, inducing the regeneration of T reg cell have been report as a mechanism of TB persistence at the site of infection where these cells suppress the inflammatory cells activity (77) . In diabetic –TB patients the frequency of Treg has been shown low and less effective with higher inflammatory response (80). Overall, most adaptive immune response studies suggest that TB-DM patients have a hyper-reactive cell-mediated response to *M. tuberculosis* antigens. This distinction from non-DM TB patients provides indirect support for dysfunctional immunity in DM patients which leads to TB susceptibility.

Hypothesis

Hypothesis 1: The frequency of CD4+ and CD8+ memory cells would be higher in TB-DM patients as compared with TB.

Hypothesis 2: The frequency of T regulatory cells would be higher in TB-DM as compared with TB.

Chapter Three

3. Objectives

3.1. General objective

To determine the prevalence of DM and assess its effect on T-cell immune response among PTB patients, from June 2014-February 2015, Addis Ababa, Ethiopia

3.2. Specific objectives

- To determine the prevalence of DM in persons with active PTB
- To characterize CD4+ and CD8 +T-cells memory phenotypes in DM-active PTB coincidence
- To characterize T regulatory cell in DM- active PTB coincidence
- To compare the frequency of memory T cell and T regulatory cells balance in DM–active PTB coincidence and tuberculosis

Chapter Four

4. Methods and Materials

4.1. Study area and period

The study was conducted from June 2014 to February 2015 in four health centers (Arada, Woreda 7, Woreda 23, Tekle Hayimanot and Alem bank health centers in Addis Ababa, Ethiopia. Based on the 2007 GC census conducted by the Central Statistical Agency of Ethiopia, Addis Ababa has a total population of 2,739,551, of whom 1,305,387 were men and 1,434,164 were women with an estimated area of 530.14 square kilometers, this chartered city has a density of 5,607.96 people per square kilometer. These populations are being served in 33 Hospitals, 28 Health Centers and 35 Health Posts. The case detection rate for new smear positive TB cases is 81% (81).

4.2. Study design

Comparative cross sectional study was conducted.

4.3. Populations

4.3.1. Source population:

Bacteriologically and clinically diagnosed PTB patients coming for tuberculosis care and treatment unit in the selected health centers

4.3.2. Study population:

Bacteriologically and clinically diagnosed active PTB 18-65 years aged patients coming for tuberculosis care and treatment unit in the selected health centers. Culture confirmed pulmonary TB-DM patient, Culture confirmed TB and Non DM were the actual study participants for T cell phenotyping.

TB with DM: Pulmonary TB diagnosed patients were screened for DM based on fasting blood glucose measurements $\geq$ 126 mg/dl and then chronic hyperglycemia were checked by HbA1c $\geq$ 6.5% in accordance with World Diabetes Association (82). Mycobacterial infection was checked by solid culture on Lowenstein Jensen medium.

Active pulmonary TB without DM: Non DM culture confirmed pulmonary TB (FBG below 110 mg/dl)

4.4. Eligibility criteria

4.4.1. Inclusion and exclusion criteria

All active PTB age 18-65 years, HIV negative patients were screened for DM and the following criteria were used to enroll patients to the immunological study.

Tuberculosis – Diabetes ,and Tuberculosis	
Inclusion criteria	**Exclusion criteria**
✓ Willing to give informed consent ✓ newly diagnosed smear and /or culture confirmed TB and diabetic patients Fasting blood glucose ≥126mg/dl and/or HbA1c ≥6.5% (76) ✓ age between 18-65 ✓ Patients who are not taking anti TB.	- HIV sero positives status - Cancer - Renal disease - Pregnancy(Based on Client response) - Taking immunosuppressive drugs - DM Complications - Known Anemia

4.5. Sample size

It was determined for the two objectives (prevalence and T cell phenotyping) independently:-

For the prevalence of DM among PTB patients a total of 205 active PTB patients were enrolled using single population proportion formulae.

$n = Z_{\alpha/2}^2 * p*(1-p) / d^2, = 205$

$Z_{\alpha/2}$ is the critical value of the Normal distribution at α/2 (e.g. for a confidence level of 95%, α is 0.05 and the critical value is 1.96), d is the margin of error= 0.5, p is the proportion of DM among PTB = 15.8% (45).

Sample size for immunological study was calculated by comparing two means by considering the mean value and standard deviation of CD4+CD25+T-cells from the study entitled 'Significance of the frequency of CD4+CD25+CD127− T-cells in patients with pulmonary tuberculosis and diabetes mellitus' (83).

$$n_1 = \frac{(\sigma_1^2 + \sigma_2^2 / \kappa)(z_{1-\alpha/2} + z_{1-\beta})^2}{\Delta^2}$$

The notations and assumption for the formulae are:

$Z_{1-\alpha/2}$ = two-sided Z value =1.96 for 95% confidence interval).
Power ($Z_{1-\beta}$) = 80%
σ_1 = standard deviation of TB-DM
σ_2 = standard deviation of Group TB
Δ = difference in group means
κ = Ratio of sample size (Group 2/Group 1) = 1
A final sample size of 17 PTB+DM+ and 20 PTB were enrolled.

Table 1- Sample size calculation and assumptions

	TB -DM	TB only	Mean difference	Size estimate
Mean	13.3	6.9	6.4	
SD	9	4.4		
Sample size of PTB+DM+				20
Sample size of PTB				20
Total sample size				40

4.6. Sampling technique

Purposive sampling for health facilities and convenient sampling technique for study units were used to recruit consecutively those who full fill the inclusion criteria.

4.7. Study variables

Dependent variables;

- Prevalence of DM
- Percentage of Effector T cells
- Percentage T regulatory cells

Independent variables

- Age
- Sex
- Marital status
- Body Mass Index
- AFB Smear result
- History of tuberculosis
- TB contact history
- Glucose level
- Dyslipidemia
- Alcohol consumption
- Family history of DM

4.8. Data collection procedures

Socio demographic data from the participants were collected by using structured questionnaire. Screening procedures of HIV test, blood glucose using diagnostic test were done by trained nurses from the respective health facility where the study participants were found.

4.8.1. Sample collection and laboratory methods

Screening of study participants

I. Sputum collection and bacilli detection

Sputum were collected from PTB patients diagnosed for TB either clinical, smear and/or culture by trained data collectors with a clear instruction to the patients. AFB staining, and/or culture of the mycobacteria were done to get tuberculosis cases.

II. Glycated hemoglobin and fasting blood sugar for the diagnosis of DM

Diabetic patients primarily diagnosed using clinical symptom and FBG $\geq$ 126 mg/dl using Senso Card Blood Glucose Meter (77 Elektronika Kft, Hungary). Two ml of EDTA blood were collected and used for testing the chronic hyper glycaemia of FBG diagnosed DM patients. HbA1c test refers to glucose modified hemoglobin A (HbA) specifically at N-terminal valine residues of hemoglobin beta chains. Glycohemoglobin is produced by non-enzymatic addition of glucose to amino groups in hemoglobin. Non-diabetic individuals have HbA1c values in the range of 3 – 6 % and controlled diabetic individuals have HbA1c values in the 6 – 9 % range. Individuals with uncontrolled DM can have HbA1c as high as 20% (82).

Laboratory procedures

I. Blood Sample Collection and Peripheral Blood Mononuclear Cell (PBMC) Isolation:

Fifteen ml venous blood was collected with sodium heparin tube for cellular /cytokine assay from each study participants. Two ml of blood with EDTA tube were also collected from TB-DM subjects for HbA1c measurement.

Separation of PBMC from whole blood was accomplished by using standard operational procedure developed for Ficoll Paque^{TM} PLUS gradient centrifugation. In brief, whole blood was resuspended in two volumes of R0 and mixed well by pipetting. Two volumes of the diluted blood were gently overlaid on top of 1 volume of Ficoll Paque^{TM} PLUS density gradient (GE Healthcare Bio-Science AB, Sweden) and centrifuged at a speed of 1800 rpm (Allegra^{TM} 6R Centrifuge, Beckman Coulter) for 30 min at room temperature with no brake. After centrifugation, the white ring of PBMC at the interface of the plasma and the Ficoll-Paque™ Plus was harvested aseptically and washed three times with R0 medium at 1500 rpm for 10 min at RT. The cells were finally resuspended in R10 (10 % sterile heat inactivated FBS (Sigma-Aldrich) and 1% penicillin-streptomycin (Sigma-Aldrich) and counted using haemocytometer under light microscope (40 x magnifications) using 0.4% trypan blue dye in PBS. Four corners of the WBC counting chambers -16 squares (Improved Neubauer cell counting chamber) were counted for refractile cells and the mean count of four chambers taken to calculate final cell number per ml by multiplying with dilution factor 10 and volume of the counting chamber (10^4) under the cover slip. Viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm the cells are cryopreserved in 10% Dimethyl Sulfoxide in Fetal Calf Serum at -150℃ until analysis (84).

II. Lipid profile:

Lipid panels (Total cholesterol, Triglyceride, Low density lipoprotein(LDL) and High density lipoprotein (HDL) for the study participant were done at AHRI Clinical trial lab from heparinized plasma sample stored at -80^{o}C. Total cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinonemine is formed from hydrogen peroxide and 4-aminophenazone in the presence of phenol and peroxidase. The triglycerides are determined after enzymatic hydrolysis with lipase. Indicator is quinonemine formed from hydrogen peroxide, 4-aminoantipyrine and 4-chlorophenol under the catalytic influence of peroxidase. The test method for HDL and LDL Cholesterol involves a combined two specific steps; in the first step chylomicron, VLDL and LDL cholesterol *(during HDL analysis)* and HDL cholesterol (*during HDL analysis*) are specifically eliminated and destroyed by enzymatic reactions. In the second step remaining cholesterol from HDL/ LDL fractions respectively was determined by well-established specific enzymatic reaction in the presence of specific surfactants. All reagent kits are from HUMAN Diagnostics, Germany and manufacturer instructions were strictly followed.

III. Flow cytometry analysis:

Thawing of cryo-preserved PBMC was performed by rotating cryovials in a 37℃ water bath until a small crystal was remaining in the cell suspension. Cells were then slowly re-suspended in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine and 100 U Penicillin/Streptomycin (all Lonza, Verviers, Belgium). PBMCs were stained with fluorochrome-labeled monoclonal mouse anti-human antibodies CD3-V500 (Clone UCHT1), CD4-FITC (Clone RPA-T4), and CD8-PE (clone RPA-T8), CD45RO PE-Cy7 (Clone UCHL1) each 5µl volume and all from BD Bioscience, USA. CD25-Percp Cy5.5 of 5µl volume (Clone BC96), and Fixable Viability Dye eFluor® 78 of 1µl/100ml PBS, both from eBiosciences, Belgium) followed by fixation/permeabilization using cytofix/cytoperm solution for 1 hour and intracellular staining with monoclonal antibodies to Foxp3- APC (eBiosciences, clone PCH101) of 5µl volume. As indicated in *Annex -1 - Laboratory protocols*, cells were incubated with the respective mAbs at 4°C in the dark for 30 minutes. Fluorescence minus one (FMO) and unstained controls were used for gating purposes. Cells were included in the analysis if the cell viability was 75% after thawing. Approximately 2.5 x 10^5 – 1x 10^6 events were acquired. Flow cytometry data were

collected on a FACS Canto II flow cytometer (BD Biosciences) using FACS Diva acquisition software and then analyzed using Flowjo 10 (Tree star).

4.9. **Quality control**

Standard operational procedures and manufacturer instructions were strictly followed throughout the procedures. Translation of questionnaire to Amharic and back translation to English to assure consistency was done. Training for data collectors were done to maintain the quality of data collection process. Pre-test was conducted on 5% of the sample size prior to actual data collection. Data checking was done daily after data collection and amendments were made before the next data collection. Data clean up and cross check was done before analysis. To check whether culture media supports growth, known ATCC strain of H37Rv was used. Randomly selected slants of LJ media inoculated with sterile distilled water incubated for the negative controls. Different optimization test on the concentration of appropriate antibody concentration to cell ratio were done. Fluorescence minus one and unstained controls was used for gating purposes and compensation controls were used to avoid spectral overlap in the cytometry analysis.

4.10. **Data analysis and interpretation**

Data were entered, cleaned and analyzed using Microsoft Office Excel, SPSS version V 22 and Graph pad Prism Version 5.03 statistical software. Descriptive statistics were used to describe the socio-demographic and clinical data. Flow cytometry T cell data acquisition was done with FACS Canto II Diva software, exported to Flowjo V10 then to Microsoft Excel. Finally statistical analysis was computed through Graph pad prism. The normality of the data was assessed by using both Kolmogorov-Smirnov and Shapiro Wilk normality tests and the immunological data was non normal. Frequency distributions, descriptive statistics (mean, SD), bivariate analyses using chi-square test was performed. For variables in which the cell values were less than 5, the Fischer's exact test, Likely hood ratio and logistic regression was used instead for the analysis of association of dependent variables with the prevalence of DM. The immunological data were quoted as median plus the interquartile range and non-parametric tests were therefore used to compare different groups. Mann-Whitney U test was used to compare two

unpaired groups. Spearman correlation was also used to demonstrate the relationship between two continuous variables. P value < 0.05 was taken as statistically significant.

4.11. Ethical consideration

The project proposal was reviewed and approved by ethical review committees of AHRI/ALERT, School of Graduate Studies College of Health Science Jimma University and Addis Ababa Health Bureau Ethics Board. Written informed consent was obtained from each study participants following a clear explanation of study purpose, benefit and possible discomfort. The privacy was maintained and information obtained throughout the study kept confidential.

4.12. Plan for dissemination of study findings

The findings of the study will be submitted and presented to Department of Medical Laboratory Science & Pathology and Armauer Hansen Research Institute .The clinical results were notified to the study participants soon after the diagnosis. The research findings will also be presented at different seminars and workshops and finally the paper will be submitted to peer reviewed journal for publication.

4.13. Operational definitions

Active pulmonary tuberculosis : Refers to any bacteriologically confirmed TB using sputum smear microscopy and clinically diagnosed case based on sign and symptoms of TB and chest X-ray findings involving the lung parenchyma or the tracheobronchial tree with no prior history of active tuberculosis and treatment (86).

Diabetes mellitus: defined by increase blood glucose when the pancreas fails to produce the insulin usually caused by an auto-immune reaction or when the body is unable to respond properly which is defined by [HbA1c $\geq$6.5% and fasting blood sugar >126 mg/dl (82).

T-cell subtypes:-defined by the combinations of cell surface markers and transcription factors they express and the cytokines they secrete. Memory cells are defined by the expression of CD45 RO, Activated cells are defined CD25 +, and regulatory cells are defined by $CD4^+$ $CD25^+$ FoxP3+ cells.

4.14. General outline of the work flow

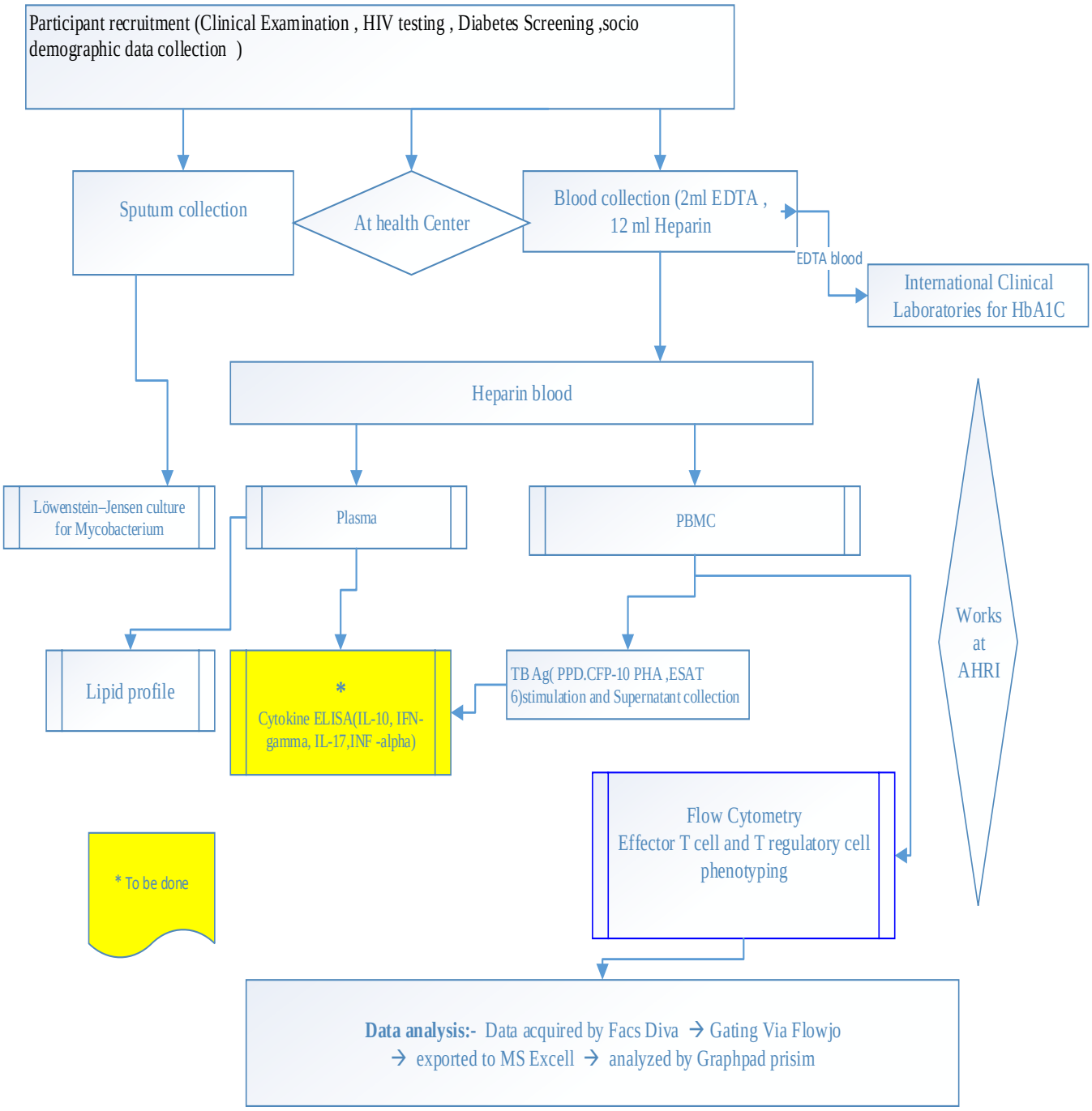

Figure 1. Flow chart showing study participant recruitment and laboratory work flow, Addis Ababa, Ethiopia from June 2014 to February 2015

Chapter Five

5. Result

5.1. Socio demographic characteristics of study participant

A total of 205 clinically and AFB microscopically diagnosed PTB patients were enrolled from June 2014 to February 2015. All of these patients were HIV negative. Out of 205 study participants, males accounted for 141(68.8%) and age ranged from 18-70 years with mean age of 36±13 years. Majority of them were from age group 25-34 years (33.2%) followed by 18-24 years (24.9%) and 93(45.4%) were married. Of these, 127(62.0 %) were self-employee and 156 (76.1%) had lower than 1000 ETB monthly income. As shown in *Table 2*, majorty of the study participans had BMI of 18.5-25 Kg/M^2 98(47.9%) and comparable proportion of participants had BMI lower than 18.5 Kg/M^2 94 (45.8%).

Table 2. Socio-demographic characteristics of the study participants from selected health centers in Addis Ababa, Ethiopia, June 2014 to February 2015 (N=205).

Characteristics		Frequency	Percentage
Sex, Male		141	68.8
Age (Years)	18-24	51	24.9
	25-34	68	33.2
Mean ((age ± SD	35-44	30	14.6
(36±13))	45-54	22	10.7
	55-70	34	16.6
BMI (Kg/M^2)	>25	13	6.3
	18.5-25	98	47.9
(18.7±2.15)	<18.5	94	45.8
Marital Status	Single	89	43.4
	Married	93	45.4
	Divorced	17	8.3
	Windowed	4	2.0
Occupation	Farmer	13	6.3
	Gov't worker	10	4.9
	Self-employee	127	62.0
	Student	20	9.8
	Retiree	4	2.0
	Merchant	6	3.1
	Others	23	11.2
Household member	1-2	57	27.8
living together	3-5	107	52.2

	6-8	32	15.6
	>9	5	2.4
Monthly income (ETB)	<1000	156	76.1
	1001-1500	24	11.7
	1501-2000	8	3.9
	2001-3000	6	3
	>3001	11	5.4

5.2. **Prevalence of DM and clinical characteristics of PTB patients**

Diabetes was diagnosed in 17 of 205 study participants giving a prevalence of 8.3% and 54(26.4 %) had impaired fasting glucose tolerance at the time of diagnosis. Most of these patients were type two DM by considering age of patients and diagnosis year. Out of the total 205 enrolled study participants, majority of them had; 143(69.80%) no contact history, 176 (85.95%) no previous history of TB illness, 177(86.3%) no smoking habit, 144(70.2%) no alcohol drinking habit. During enrolment, 110 (53.7%) of the study participants were diagnosed for smear negative TB. as shown in *Table 3***.**

Table 3. Clinical characteristics and health factors of the study participants from selected health centers in Addis Ababa, Ethiopia, June 2014 -February 2015 (N= 205)

Clinical characteristics		**Frequency**	**Percentage**
TB Contact History	Yes	40	19.5
	No	143	69.8
	Don't remember	22	10.7
BCG vaccinated (200)	Yes	67	32.7
	No	133	64.9
Smoking habit	Yes	28	13.7
	No	177	86.3
Alcohol drinking	Yes	61	29.8
	No	144	70.2
Previous history of TB	Yes	29	14.1
	No	176	85.9
Pulmonary Diagnosis of AFB	Smear Positive	95	46.3
	Smear Negative	110	53.7
Diabetes	Yes	17	8.3
	No	188	91.7
Fasting Blood Glucose(mg/dl)	DM (≥126)	17	8.3
	IFG (110-125)	54	26.4
	Normal (<110)	134	65.3

Key: DM= Diabetes mellitus IFG= Impaired Fasting Glucose. BCG: Bacillus Calmette Guerin

Increased in BMI and family history DM were associated with occurrence of DM when compared to patients with pre-DM and normolycaemia (p=0.00). Alcohol drinking habit was also found to be associated to tuberculosis with DM (p=0.029) and the odds of becoming DM was almost three times higher among alcohol drinker. Tuberculosis patients with DM were also have three times more likely to be smear positive at the time of diagnosis than tuberculosis alone (p=0.036) *Table 4*.

Table 4. Association between background characteristics and DM among PTB patients from selected health centers in Addis Ababa, Ethiopia, June 2014-February 2015 (N =205)

Variable		**DM**		**P-Value**	**OR(95%CI)**
		Yes	No		
Sex	M	13	128	0.475*+	NS
	F	4	57		
Age (Year)	18-24	1	50	0.051**	NS
	25-34	4	64		
	35-44	5	25		
	45-54	3	19		
	55-70	4	30		
BMI (Kg/M^2)	>25	8	86	0.000+	14.687[3.278-65.818]
	18.5-25	4	94		
	<18.5(ref)	5	8	0.002	6.719[1.774-25.446]
Marital status	Single	5	84	0.321**	NS
	Married	9	84		
	Widow	1	3		
	Divorced	1	16		
Contact History	Yes	3	37	0.281*	NS
	No	10	133		
	Don't remember	4	18		
Smoking habit	Yes	3	25	0.418***	NS
	No	14	163		
Alcohol drinking	Yes	9	52	0.029*+	2.942[1.077-8.035]
	No	8	136		
Previous history of TB	Yes	0	29	0.067***	NS
	No	17	159		
Pulmonary Diagnosis	Smear positive	12	83	0.036*+	3.036[1.029-8.961]
AFB	Smear negative	5	105		
BCG vaccination	Yes	4	63	0.416**	NS
	No	13	120		
Family history of DM	Yes	8	9	0.000+	17.679[5.519-56.631]
	No	9	179		

** Likely hood ratio, * X^2 test, *** Fisher exact test, +binary logistic

5.3. T- Cell phenotyping

5.3.1. Demographic characteristics, clinical and biochemical features of patients

We studied a group of 37 individuals with active PTB: 17 had DM and 20 did not have DM. PTB was diagnosed on the basis of both sputum smear and culture. The culture result and smear result of 3 DM diagnosed patients were negative and they were followed for the prognosis and finally included in the study. DM was diagnosed on the basis of HbA1c levels and fasting blood glucose level. All individuals were human immunodeficiency virus negative and naive to anti-tuberculosis treatment. .

As indicated in *Table 5***,** , those with DM had higher BMI, fasting blood glucose, cholesterol, low-density lipoprotein cholesterol, and triglyceride levels but lower high-density lipoprotein cholesterol levels as compared with subjects without DM.

Table 5. Demographic characteristics, clinical and biochemical features PTB –DM (N=17), and PTB (N=20) participated in T cell phenotyping from June 2014- February 2015, Addis Ababa, Ethiopia

Characteristics		**PTB - DM**	**PTB - No DM**	**P-value**
Age in years		43 ± 13.11 (20-65)	32.48 ± 11.8 (18-65)	0.036
Sex	Male	13	14	0.086
	Female	4	6	
BMI (Kg/M^2)		21.01± 4.19 (15.43-28.90)	19.49 ± 5.32 (14.04-27.37)	0.000
DM duration	New	9		------
	Previously Dx	8	NA	
Smear Result	Smear Positive	12	20	0.000
	Smear Negative	5	0	
Fasting glucose level, mg/dl		186.76+63.73 (127-356)	102.27+13.71(77-118)	0.000
Glycated hemoglobin level,%		9.98+2.77 (6-14.5)	ND	--------

Total cholesterol level, mg/dl	146.59 ±38.29(89-211)	143.56±33.63(87-218)	0.780
Triglyceride level, mg/dl	113.12±38.48 (40-209)	83.08±30.68(36-144)	0.026
High-density lipoprotein cholesterol level, mg/dl	34.4± 9.03(20-53)	33.38± 10.52(16-65)	0.939
Low-density lipoprotein cholesterol level, mg/dl	94.18+35.56 (55-164)	94.84 +30.57(46-149)	0.719

The values in the table shows Mean± SD (Range) or frequency

Abbreviations: Body mass index (BMI) is calculated as the weight in kilograms divided by the square of the height in meters. PTB= Pulmonary tuberculosis, ND= Not Done, DM= Diabetes Mellitus; NA=Not Applicable, Lipid profiles are measured from stored plasma.

5.3.2. **Gating strategy**

The following gating strategies for memory T cell and T regulatory cell phenotyping were used: Primarily live cells were gated (Live dead Vs FSA) and single cells were drawn using FSA Vs FSH then PBMC were electronically gated (SSA vs FSA on lymphocyte population, followed by gate on total lymphocyte (CD4 vs CD8). CD4+ cells were analyzed for the expression of CD25 and FOXP3; CD25+ FOXP3+ cells were identified as Treg, whereas CD25 +counted as T activated. Memory cell were defined by expression of CD 45RO; CD4 memory (CD4+ CD45RO+), CD8 memory (CD8+ CD45RO+) and Treg memory is defines as CD4+CD25 +FOP3+ CD45RO+.

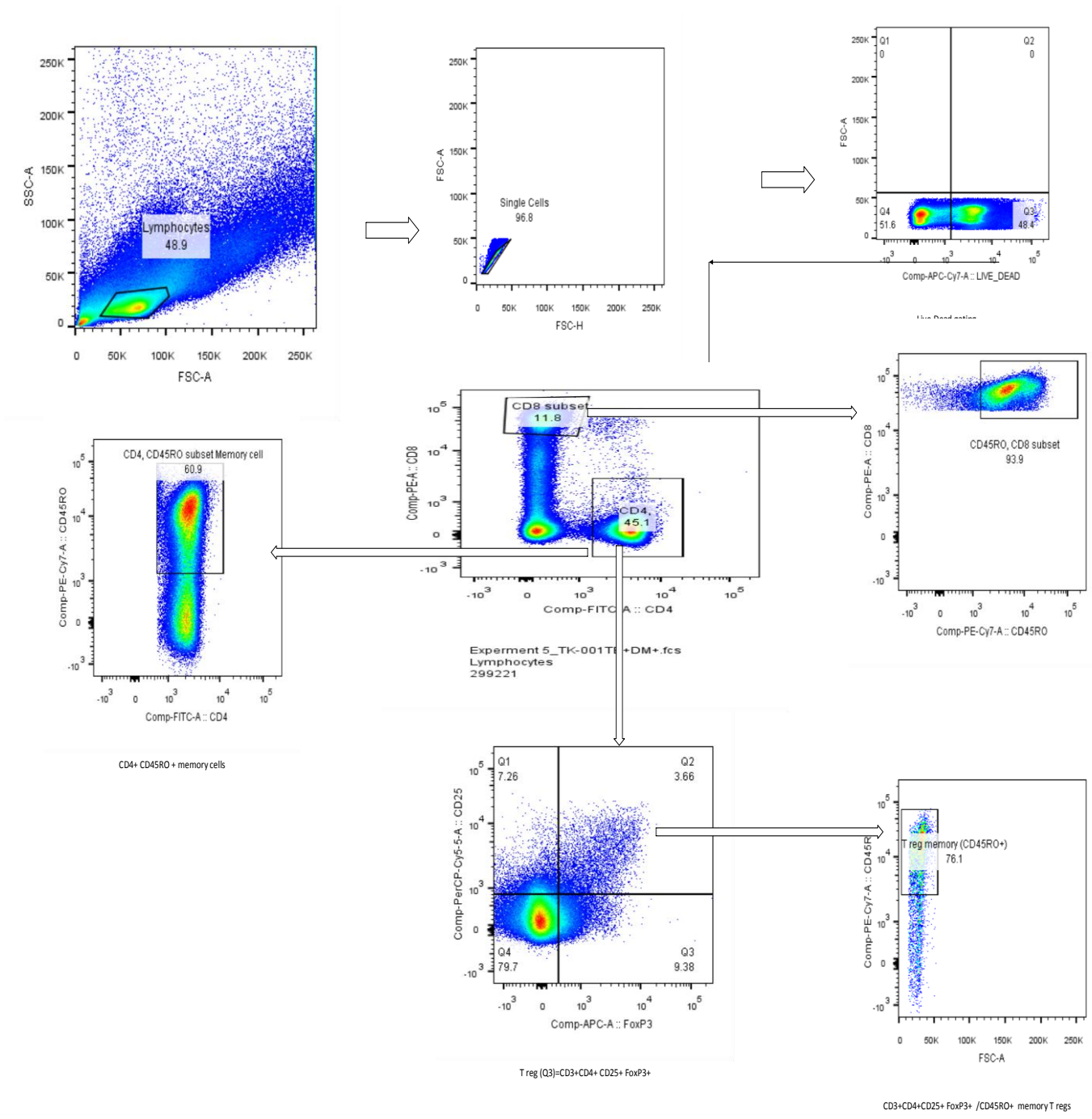

Figure 2. Flow cytometry gating strategy of the different T cell population.

5.3.3. The frequency of CD4+ memory cell of PTB, and PTB-DM comorbid patients

The CD3+ T cell compartment was divided into CD4+ and CD8+T cells and naive and memory CD4+ and CD8+ T cells. In this study we performed generally as memory and naive T cell subset phenotyping using surface expression of CD45RO.

To determine whether there was a difference in percentage of CD4+ and CD4 memory cells in PTB- DM and PTB alone, we quantified the cells using CD4+ CD45RO+ as memory marker. As depicted in figure 3 below , there was no statistically significant difference in percentage of CD4 + cells in between the two groups: PTB-DM (Median, 46.65 % ; IQR, 37.98-55.85) and PTB (Median, 51.50; IQR, 40.25-64.55) groups, p = 0.2792. Similarly, median percentage of CD4 memory -cells had no statistically significant difference between PTB-DM (Median, 56.30 %; IQR,46.35-66.55) and PTB cases (Median, 53.25 %; IQR, 42.25-60.88), p= 0.4371.

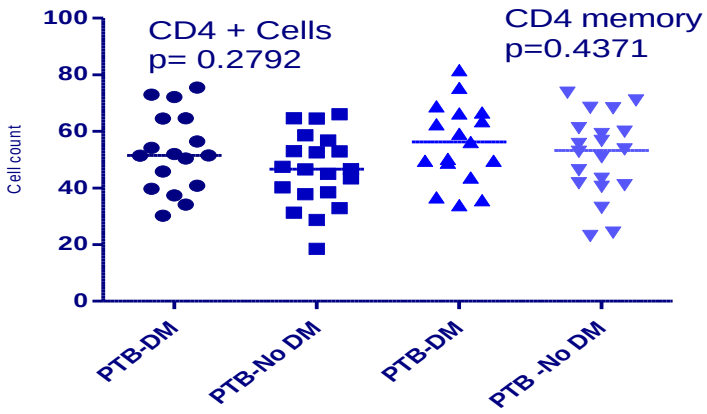

Figure 3. CD4+ and CD4 memory cells frequency among pulmonary tuberculosis and DM comorbid patients from June 2014- February 2015, Addis Ababa, Ethiopia

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5.3.4. The frequency of CD8+ memory cell of PTB and PTB-DM comorbid patients

The frequency of CD8+ memory cell among the two groups is shown below in figure 4. There was no statistically significant difference in the percentage of $CD8^+$ between the two groups: PTB-DM (Median, 28.23 %; IQR, 20.45 -38.90) and PTB (Median, 28.00 %; IQR, 24.08 - 40.48), p = 0.7808. Similarly, median percentage of CD8 memory -cells had no statistically significant difference between PTB-DM (Median, 91.40 %; IQR, 42.40 -94.60) and PTB cases (Median, 93.15 %; IQR, 32-96.73,), p =0.8549.

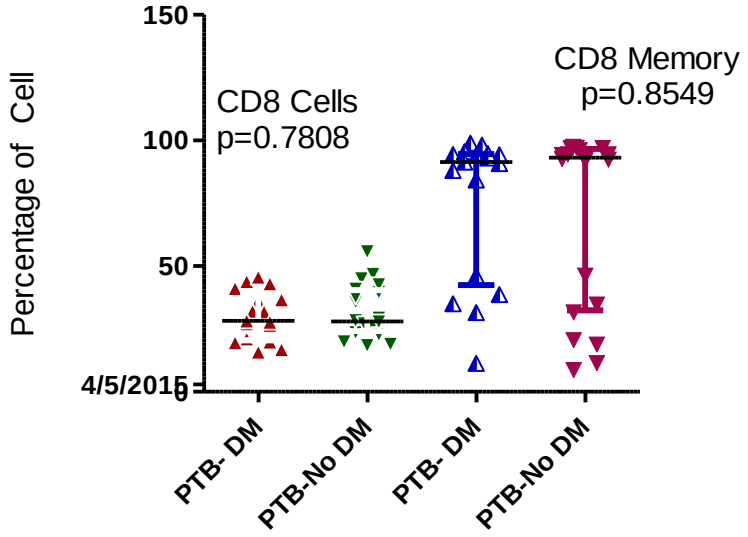

Figure 4. CD8 memory cells frequency among pulmonary tuberculosis and DM comorbid patients from June 2014- February 2015, Addis Ababa, Ethiopia.

5.3.5. The frequency of T regulatory cells of PTB and PTB-DM comorbid patients

As shown in *Figure 5* , there was statistical significant difference between the two groups where PTB-DM patients had shown higher expansion of naturally occurring or induced T regulatory cells p=0.0174 with a median of 3.660; IQR, 2.445 -4.010) in PTB - DM cases and a median of 2.165; IQR, 1.943-2.858 in PTB cases. Similarly the PTB DM (Median 85.70; IQR, 76.00-

89.35) group had slightly higher but statistically insignificant Treg memory than the PTB DM (78.65; IQR, 66.38-88.33), p=0.1130.

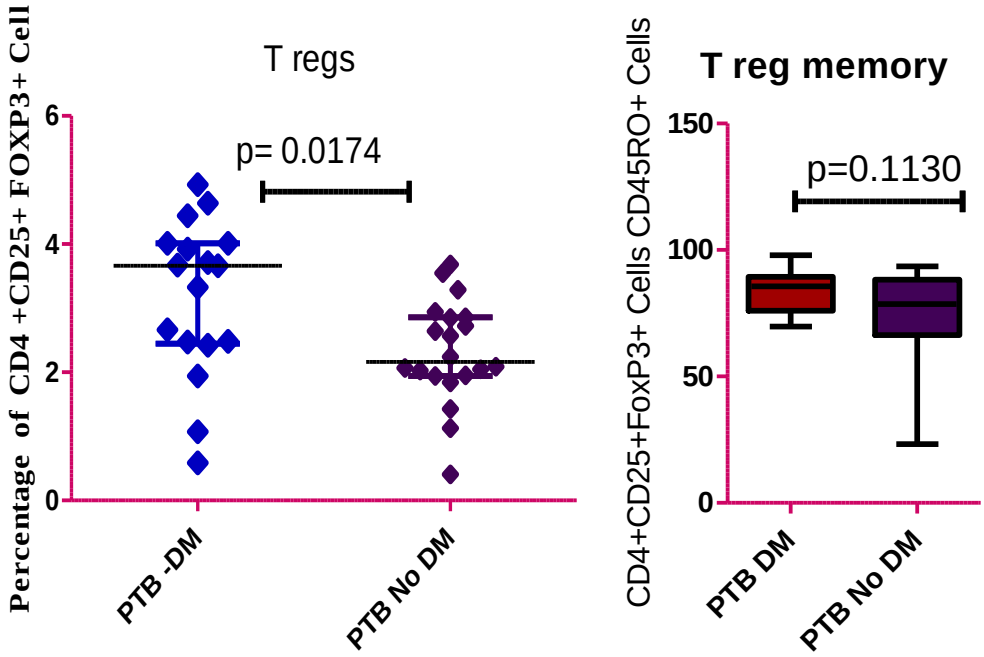

Figure 5. Comparison of T regulatory cell and T regulatory memory cells between PTB-DM and PTB patients from June 2014- February 2015, Addis Ababa, Ethiopia

5.3.6. **The percentage of activated T cells among groups**

CD 25 expression was used to determine baseline activation of CD4+ T cells using CD4 CD25+ gating (We found significant difference between the two groups, p= 0.0373 with a median of 3.360; IQR, 2.515-3.91in PTB-DM cases vs 2.605 IQR 2.130-3.078 in PTB cases.

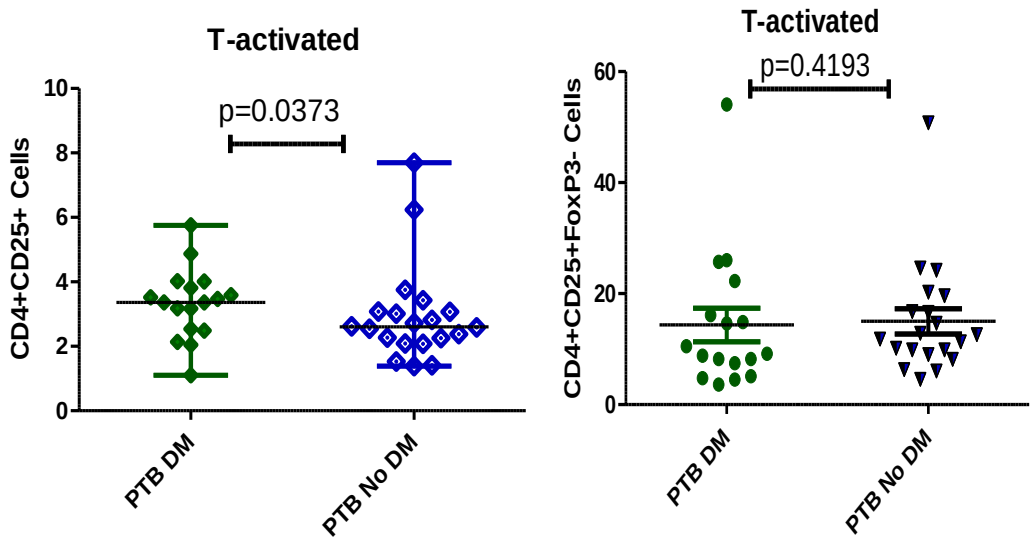

Figure 6. Activated T-Cell percentage among groups

The T activated cells are characterized by CD4+ CD25+ and CD4+ CD25+ FoxP3- cells. There was significant difference among groups in the first definition (CD4+ CD25+) which including CD25 ^{hi} activated T reg subset. But there was no any significant difference in the conventional definition of T activated inflammatory cell which avoids the FoxP3 expressing cells.

5.3.7. **Correlation of glucose leve1 with frequency of cells of PTB -DM patients**

Table 6. Summary of correlation between HbA1C and cell percentage of PTB-DM patients

Statistics	CD3 CD4+	CD3/CD4/ CD4CD45 RO+	CD4 FoxP3+	CD4 CD 25+	CD4 CD25+ FOXP3-	CD4CD25 FOXP3+	CD4CD25+ FOXP3 CD45RO+	CD3 /CD8 CELLS	CD3 /CD8 /CD8CD45R O+
Spearman r	-0.1952	0.01351	0.08717	0.5504	-0.2640	0.1321	-0.2824	0.1375	0.4813
95% confidence Interval	-0.6274 to 0.3290	-0.4823 to 0.5027	-0.4236 to 0.5559	0.07930 to 0.8205	-0.6695 to 0.2628	-0.3856 to 0.5865	-0.6803 to 0.2441	-0.3809 to 0.5901	-0.01479 to 0.7872
P value (two-tailed)	0.4527	0.9589	0.7394	0.0221	0.3059	0.6134	0.2721	0.5987	0.0252*(one tailed)*
P value summary	Ns	Ns	Ns	*	Ns	Ns	Ns	Ns	*
Is the correlation significant? (alpha=0.05)	No	No	No	Yes	No	No	No	No	yes

Activation marker CD25+ and CD8 memory are found to be positively correlated to an increase to HbA1C concentration r_s=0.5504, p=0.022 and r_s=0.4813, p= 0.0252 respectively (Figure 7)

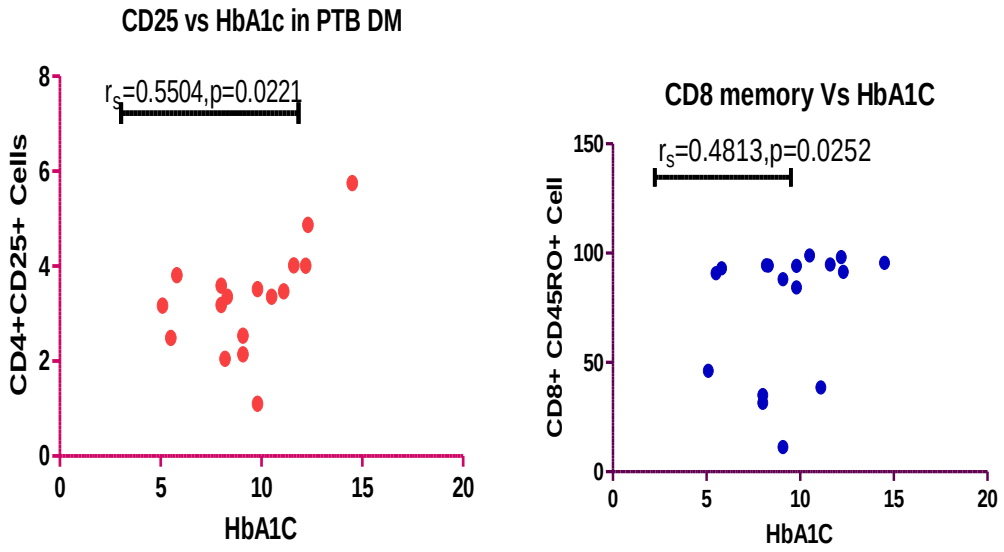

Figure 7. Spearman Correlation of HbA1C Vs CD4 CD25+ Cells and CD8 memory cells of PTB-DM patients June 2014 -February 2015, Addis Ababa, Ethiopia

As depicted above the percentage of activated T cells and CD8 memory are positively correlated with HbA1C concentration in PTB-DM patients.

Chapter Six

6. Discussion

In recent decades, tuberculosis has increasingly become a problem in low-income countries. Diabetes, particularly type 2 DM, is rampant in low- and middle-income countries, affecting the poor and rich alike, and increasing the risk of TB across all population segments**.** This study also demonstrated that DM (8.3%) and Impaired Fasting Glucose (26.4%) were highly frequent among pulmonary TB patients in Addis Ababa. The reported prevalence of DM among the TB patients of 8.3% was significantly higher than the estimated prevalence of DM among the general population in Ethiopia (2-5%) (87). this link may become even more meaningful in coming years, as the prevalence of DM is expected to rise dramatically in the resource-poor areas where TB thrives including Ethiopia.

The observed prevalence of DM in this study is very low as compared to other cross sectional studies conducted in other world ; 39% in Texas and 36% Mexico (32) , 15.9 % San Francisco USA (34) and 25.3 % (34) and 44 % (38) from India, but higher as compared with a large community cohort study conducted in 6.3% china (35). This variation could arise from the population genetics and variation in sample size as well as the difference in the proportion of susceptible population; as both china and India are leading in prevalence of both TB and DM.

The documented prevalence of DM among TB patients in the African studies published between 1980 and 2006 varies between 3.35%-29.8% (39-42). The observed prevalence of DM among PTB patients in our study is comparable to 8.5% prevalence that reported from Uganda (41); higher than the 3.35% report from Guinea Conakry (40) but lower than what was noted, 16.4%, in Tanzania (42) and 29.8% from South Africa (39). Differences in among these studies might also be due to variations in socio-demographic factors, geographical areas, methodology; applied different screening method of diagnosing diabetes (HbA1C, oral glucose tolerance test and FBG).

As compared with other studies conducted across Ethiopia; the present figure is lower than the recently conducted studies in St Peter's hospital (45). In our study we only included HIV negative PTB patient, but in this comparable study, HIV had been reported as frequent

comorbidity on PTB DM patients with (15.9% of TB DM patient having HIV) that could be a possible explanation for this high reporting of DM in those studies. HIV treatments may raise the risk of developing diabetes (88) as well, this difference might be due to variation in screening modalities as they have used enzymatic colorimetric test for glucose (GOD-PAP). But it is comparable with a study conducted in Gondor hospital, 8.5%, (14).

Previous studies revealed that different socio-demographic characteristics had been associated with the occurrence of DM in tuberculosis patients such as increased age (38), family history of DM (45,) BMI (32). In this study, majority of them were males and age group 25-34 years. Although DM is seen most frequent in 35-44 years of age and male sex categories, it's not found to be statistically significant. It is evident that type 2 DM is highly associated with age and physical activity (29). Although not statistically significant, our study also showed an increase DM with age and men sex , which is in line with the global prevalence of DM (87) and other similar studies (34) which is higher in men than women. It is well-known that increased in waist circumference, body weight ratio associated with DM and similarly PTB-DM comorbid group of population, which an inherent characteristics of type 2 DM, had shown to be more obese (43). Likewise, in the present study we have shown strong association of increased BMI with DM occurrence in PTB (p=0.000) but we realized that majority/comparable segments of the study population were under weight and nutritional deficiency might also be associated negative confounding factor.

A number of studies appraised the clinical and social factors associated with frequent DM in tuberculosis patients such as contact history, previous history of tuberculosis, smoking habit and smear positive PTB (14, 38, 45, 89). Likewise, the present study was assessed those factors and we found smear positive diagnosis had statistically significant association. Our findings revealed that 12 of 17 pulmonary patients with DM had smear positive PTB. There was also statistically significant association between AFB smear positive result and the occurrence of DM (p=0.036). Similar findings were reported from other studies in Ethiopia (45) and other parts of the world (35, 37). Due to high rate expulsion of the bacilli from smear positive patients, these groups of patients are highly infectious and failure to address the DM epidemics would instead offset the downward trajectory of global tuberculosis incidence**.** In addition to the factors mentioned above,

this study also showed that consuming alcohol was associated with PTB-DM (p= 0.029) which is the case in other major disease as well in DM alone.

Despite the clinical and public health significance posed by the dual burden of TB and DM, very little is known about the immunological and biochemical mechanisms of susceptibility. Even though, a growing body of research suggested that a defective immune response, metabolic effect of fat tissue in DM may contribute to an increased susceptibility.

The adaptive immune system, largely orchestrated by lymphocytes, is central to the development of acquired immunity against current and subsequent infection with pathogens. T lymphocytes are key regulators and effectors of the adaptive immune responses. Upon contact with specific antigen (through natural infection or vaccination), they differentiate and expand into two populations: effector and memory cells. The generation and persistence of the latter provides the basis for an efficient immune response in subsequent encounters with the pathogen preventing or reducing re-infection. Similarly these cells have been shown to give a either the protection or damage to the host due to unbalanced response up infection and disease during comorbidities like TB-HIV and TB- DM (80, 90).

A number of studies have evaluated the immunological mechanisms contributing the dual burden of DM and TB both in animal model and human studies (26, 56, 60, 83). Our data revealed that DM is not associated with significant alterations in the frequencies of CD4, CD8 and memory cells among groups which is in agreement with a study conducted on Indian PTB-DM patients (70). Although it is not significant, there was slightly higher memory cell in TB coincident DM patients which is related is a rapid response to recurrent infection following challenge.

This study had shown an elevated percentage of T regulatory cells. Since natural Tregs have been shown to be associated with down regulation of inflammatory responses in active *M. tuberculosis* infection, we analyzed the activation of T cell and frequency of T regulatory cells. There is emerging evidence regarding the influence of Tregs and associated cytokines during *M. tuberculosis* infectious diseases in facilitating pathogen persistence and modulating the host immune response to infection (77, 91). There was significant difference in the activation of T cell and frequency of T regulatory cells between groups. The significant increase in Tregs in

PTB-DM patients in this study strengthens this notion. We observed a significant increase in the frequency of Tregs in PTB-DM patients (Median, 3.660; IQR, 2.445 -4.010) compared to Non DM TB patients (2.165; IQR, 1.943-2.858).This expansion of T reg population might be associated with more frequent smear positive tuberculosis, as shown in different epidemiological studies (35, 37) including the present study. T reg cells proliferate and accumulate at sites of infection, and have the capacity to suppress immune responses that contribute to the control of *M. tuberculosis.*

This finding was in line with study done by Sun *et al* (83) but to the contrary with, *Kumar et al* in which DM were associated with diminished Treg and hyper inflammatory response (65). Recently, but to the contrary of active PTB-DM, *Kumar* (71) had also indicated a decrease in inflammatory Th1 and Th17 cells in latent PTB-DM patients This variations could arise from the methods employed, study population and other underline factors such as extent of chronic hyperglycemia. This variation warrants further controlled investigations.

Our finding taken together with other studies indicated that poorly controlled DM is more associated with alteration of immunity during infection (27) and its evident that chronic hyperglycemia is a culprit mechanism of diabetes (53, 92). In line with these reports, we found a strong correlation between HbA1C and activation of T cell (p=0.02219) and CD8 memory cells frequency (p=0.0251) in PTB DM patients. This could also be explained, due to the inherent nature of the accumulation of advanced end product in DM (58) would result in delayed hyperactive and defective immunity.

Limitations of the study

This study was only done on pulmonary tuberculosis patients and adult age group which may not reflect the actual situation of DM for all tuberculosis cases. In addition, this study was done on total populations of Treg and memory cells rather than pathogen -specific T cell *in vitro* and may not tell the exact mycobacterium specific response. A more informative approach would have been to follow a cohort of PTB-DM and PTB patients and compare the immune parameters at different time point.

Chapter Seven

7. Conclusion and Recommendation

7.1. Conclusion

The present study has shown a high (8.3%) prevalence of DM and 26.4% had impaired fasting glucose tolerance at the time of diagnosis which is higher than the estimated general population. It was also noted from this study that BMI, alcohol drinking, and smear positive TB had statistically significant association with the occurrence of DM, and smear positive patients could make a disproportionately higher contribution to TB transmission.

The memory and regulatory T cells in PTB- DM and TB alone patients did not show any statistically significant difference in CD4, CD8 and memory cells, but there was an increased frequency of regulatory and activated T cells in PTB-DM patients. Concentration of HbA1C had a significant correlation with increased frequency of regulatory and activated T cell. Therefore, this study revealed that pulmonary tuberculosis in persons with DM is characterized by elevated frequencies of regulatory T cells, indicating that immune alteration in underlying DM is associated with expansion of Tregs in favor of pathogen survival and would result more smear positive infectious patients.

7.2. Recommendation

There are many challenges in screening and diagnosis of tuberculosis in DM patient and vice versa; as the two disease managed in two different clinics. Joint planning and better collaboration between DM and TB care units could help for better identification, earlier treatment and case management. Since this study was conducted in limited sample populations in Addis Ababa, further country wide studies with a comprehensive study design are warranted to substantiate this claim and would provide a representative figure.

This study was conducted on total populations of Treg and memory cells rather than pathogen - specific T cells to determine if an association with mycobacterium infection could be determined at the T cell population level. More prospective studies are needed to assess the suppressive role of Treg and mycobacterium specific functional response in DM-TB patients.

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Annexes

Annex -1 - Laboratory protocols

Sample transportation

Blood specimen and sputum containing tubes were be transported upright and secured in a screw cap container or in a rack in transport box. Enough absorbent paper was around them to sock up all the liquid in case of spillage.

An emergency kit was ready for every specimen transport. It contains latex glove, gown, disposable absorbent material, disposable gauze/paper towel and alcohol hand rubs. Any injury sustained while handling patient specimen inquire about medical treatment where appropriate.

i. **Packaging of specimen for transport**

- ✓ All specimens were collected in a container that is water tight and leak proof. The cap will be correctly used and securely closed.
- ✓ The container kept in upright position in a rack during transport and the rack holding the container will be put in a cold box.
- ✓ There should be an adequate cushion material inside the box so as to absorb shocks during transport, and adequate absorbing material to absorb any spillage.

ii. **Proper specimen handling during transport**

- ✓ The outer container (cold box) should be handled gently with care. Throwing or dropping of the transport box is prohibited.
- ✓ Good personal hygiene should be maintained during transporting these boxes. Hands should be washed after each session of work, when contaminated or soiled ,or after removal of gloves (staff must not touch mouth ,eye, nose and mucosal membranes prior to hand washing and definitely not with gloved hands)

iii. Handling of specimen leakage and spillage

- ✓ Leaking specimens are hazardous to all staffs involved in their handling. Such specimen could be rejected or discarded according to the laboratory practice.
- ✓ When leakage of specimen contains within or outside of the cold box is encountered during transport, the spill should be decontaminated as soon as possible according to the spill clean-up procedure.

iv. Spill clean-up procedure

- ✓ Glove and appropriate personal protective equipment should be worn, including disposable gown.
- ✓ The spill should be covered with cloth, gauze or paper towels to contain it.
- ✓ Freshly prepared solution of 1 in 5 dilution of domestic bleach should be poured over gauze/paper towel. After 30 minutes, the material will be cleared away. A dust pan will be used to collect any broken glass or sharp and deposit it into a puncture resistant container. All the material should be discarded as clinical waste and the dust pan should be disinfected after use.

Whole blood peripheral blood mononuclear cells isolation, freezing and thawing using Leucosep tubes 227 289

1. Principle

PBMC can be isolated from heparinized whole blood by use of Ficoll density gradient centrifugation.

2. Materials and equipment

- ✓ Ficoll
- ✓ 7ml tubes of blood collected in green top (heparin) tubes
- ✓ Freezing can(Mister frosty)
- ✓ PBS without Ca/Mg
- ✓ Pipette with Aid
- ✓ 50 ml conical centrifuge tubes,
- ✓ Leucosep tube
- ✓ Biosafety cabinet BSL-2
- ✓ Hemacytometer
- ✓ 0.4% trypan blue
- ✓ 10% DMSO/90% FBS=freezing medium
- ✓ Cell culture media cRPMI

3. Procedure

1. Warm –Up Separation Medium (Ficoll-Paque Plus) to Room temperature.
2. Centrifuge the sample at 1500 rpm for 5 minutes then collect the plasma using pasteur pipette in to 2 cryotubes and keep at -20
3. Fill the Leucosep tube with separation medium (15ml Ficoll-Paque) and centrifuge for 1 minute at 900 rpm at RT then fill with the remained anticoagulated blood (15-20ml)

4. Centrifuge for 25 minutes at 1800 rpm or 800g **(Break off)**
5. Collect the PBMC using Pasteur pipette in to another centrifuge tube
6. Wash the enriched cell fraction (lymphocyte/PBMC's) with 10 ml Phosphate Buffered Saline(PBS), Subsequently centrifuge for 10 minutes at 1800 rpm (**Break off)**
7. Repeat washing at 1520 rpm for 10 ' using RPMI (**Break at low)**
8. Re-suspend cells for counting in 1ml Cell culture medium (cRPM)**.**

   Note: Here after place cell containing tube with Ice
9. Count live cells using trypan blue exclusion test

   (1:10 dilution, 90µl trypan blue: 10 µl cell suspension)

   No cell X dilution factor X Chamber factor X volume of sample

   No cell X 10 $X10^4$ x volume of cell suspension (1ml)
10. Centrifuge the cell suspension at approximately 100–200 × g for 5 to 10 minutes. Aseptically decant supernatant without disturbing the cell pellet.
11. Resuspend the cell pellet in cold freezing medium at the recommended viable cell density. Dispense aliquots of the cell suspension into cryogenic storage vials.

PBMC freezing

1. Pour off supernatant and resuspend cell pellet by gently tapping the tube by hand (with fingers)before adding the appropriate amount of freezing medium (10%DMSO in FBS) to tube to yield 1 X 10^6 cells per ml
2. Immediately pipet 1 mL of cells in cryprotectant medium into labeled cryovials,close caps tightly and place the vials into ice.
3. Let vials stand in ice bath for 15 minutes before moving to a chilled Mr. Frosty controlled rate freezing container*. Do not let vials stand in ice for longer than 30 minutes as cell viability will be compromised.
4. Place the freezing container with the vials in a -80^0c freezer and let freeze for 24 hours before transferring to -150^0c for long term storage.

Procedure for thawing:

1. Move tubes quickly to dry ice from liquid nitrogen freezer for transporting to laboratory. Use protective freezer gloves and a face shield when removing tubes from the liquid nitrogen freezer.

2. Thaw only 1-2 tubes at a time in 37^{o}C water bath while shaking continuously just until the last ice crystal remains. This should take 65-70 seconds for vials containing 1 mL of freezing medium.

3. Using a one mL pipet, transfer the entire contents from the tube to a15ml conical polypropylene tube at ambient room temperature. Slowly add 5ml of warm complete medium (RPMI 1640 +8% FCS at 25 to 37^{o}C) 1-2 drops at a time, while mixing carefully and thoroughly by swirling tube. Repeat for the second vial (if applicable). Add an additional 8ml of warm R8 to each tube before proceeding directly to next step.

4. Centrifuge tubes at 300 X g for 10 min at 20^{0}C, remove supernatant, gently tap tube to resuspend cell pellet and add 10 ml warm cRPMI. Centrifuge the tube again as above. Discard supernatant.

5. Resuspend to desired volume in medium. An aliquot of cell suspension is diluted in trypan blue exclusion dye for counting in a hemacytometer to determine recovery and viability.

TB culture using LJ medium

1. Procedure

This protocol is for preparation of a total volume of 1600ml

1.1: Mineral Salt Solution

LJ Glycerol (LJG):

- Weigh 37.2g of LJ medium base powder * (DIFCO/Fluka).
- Measure 600 ml of distilled water into a 1000ml beaker.
- Dissolve the Lowenstein-Jensen Medium base in the water.
- Mix well and bring to the boil into a microwave oven with constant agitation until the reagents are completely dissolved.
- Using a single sterile pipette (25ml), measure 12ml of glycerol and add it to the mixture.
- Mix and autoclave the mixture at 121°C for 15 minutes.
- Let cool to room temperature.

1.2. Egg fluid

- Break 1000ml of eggs into a sterile beaker (2000ml).
- Add the fluid eggs to the autoclaved mineral solution.
- Using a Homogeniser, mix until homogeneous.
- Add 20ml of 2% Malachite Green.
- Stir for at least 10 minutes before dispensing.
- Filter the mixture through sterile muslin into a sterile round glass flask (2000ml) containing a sterile magnetic bar.
- Add: 3.0ml of Polymyxin B (100,000 iu/ml), 0.75ml of Carbenicillin (0.2g/ml), 15ml of Fungizone (5mg/ml) and 3.0ml 1% Trimethoprim.
- Mix well and slowly on a magnetic mixer for at least one hour under UV light in a biosafety cabinet (without running the cabinet).
- Decant into two separate sterile Duran glass flasks and close each flask with sterile lids surmounted by a sterile dispenser.

1.3 Aliquoting the medium

- Dispense 8ml into each sterile test tube or 10ml into each universal tube. Avoid air bubbles.

- Clean the dispenser top with paper tissue impregnate with 70% ethylic alcohol after every set of 10 tubes.
- Transfer the tubes containing the medium into a sterile crate.
- After dispensing the entire medium, range the tubes on a special rack and lean them to give a slope when placing in an oven.
- Tighten screw caps, slant them and coagulate by inspissations at 85 ^{o}C for 50 min.
- Let cool.
- Give the medium batch a lot number and label the racks with this number.
- Label the universal lids or culture tube with colour codes.

2. Sputum processing

Except for the simple culture method (the modified Kudoh method), specimens must be processed in centrifuge tubes. If collected in standard containers, sputa must be transferred into centrifuge tubes, which increase the risk of cross-contamination and labelling error. It is thus practical to use 50ml centrifuge tubes to collect specimens for culture.

Sputa should not be processed in sets of more than 6–8 because the methods described here are strictly time-dependent. Larger sets cannot be handled in time (except in the trisodium phosphate and CPC procedures). The first step of these less time-dependent methods can be performed in microscopy laboratories, outside a BSC. Transport to a biosafety level 2 laboratory must be organized within less than one month for samples stored without preservative via cold chain, one day for the trisodium phosphate method and within one week for the CPC method.

The mucolytic agent *N*-acetyl *L*-cysteine (NALC) enables the decontaminating agent, sodium hydroxide, to be used at a lower final concentration. Sodium citrate is included to bind the heavy metal ions that might be present in the specimen and that could inactivate NALC. The method is suitable for inoculation in liquid media and can be used in egg based solid medium.

Reagents

1. Sodium hydroxide (NaOH) solution, 4%
2. Trisodium citrate·$2H_2O$ solution, 2.9% (or anhydrous trisodium citrate solution, 2.6%)
3. *N*-acetyl *L*-cysteine (NALC)
4. NALC-NaOH solution, freshly prepared for daily use only:
   - Mix equal volumes of (1) and (2);
   - Add 0.5 g NALC per 100 ml of Trisodium citrate–NaOH solution just before use.

5. Phosphate buffer, 0.067 mol/litre, pH 6.8

Procedure

1. Mark the volume of sputum on the centrifuge tube (at least 2 ml, not more than 5 ml). Add an equal volume of the NALC–NaOH solution and tighten the screw-cap.
2. Vortex for not more than 20 seconds.
3. Keep at 20–25 °C for 15 minutes for decontamination.
4. Fill the tube to within 2 cm of the top (e.g. to the 50-ml mark on the tube) with PBS
5. Centrifuge at 3000*g* for 15 minutes.
6. Carefully pour off the supernatant through a funnel into a discard can containing 5% phenol or other mycobacterial disinfectant.
7. Resuspend the deposit in approximately 0.3 ml phosphate buffer (when using only solid media) or approximately 0.8 ml phosphate buffer (when using liquid media).
8. Inoculate deposit on two slopes of egg-based LJG medium + one slope of LJP medium and/or into a vial of liquid medium labelled with the ID number. Use a pipette to inoculate each slope/vial with 3–4 drops (approximately 0.1–0.15 ml).
9. Smear one drop on a slide, marked with the ID number, for microscopic examination.

Follow the growth of mycobacterial species three days post inoculation and every week for 8 weeks. Record each reading at every week interval as per the format prepared for culture reading. After eighth week produce the final report and store isolates and/or proceed for the next step or procedure.

Flowcytometry

Materials

- RPMI
- FCS
- Penicillin-Streptomycin
- L-Glutamine
- BSA
- EDTA
- PBS
- Paraformaldehyde (4%)
- dH2O
- Antibodies and isotype controls
- Compensation beads
- 96 well round bottom plate

Preparation:

Reagents:

- Complete media (RPMI + 10%FCS+1%Pen/Strep + 1mM L-Glut)
- FACS buffer: (1%BSA, 1mMEDTA, in PBS)
    - Make a 0.5M EDTA by adding 14.6g of EDTA [or 18.6g of EDTA disodium] to 100ml PBS
        - Use NaOH (10M or pellet) to bring pH to 8 so it dissolves
    - To make 500mL FACS Buffer
        - Add 1ml of 0.5M EDTA and 5g BSA in 500ml PBS.
- FOXP3 staining buffer set:
    - Prepare 1X Permeabilization buffer from 10X concentrate using dH_2O. 6ml required for each staining tube.
    - Prepare 1X Fixation/Permeabilization solution by diluting 4X concentrate with diluent. 1ml per tube required.
- BD Cytofix/Cytoperm:
    - Use Cytofix/Cytoperm solution 250µl per tube.
    - Prepare 1x Perm Wash buffer from the 10X concentrate using dH_2O.

Fluorochrome Panels

- Use the following quantities per tube. Multiply by number of tubes to prepare total cocktail for one day. Use 50µl antibody cocktail per tube prepared in staining buffer.

Staining Panel

Antibody	Marker	Volume (µl)
CD4	FITC	5
CD8	PE	5
CD25	PerCP-Cy5.5	5
CD45RO	PE CY 7	10
CD3	V500	5
FoxP3	APC	5
Live/Dead	APC- CY 7	1µl/100µl PBS

Procedure:

Staining

1. Collect cells 0.2- 1x10^6 and transfer to FACs tubes
2. Spin at 400g(1400rpm) for 5' at RT
3. Discard the supernatant
4. Resuspend in 1 ml PBS
5. Spin as above
6. Discard the supernatant
7. Resuspend cells in lit bit buffer(approximately 50 µl
8. Add your extracellular Abs(CD3,CD4,CD25,CD8,CD45 RO) to the cells

   NB: the total volume shouldn't be greater than 100 µl
9. Incubate in dark at 4 ^oC for 30'

   In the meantime prepare beads for compensation controls. Compensation controls need extracellular single staining only
10. Add 1 ml PBS
11. Spin as above and discard the supernatant
12. Add live/dead stain: to the final concentration of 1µl/ml
13. Incubate in dark at 4 ^oC for 30'
14. Add 1 ml FACs buffer
15. Spin as above and discard the supernatant
16. Resuspend cells in lit bit buffer

17. Add 1ml of Foxp3 fixation/Permeabilization working solution to each tube and Pulse vortex
18. Incubate for 60' at 4 ^{o}C in the dark
19. Without washing, add 2ml of 1X Permeabilization buffer to each tube
20. Spin as above (700g,7 minutes) and discard the supernatant
21. Resuspend pellet in 50µl 1x permeablization buffer(FoxP3 permeablization wash)
22. Add your intracellular Ab(FoxP3) to cells: fine max Vol 100 µl

(To each tube add 5 µl anti FOxP3 Ab)

23. Incubate in dark at 4 ^{o}C for 30'

24. Without washing, add 2ml 1x permeablization buffer

25. Spin as above and discard the supernatant
26. Add 2ml 1x permeablization buffer
27. Spin as above and discard the supernatant
28. Resuspend cell in lit bit buffer
29. Add 400 µl FACs buffer and read

Lymphocyte stimulation test

Principle

PBMC are stimulated with different antigens. Lymphocytes recognizing the antigens will proliferate and produce IFN-γ, IL-10,IL-17 and TNF-alpha which will be secreted in the medium. As a positive control, PBMC are stimulated with PHA (a mitogen). As a negative control, PBMC are incubated with medium alone.

Materials

Samples

- Isolated PBMCs
- RPMI-1640 Medium
- Penicillin-Streptomycin

Antigens

- PHA at 4µg/ml (=2X final concentration)
- ESAT6 peptide pool at 10µg/ml/peptide (=2X final concentration) + CFP-10 at 10µg/m
- PPD at 10µg/ml (=2X final concentration)

Plastic disposables

- centrifuge tubes, 15ml polypropylene, conical bottom, sterile
- centrifuge tubes, 50ml polypropylene, conical bottom, sterile
- pipette tips

- 96 well culture plate, flat bottom

Equipments

- Pipet aid
- Pipetman (P20, P100, P200, P1000)
- Multichannel pipette
- Incubator (37^{o}C and 5%CO_2)

Specimen and reagent management

The 4 stimuli (medium alone, PHA, , PPD , ESAT6 / CFP-10 peptide pool in that prioritized order) stored at -20^{o}C.

Stimulation of PBMC

1. For each sample/study subject, suspend the thawed PBMCs to a concentration of 2 * 10^{6} cells/ml PBMC suspension in culture medium.
2. Add 100µl/well of cell suspension in dupplicates for each of the 4 stimuli in the prioritized order as indicated in the plate-layout shown below. Thus: add 2 times 100 µl cell suspension in the first 2 Medium alone (MED), and then proceed to the 2 PHA wells next and so on.

Add 100µl/well in triplicates of PHA (4µg/ml), Medium alone (MED), (ESAT6 /CFP-10 peptide pool at 10µg/ml/peptide, PPD at 10µg/ml (PPD) to all the wells with cell suspensions.)

3. Incubate for 6 days at 37^{o}C and 5% CO_2 in a humidified incubator.
4. On day 6, pool the supernatant per stimulation (2*100 µl), transfer half (=100µl) to a second 96 wells flat bottom plate as a backup.
5. Store the supernatants at -20^{o}C.
6. Label each plate with the pooled supernatant with the subject and study number and current date.

Annex- 2- Respondents information sheets consent forms

Respondent's information sheet -English version

Title of the project: Effect of diabetes mellitus on T-cell immune response of pulmonary tuberculosis in Addis Ababa, Ethiopia.

Name of principal investigator: Zebene Andargie

Organization: Jimma University (Medical laboratory science & Pathology department)

Name of sponsor: Armauer Hansen Research Institute and Jimma University

This information sheet is prepared for pulmonary tuberculosis and diabetic mellitus patients who will be involved in project entitled above. We are going to tell you about the whole process that will happen in the study and requesting you to participate voluntarily.

Description and purpose of the study

Tuberculosis is the most common infectious disease worldwide, especially in a developing world like our country, Ethiopia. The disease is mostly occurred from previous latent tuberculosis that contained by the immune cells. But when the immunity is suppressed or altered due to other underlying conditions like diabetes mellitus the infection will be reactivated and cause the disease occurrence. So this study is designed to characterize the effect of diabetes mellitus on the immune response of pulmonary tuberculosis.

Procedures

If you are willing to participate in the study, you will be asked to sign a consent form and the following procedures will be done.

- ✓ Your medical history will be reviewed
- ✓ You will provide us a about 20 minutes interview
- ✓ You will be tested for HIV and Diabetes
- ✓ We will take sputum and approximately 3 teaspoon full of blood sample
- ✓ The collected sample will be sent to AHRI and
- ✓ Immunological studies will be done on the sample and preserved at AHRI laboratory for 5 years to study other cytokine biomarkers related with the current study.

Risks and discomforts

During all sample collection we will follow Standard operational procedures. The blood drawing may cause minor pain, at the place where blood is taken. However, this pain will disappear in

few hours. To minimize these discomforts, the procedure will be performed by an experienced nurse with standard aseptic and serial procedures.

Benefits and compensation

By participating in this study, there will not be direct financial benefit, however, you will be provided 50 Birr as a reimbursement for your transportation cost. You will get pre and post counsing on Information about the HIV test—what it tests for, what it might not tell you, and how long it will take you to get your results, how HIV is transmitted and how you can protect yourself from infection, confidentiality of your test results and a clear, clear communication of what your test results mean. In addition, if you are HIV,Diabetes positive, you will be referred to the respective health department or health facility for further care and treatment.

Confidentiality

All information that all be collected from the study subjects will be kept confidential. Any information about the participant that will be collected from the study will be stored in a file that will not bear a name on it, but only a number assigned to it instead.

Voluntary participation and withdrawal:

Your participation in this study is voluntary. You may decide not to participate or you may leave the study at any time. Your decision will not result in any penalty or loss of benefits to which you are entitled. Your decision will not put at risk any present or future medical care or other benefits to which you otherwise entitled. You should ask the study investigators listed below any questions you may have about this research study. You may ask questions in the future if you do not understand something that is being done.

Use the following address for any question.

Zebene Andargie, Phone No +251 910 45 84 34, Email: zbnandargie9@gmail.com

Dr Adane Mihret, Phone No +251 911 40 89 84, Email: adane_mihret@yahoo.com

Dr Tesfaye Kassa, Phone No +251 931 05 71 95, Email: ktes36@gmail.com

Dr Getnet Beyene, Phone No +251 911 64 40 93: Email: rgetenet@yahoo.com

Prof. Yeweyenhareg Feleke Phone No +251 911 16 95 13: Email: yeweyenharegf@yahoo.com

AHRI/ALERT Ethics Review Committee, Phone Number 0113-481285

For the success of our study, we will be asking you to give the correct answer for the respective questions. Thank you for your assistance. Continue answering the questions.

የጥናቱ ተሳታፈዎች መረጃ ቅፅ (የአማርኛ ግልባጭ)

የጥናቱ ርዕስ፡ የስኳር ህመም በሳንባ በሽታ በሰውነታችን ቲ_ህዋሳት የመከላከያ ምላሽ ላይ የሚያመጣው ተፅዕኖ
የዋና ተመራማሪ ስም: ዘበነ አንዳርጌ
የድርጅቱ ስም፡- ጅማ ዩኒቨርሲቲ / የህክምና ላቦራቶሪ ሳይንስ እና ፓቶሎጅ ትምህርት ክፍል)
ድጋፍ ሰጭ ተቋም:_አርማወር ሃንስን የምርምር ተቋም እና ጅማ ዩኒቨርሲቲ

ይህ የመረጃ ቅፅ የተዘጋጀው ከላይ በተጠቀሰው ጥናት ለሚሳተፉ የስኳር እና የሳንባ ነቀርሳ በሽታ ህሙማ እና ጤነኛ ሰዎች ሲሆን በአጠቃላይ በጥናቱ ውስጥ ልናካሂዳቸው ስለፈለግናቸው ጉዳዮች እና ስለጥናቱ ጠቅላላ ማብራርያ ይሰጣል::በመሆኑ በጥናቱ የሚሳተፉት በራስዎ ፍላጐት ብቻ መሆኑን በትህትና እንገልፃለን፡፡

<u>ስለ ጥናቱ በጥቂቱ</u>

የሳንባ ነቀርሳ በሽታ በአለማችን ላይ በከፍተኛ ደረጃ ላይ ይገኛል፡በተለይም እንደ ኢትዮጵያ ባሉ ታዳጊ አገሮች ጉዳቱ ከፍተኛ ነው፡፡ በሽታው በአብዛኛው በትንፋሽ ከሠው ወደ ሰው የሚተላለፍ ሲሆን ሳንባ ውስጥ በሰውነታችን የመከላከያ ህዋሳት ተወስኖ ይኖራል::ነገርግን የሰውነታችን የመከላከል አቅም በተለያዩ እንደ የስኳር በሽታ ባሉ በሽታዎች ሲዳከም በሽታው እንደገና ይነሳና ወደ ሙሉ የቲቢ ህመምነት ይቀየራል፡፡ስለሆነም የዚህ ጥናት ዋና አላማ የስኳር በሽታ የሳንባ ነቀርሳ በሽታን ለመከላከል ሰውነታችን በሚያደርገው ስራ ላይ ያለውን ተፅዕኖ ማጥናት ይሆናል፡፡

<u>የጥናቱ ሂደት ዝርዝር</u>

በጥናቱ ለመሳተፍ ከተስማሙ የሚከተሉትን መረጃዎችና ናሙና እንወስዳለን፡፡

- የህክምና መዝገብዎ ይታያል :: እንዲሁም ከራስዎ አንደበት ወደ 20 ደቂቃ የሚደርስ ቃለ መጠየቅ ይደረግሎታል፡:
- የ ኤችአይቪ እና የስኳር በሽታ ምርመራ ይደረግሎታል።
- 3 የሻይ ማንኪያ ያክል የደም ናሙና እጅግ በጣም አነስተኛ በሆነና ንፅህናው በተረጋገጠ ሲሪንጅ መርፌ ይወሰዳል፡:
- የአክታ ምርመራ ይደረጋል::
- የተሰበሰበው የደምና የአክታ ናሙና ወደ አህሪ ላብራቶሪ ይወሰዳል :: እንዲሁም ሌሎች በዚህ ጥናት የማይጠኑ ነገር ግን ከዚህ ጥናት ጋር የሚያያዙ በሰውነታችን የሚገኙ ቅመሞችን ለማጥናት ናሙናው ለ5 አመት ያክል በአህሪ ላቦራቶሪ ይቀመጣል።

<u>ስጋትና ጉዳት</u>

በአጠቃላይ ከላይ የተጠቀሱት ናሙናዎች በሰጠነ የነርስ ባለሙያ የሚወሰዱ ሲሆን ህክምናው የሚያስገድደውን የአሰራር ሂደት ስለምንከተል ሊያጋጥሙ የሚችሉ የህመም ስሜት በጣም አነስተኛ ነዉ :: ቢሆንም የደም ናሙና በሚወስድበት ጊዜ ትንሽ የህመም ስሜት ሊያጋጥም ይችላል፡፡ነገር ግን ይህ ህመም በአጭር ጊዜ ይጠፋል፡፡

<u>ሊያስገኛቸው የሚችሉት ጥቅሞች እና የካሳ ክፍያ</u>

በዚህ ጥናት ውስጥ በመሳተፍዎ በጥሬ ገንዘብ የሚደረግ የካሳ ክፍያ አይኖርም:: ነገር ግን ለመጓጓዣ ወጭዎ የሚሆን ፬ ብር ማካካሻ ይሰጥዎታል፡፡የ አኤች አይቪ የ ደም ምርመራ ና የምክር አግልግሎት ስለ አጠቃላይ የምርመራው አካሄድ፡-ምን እንደሚነግረን፣ምን ያክል ጊዜ እንደሚወስድ፡እንደት ራስዎን ከበሽታው መከላከል እንዳለብዎት እንዲሁም ከጤና ባለሙያው ጋር ማንም የርስዎን መረጃ በማያገኝበት መልኩ ውጤትዎን ይዎያያሉ። በተጨማሪም የስኳር በሽታ እና ኤችአይቪ በደመዎ ከተገኘ ለበለጠ ህክምና እና እንክብካቤ በአቅራቢያዎ ወደሚገኝ የጤና ተቋም ይላካሉ፡፡

<u>የጥናቱ ምስጢራዊነት</u>

ማንኛውም በጥናቱ የሚገኙ መረጃዎች በምስጢር ይጠበቃሉ:: የጥናቱ መረጃዎች በሙሉ የሚቀመጡት ከእርሶ ስም ጋር ሳይሆን ለጥናቱ ተብሎ በሚሰጠው ስውር ቁጥር ሲሆን ጥናቱን ከሚያስከሄዱት ባለሙያዎች በስተቀር ማንም ሊያውቅ አይችልም፡; የእርስዎን ማንነት በሚገልጥ መልኩ የተዘጋጀውን መረጃ በፌርማዎ የተረጋገጠ ፍቃድ ሳናገኝ ይፋ አናደርግም::ይህ ጥናት ሳይንሳዊ መረጃ እንደመሆኑ መጠን በወረቀት ታትሞ ቢወጣ ወይንም በሚድያ ቢነገር የእርስዎ ስም በምንም መልኩ አይጠቀስም፡:

<u>*ያለመቀበል ወይም ጥሎ የመውጣት መበት*</u>

በዚህ ጥናት ውስጥ የሚኖርዎት ተሳትፎ ሙሉ በሙሉ ፈቃደኝነት ላይ የተመሰረተ ይሆናል:፡በማንኛውም ጊዜ ይህንን ጥናት የማቋረጥ መብትዎ ሙሉበሙሉ የተጠበቀነው:: በጥናቱ ባለመሳተፍዎ ወይም ከጥናት በመገለልዎ ምክንያት በአሁኑ ወይም የወደፊት የህክምና እርዳታ ላይ ተፅዕኖ አይኖረውም፡ከዚህ በፊት ሲያገኙ ከነበሩት ጥቅሞች አንዳች ነገር አይጐሎቦትም፡ጥናቱን የሚያከናውነው አካል ወይም ድጋፍሰጭ አካል ከእራስዎ ጥቅም ሲባል በጥናቱ እንዳይሳተፉ ሊከለከል ይችላል፡፡

ጥያቄ ካለወት

ስለ ጥናቱ ማንኛውንም ጥያቄ ወይም እርስዎ በዚህ ጥናት ውስጥ ለሚኖርዎት ድርሻ፣አሳሳቢ ጉዳት ወይም ቅሬታ ካለዋት የሚከተሉትን ስልኮች ወይም ኢሜል አድራስ መጠቀም የጥናቱን ባለቤቶች ማነጋገር ይችላሉ፡፡

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አህሪ/አለርት የምርምር ስነ ምግባር ኮሚቴ፡ ስልክ 0113-481285

Patient consent form for study participant- English version

Participant code _____

Participant full name _____

I am informed fully in the language I understand about the aim of above mentioned research. I understood the purpose of the study entitled with " Impact of diabetic mellitus on the immune response to pulmonary tuberculosis in Addis Ababa ,Ethiopia ."I have been informed that medical history, HIV test, diabetes test, sputum and around 3 teaspoonful of blood samples will be taken with minimal risk and stored at AHRI laboratory, if future analysis is needed related with the study. I have also informed that I will be Paid 50 ETB for a reimbursement for transportation cost. In addition I have been told all the information collected throughout the research process will be kept confidential. I understood my current and future medical services will not be affected if I refused to participate or with draw from the study.

I have also been asked for the left over samples taken from my body for the research to be stored at AHRI to answer relevant questions that might arise after a research period.

_____Agree Not agree_____

Therefore I give my consent freely for my participation in this study.

Patient Name _____ signature _____ Date_____

Investigator name _____signature _____Date_____

Witness

1. Name _____signature _____date _____
2. Name _____signature _____date_____

የስምምነት ቅፅ (የአማርኛ ግልባጭ)

የተሳታፊው ልዩ መለያ ቁጥር _____

የተሳታፊው ስም _____

እኔ ስሜ ከላይ የተጠቀሰው ተሳታፊ የስኳር በሽታ በሳንባ ነቀርሳ የቲ-ህዋሳት የመከላከያ ምላሽ ላይ የሚያመጣው ተፅዕኖ በሚል ርዕስ በታሰበው ምርምር ላይ በሚገባኝ ቋንቋ በቂ መረጃ አግኝቻለሁ ፡፡ የህክምና መረጃ፣HIVመረመራ ፣ የስኳር ምርመራ ና የአክታ አንዲሁም 3 የሻይ ማንኪያ የሚሆን የደም ናሙና ምንም አይነት ጉዳት በማያደርስ መልኩ እንደሚወሰድ ተረድቻለሁ ፡፡ በተጨማሪም የሚወሰዱ ማናቸውም መረጃዎች በሚስጥር እንደሚያዙ ተነግሮኛል ፡፡ለመጓጓዣ ወጪ የሚሆን ፱ ብር ማካካሻ እንደሚሰጠኝ ተነግሮኛል። እንድሁም የምጠየቀውን መረጃ ያለመስጠትና ለጥናቱ ያለመሳተፍ ከጥናቱ በማናቸውም ወቅት ራሴን ማግለል እንደምችል የተገለፀልኝ ሲሆን ይህንንም በማድረጌ ወደፊትም ሆነ አሁን የማገኛቸው የህክምና ግልጋሎቶች እንደማይጓደሉብኝ ተገንዝቤአለሁ፡፡

ከሚወሰዱት ናሙናዎች የተረፉት በአህሪ ላቦራቶሪ እንደሚቀመጡና አስፈጊ ቀጣይ ምርመራዎች ሊደረግ እንደሚችል ተነግሮኛል ፡፡

_____እስማማለሁ _____አልስማማም

በመሆኑም ለዚህ ምርምር ለመሳተፍ ወስኛለሁ ፡፡

የታካሚ/ የተሳታፊ ስም _____ፊርማ ----------ቀን ----------

የተመራማሪ ስም ----------ፊርማ ----------ቀን ----------

ምስክሮች

1.ስም ----------ፊርማ ----------ቀን ----------

2.ስም ----------ፊርማ----------ቀን----------

Annex- 3 - Questionnaire

Jimma University

College of Health Science

Department of Medical Laboratory Sciences & Pathology

Interviewer administered questionnaire for Masters research project on the effect of diabetes mellitus on T-cell immune response among pulmonary tuberculosis patients in Addis Ababa, Ethiopia.

Directions for Data Collectors: - Please mark the answers with '√'symbol and put the phrases or words on the space provided accordingly.

Participant Code ☐☐☐		Date of interview (dd/mm/yyyy)_____/_____/_____
Address: Sub city_____ Woreda		Institution Name
Phone Number		
Interviewer Name and Sign _____		AHRI Lab No

Part I. Socio-demographic characteristics

1. Sex ☐ 1. Male ☐ 2.Female
2. Age ☐☐
3. Marital status ☐ 1.Single ☐ 2 . Married ☐ 3. Divorced ☐ 4. Widow ☐ 5.Cohabit
4. Body Mass Index Calculation

4.1. Height (Meter) ☐.☐☐

4. 2. Weight(Kg)) ☐☐.☐☐

4.3. Body Mass Index (Kg/m^2))☐☐.☐☐

5. Occupation: ☐ 1.Farmer ☐ 2.Government worker ☐ 3.Self employee ☐ 4.Student ☐ 5.retire ☐ 6.Merchant/Business man ☐ 7. Others (specify)
6. Education level: ☐ 1.Illiterate ☐ 2. 1-4 grade ☐ 3. 5-8 grade ☐

4. 9-10 completed ☐ 5. 11-12 ☐ 6.College / University

7. Residence: ☐ 1.Urban ☐ 2. Rural

8. Monthly income (ETB): ☐ 1. < 1000 ☐ 2. 1001-1500 ☐ 3. 1501-2000 ☐ 4. 2001-2500 ☐ 5. 2501-3000 ☐ 6.3001-3500 ☐ 7.> 3500

9. Number of household members lives in together.

☐ 1. 1-2 ☐ 2.3-5 ☐ 3.6-8 ☐ 4.> 9

Part II. Patient Medical History

1. Do you have any contact history with a known TB patient or with chronic cough?

   ☐ 1. Yes ☐ 2.No ☐ 3. I don't remember

2. Have you been vaccinated with BCG? (*look for the visible scar on the deltoid*) ☐

   1.Yes ☐ 2. No

3. Do you have asmoking habit? ☐ 1.Yes ☐ 2.No

4. Do you have habit of consuming alcohol? ☐ 1. Yes ☐ 2. No

5. Did you have a history of TB disease previously? ☐ 1. Yes ☐ 2. No

5.1. If yes, have you been treated? ☐ 1. Yes ☐ 2. No

5.1.1 If yes, how was the treatment outcome?

☐ 1. Completed ☐ 2.Defaulted ☐ 3. Retreatment ☐ 4.Relapsed

6. Current manifestations of Tuberculosis symptoms

Check all that apply and state duration of symptoms (actual weeks)

Symptoms	**1.Yes**	**2.No**
6.1.Cough		
6.2.Unexplained fever		
6.3.Chest pain		
6.4.Sputum production		
6.5.Coughing up blood		
6.6.Shortness of breath		
6.7.Night sweats (persistent that leaves sheet and bedclothes wet		
6.8.Loss of appetite		
6.9.Unexplained weight loss		
6.10.Unexplained Fatigue (very tired for no reason)		

7. Chest X-ray ☐ 1. Yes ☐ 2. No

If yes,

7.1.Result: ☐ 1. Suggestive of TB ☐ 2.Not suggestive of TB

8. HIV status (*have to be tested*): ☐ 1. Positive ☐ 2. Negative
9. Pregnancy status (*Based on regularity of menstruation cycle*)? ☐ 1. Yes ☐ 2. No
10. Have ever diseased for intestinal parasites for the last 6 months? ☐ 1. Yes ☐ 2. No
11. Do you have a chronic disease? (*Review the patient records)* ☐ 1. Yes ☐ 2. No

11.1 If yes, what is that? 1 ☐ Diabetes mellitus ☐ 2.Hypertension

☐ 3. Renal disease ☐ 4. Malignancies ☐ 5. Others (specify) ----------

12. Are you taking medication now? (*The interviewer should look the records / sample drugs*) ☐ 1. Yes ☐ 2. No

12.1. If yes what type? ☐ 1. Immunosuppressive drugs/steroids ☐ 2. Antibiotics

Part III. Diabetes Mellitus status

1. Have you been previously identified as having impaired glucose tolerance or gestational Diabetes**?** ☐ **1.** Yes ☐ 2. No ☐ 3. I don't remember

2. Was there a family history of diabetes mellitus? ☐ 1. Yes ☐ 2. No
3. Do you have the following symptoms of diabetes

Symptoms	**1.Yes**	**2.No**
3.1.Polyuria		
3.2. Polydipsia		
3.3.Polyphagia		
3.4. Blurred vision		
3.5.Unexplained weight loss		
3.6.Extreme tiredness		
3.7. Slow healing of cut /wounds		

4. Do you have confirmed Diabetes? ☐ 1. Yes ☐ 2. No
5. If yes for Q 4 , types of Diabetes mellitus

   ☐ 1. Type 1 DM ☐ 2. Type 2 DM

6. How often do you check your glucose level?

☐ 1. Daily ☐ 2. Weekly ☐ 3.Monthly ☐ 4. Other

specify_____

7. Diabetic complication if available

Common complications	1.Yes	2.No
7.1.Neuropathy		
7.2.Ketoacidosis		
7.3.Diabetic retinopathy		
7.4.Nephropathy		
7.5. Heart disease		
7.6. Chronic wound infections		
7.7.Sexual impotency		

8. Type of treatment/Medication you are taking for control of your diabetes status

Medication /control measure	**1.Yes**	**2.No**
8.1. Insulin injection		
8.2.Oral Anti-diabetics (specify the name of the drug)		
8.3.Diet control alone		
8.4.Physical activity		

Remarks:_____

_____

Sample request form

Participant ID No----------

Sample type	**Volume (ml)**	**Date/time of collection**	**Approval/remark**
Blood			
Sputum			

Blood glucose test result

Type of test	**Time of test**	**Result in mg/dl or %**	**Approval /Remark**
Random blood glucose			
Fasting blood Test			
HbA1C			

ጂማ ዩኒቨርሲቲ

ጤና ሳይንስ ኮሌጅ

የህክምና ላቦራቶሪ ሳይንስ እና ፓቶሎጅት/ትክፍል

የስኳር ህመም በሳንባ ነቀርሳ በሽተኞች የቲ ህዋሳት መከላከያ ምላሽ ላይ የሚያመጣውን ተፅዕኖ ለማጥናት የተዘጋጀ መጠይቅ፡፡

ለመረጃ ስብሳቢ መመሪያ፡እባክዎ ለሚሰጡት መልሶች የ √ ምልክት በማድረግና በተሰጠው ባዶ ቦታ ላይ በመጻፍ መልሶቹን ያሰቀምጡ፡፡

የተሳታፊ መለያ ቁጥር		የ ቃለ መጠይቁ ቀን
አድራሻ		የተቻሙ ስም
ስልክ ቁጥር		
የጠያቂዉ ስም እና ፊርማ		የ አህሪ ላቦራቶሪ ቁጥር

ክፍል አንድ፡አጠቃላይ መረጃ

1. ጾታ ☐1.ወንድ ☐2.ሴት
2. እድሜ ☐☐
3. የጋብቻሁኔታ ☐ 1. ያላገባ/ች ☐ 2. ያገባ/ች ☐ 3. አግብቶ/ታየፈታ/ች ☐ 4. ሳይጋቡ አብሮ የሚኖሩ
4. ክብደት በቁመት ሲለካ(ኪ.ግ/ሜ$^{2)}$ ☐☐.☐☐

   ቁመት(ሜትር) ☐.☐☐

   ክብደት(ኪ.ግ) ☐☐.☐☐
5. ስራ: ☐ 1.ገበሬ ☐2. የመንግስትሰራተኛ ☐ 3. የግል ☐ 4. ተማሪ ☐ 5. ጡረተኛ ☐ 6. ነጋዴ ☐ 7. ሌላ ካለ ይገለጽ----------
6. የትምህርት ደረጃ ☐ 1.ያልተማረ ☐ 2. 1-4 ክፍል ☐3.5-8 ክፍል ☐4.9-10ክፍል ☐ 5.11-12 ክፍል ☐ 6. ኮሌጅ ወይም ዩኒቨርስቲ ደረጃ
7. የመኖሪያ አድራሻ፡ ☐ 1. ከተማ ☐ 2. ገጠር
8. ወርሀዊ ገቢ: ☐< 1000 ☐1001-1500 ☐ 1501-2000 ☐ 2001-2500 ☐ 2501-3000 ☐ 3001-3500 ☐> 3500

ክፍል ሁለት፡-የታካሚ ሁኔታ /የህክምና መረጃ

1. በቲቢ በሽታ ከተያዘ ወይም ለብዙ ጊዜ የቆየ ሳል ካለባቸው ስዎች ጋር ግንኙነት/ አብርው የመኖር አጋጣሚ ነበረዎት? ☐1. አዎ ☐ 2. የለም ☐ 3. አላስታዉስም
2. የቢሲጅ (የቲቢ ክትባት) ወስደዋል? ☐ 1. አዎ ☐ 2. አልዎሰድኩም *(ጠባሳዉን ጡንቻ ላይ ይመልከቱ)*
3. ሲጋራ ያጨሳሉ? ☐ 1.አዎ ☐ 2. አላጨስም
4. የአልኮል መጠጥ ይጠጣሉ? ☐ 1. አዎ ☐ 2. አልጠጣም
5. ከዚህ ቀደም በቲቢ በሽታ ታመው ያዉቃሉ? ☐ 1.አዎ ☐ 2.ታምሜ አላውቅም

ሀ፡አዎ ካሉ ታክመዋል? ☐ 1.አዎ ☐ 2. አልታከምኩም

ለ፡አዎ ካሉ የህክምናው ዉጤት እንዴት ነበር? ☐ 1. አጠናቅቄለሁ ☐ 2. አቋርጫለሁ

☐ 3. እንደገና መድሃኒት እየዎሰድኩ ነው ☐ 4.አግርሽቶብኛል

6. በአሁኑ ወቅት የሚታዩ የቲቢ ህመም ምልክቶች፤ ሁሉም ምልክቶች መኖራቸውን ያረገግጡ

የህመም ምልክት	አለ	የለም
ሳል		
ከመጠን በላይ የሰውነት ሙቀት መጨመር		
የደረት ህመም		
የአክታ መኖር		
ደም የተቀላቀለበት አክታ •		
የትንፋሽ ማጠር		
ማታ ማታ ማላብ •		
የምግብ ፍላጎት መቀነስ		
ከመጠን በላይ የሰውነት ክብደት መቀነስ		
ምክንያት የሌለዉ የድካም ስሜት		

7. የደረት ራጅ ☐ 1.ተሰርቶአል ☐ 2.አልተሰራም

ከተሰራ፡ ዉጤቱ ☐ 1.የቲቢ በሽታን ያመላክታል ☐ 2.የቲቢ በሽታን አያመለክትም

8. የ ኤችአይቪ ምርመራ፡-ቫይረሱ በደም ዉስጥ ☐ 1.አለ ☐ 2. የለም
9. የእርግዝና ሁኔታ(የወር አበባ ዑደት በመጠየቅ) ☐ 1. እርጉዝ ☐ 2.ያላረገዘች

10. ላለፉት 6 ወራት የአንጀት ጥገኛ ተህዋሲያን በሽታ ታመዉ ያዉቃሉ? ☐ 1. አዎ ታመሜ ነበር.

☐ 2. አልታመምኩም

11. ለረጅም ጊዜ የቆየ ህመም አለብዎት? (*የታካሚ ማህደር ይታይ)* ካለብዎት ምንድን ነው; ☐ 1. የስኳር በሽታ ☐ 2.የደም ግፊት ☐ 3.የኩላሊት ህመም ☐ 4. ካንሰር ወይም እጢ ☐ 5. ሌላ ካለ ይጠቀስ

12. አሁን የሚዎስዱት መድሃኒት ካለ፤ምን አይነት ነው (*መድሃኒቱ ወይም የታካሚ ማህደር ይታይ)*?

☐ 1.የሰዉነት የመከላከል አቅምን የሚቀንስ መድሃኒት ☐ 2.አንቲ-ባዮትክ (ለበሽታ አምጭ ተህዋሲያን የሚሰጥ)

ክፍል ሶስት፡የስኳር ህመምን በተመለከተ

1. ከዚህ በፊት የደም ስኳር የመጨመር ወይም ከእርግዝና ጋር በተያያዘ የሚመጣ የደም ስኳር የመጨመር ሁኔታ አጋጥምዎት ያውቃል? ☐ 1.አዎ ☐ 2.አጋጥሞኝ አያውቅም ☐ 3.አላስታዉስም
2. ከቤተሰብዎ አባል/ የዘር ግንድ የስኳር ህመም ያለበት አለ? ☐ 1. አዎ ☐ 2.የለም
3. የሚከተሉት የስኳር ህመም ምልክቶች አለብዎት?

ምልክቶች	አለ	የለም
1.ቶሎ ቶሎ ሽንት መሽናት /ሽንት ቤት መጎብኘት		
2. አሁንም አሁንም ዉሀ ማለት/ከፍተኛ የዉሃ ጥም		
3.ከፍተኛ የረሀብ ስሜት		
4.የማየት ችሎታ ለውጥ/ ብዥብዥ ማለት		
5.ምክንያቱ ያልታወቀ የክብደት መቀነስ		
6.ድካም /አቅም ማጣት		
7. ሰዉነት ሲቆረጥ፣ሲቆስል፣ሲያብጥና ሲያሳክክ ቶሎ አለመዳን		

4. የስኳር ህመም አለብዎት; ☐ 1 .አዎ ☐ 2. የለብኝም
5. ለ4ኛ ጥያቄ አዎ ካሉ 5-8 ያሉት ጥያቄዎች ይመልሱ፡ የስኳር ህመም አይነት ☐ 1.አንደኛው ☐ 2. ሁለተኛው
6. በምን ያህል ጊዜ ነው የደም ስኳር ደረጃዎን የሚለኩት----------
7. በስኳር ህመምጋር በተያያዘ የሚመጡ ዉስብስብ ችግሮች ካለብዎት

ዉስብስብ ችግሮች	አለ	የለም
1.የነርቭ መደንዘዝ/ህመም		

2.ኬቶአሲዶሲስ /የኢንሱሊን እጥረት		
3. የአይን ህመም/ሬቲኖፓቲ		
4.የኩላሊት ህመም		
5. የደምግፊት እና የልብ ህመም		
6. ለብዙ ጊዜ የቆዬ ኢንፌክሽን		
7.የግብረ ስጋ ግንኙነት ማድረግ አለመቻል		

8. የሚዎስዱት የመድሃኒት አይነት/ህመምዎን የሚቆጣጠሩበት ዘዴ

የሚዎስዱት የመድሃኒት አይነት/ህመምዎን የሚቆጣጠሩበት ዘዴ	አለ	የለም
1. ኢንሱሉን		
2. በአፍ የሚወሰድ ኪኒን (የመዲሃኒቱ ስም----------)		
3. አመጋገብን ማስተካከል ብቻ		
4. በአካልብቃት እንቅስቃሴ		

አስተያየት ካለዎት----------

Annex- 4- Declaration Sheet

I, the undersigned, MSc Medical Microbiology student declare that this thesis is my original work in partial fulfillment of the requirement for the degree of Master of Science in Medical Microbiology. Where others work has been used, it has been carefully acknowledged and referenced in accordance with the regulations of the University and meets the accepted standards with respect to originality and quality of the requirements.

Student

Zebene Andargie Signature _____ Date_____

This thesis has been submitted for examination with my approval as an adviser

Advisors/ Co-investigators

1. Dr. Tesfaye Kassa (Jimma University)
   Signature _____Date_____

2. Dr. Getenet Beyene (Jimma University)

   Signature_____ Date_____

This thesis has been submitted with my approval as an examiner.

Name of examiner: Mulualem Tadesse (PhD fellow)

Signature: _____ Date: _____