ADDIS ABABA UNIVERSITY

COLLEGE OF HEALTH SCIENCES, SCHOOL OF ALLIED HEALTH SCIENCES,

DEPARTMENT OF MEDICAL LABORATORY SCIENCES

Immunological and lipid profile among leprosy patientsat ALERT Centre, Addis Ababa Ethiopia

A thesis submitted to the school of graduate studies of Addis Ababa University in partial fulfilment of the requirements for the Degree of Masters of science in clinical laboratory science, Diagnostic and Public Microbiology special track.

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Feb, 2016

Acknowledgement

I am very grateful to my advisors, Ato Edessa Negera, Ato kassuDesta and Dr. Ibrahim Ali. And I also like to thank Dr. KidistBobosha, BirtukanEndale,YonasBekele for theirunreserved support and giving me constructive advice and guidance that helped me to materialized this document.

I would also like to express my gratitude to All African Leprosy, Tuberculosis and Rehabilitation Training Centre and Armauer Hansen Research Institute for the financial support of my thesis work.

My appreciation also extends to MulugetaWoji, RobelGesesse, MetasebiaTegegn and to all ALERT Laboratory staffs.

Acronyms

BB – mid-borderline

BL – borderline lepromatous

BT – borderline tuberculoid

CMI – cell-mediated immunity

CRP – C-reactive protein

ENL – erythema nodusum leprosum

HDL – High-density lipoprotein

HLA – Human leukocyte antigen

LDL – Low-density lipoprotein

LL – lepromatous leprosy

MB – Multi-bacillary

MDT – Multidrug therapy

PB – Pauci-bacillary

RR – reversal reactions

SAA – serum amyloid A

TB – tuberculosis

TG – triglyceride

TNF – tumor necrosis factor

TT – polar tuberculoid

VLDL – very low-density lipoprotein

Operational definitions

C Reactive protein – is a pentameric protein found in blood plasma known as acute-phase proteins, produced in the liver: whose plasma concentrations increase by 25% or more during inflammatory disorders

Lipid – Lipid is a molecule that contains hydrocarbons and make up the building blocks of the structure and function of living cells any substance that contains fat and that is important parts of living cells and also soluble in nonpolar organic solvents but insoluble in water.

Lepromatous leprosy (LL) –a patient has LL when he/she has widely disseminated nodular lesions with ill-defined borders and BI above 2

Tuberculoid leprosy (TT) – patients with either one large red patch with well- defined raised boarders or a large hypopigmented few asymmetrical spots.

Paucibacillary: A patient classified as PB when he/she has two to five skin lesions with negative skin smear result.

Multi-bacillary: A patient is classified as MB when he/she has more than five skin lesions or positive skin smear results at any site with BI above 2.

Erythema nodusum leprosum (ENL) - a patient has ENL when he/she has tender erythematous skin lesions and systemic features of disease often fever, neuritis and bone pain.

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Abstract

Background: Leprosy is an old chronic infectious disease that continues to be an important public health problem in several developing countries.Host immunologic factors contribute to the clinical outcome in leprosy. This infection is also accompanied by several alterations in lipid metabolism and changes inthesynthesis of a number of acute phase proteins.

Objective: To assess the immunological and lipid profile of leprosy patients across the disease spectrum

Material and Methods: A crossectional study was conducted at ALERT center which included 30 leprosy patients and 21 healthy controls. An 8 ml of blood sample was collected from30 patients and 21 healthy controls and used for immunological and lipid profile assays. About 200µl of whole blood was stained with antibody conjugated flourochromes and data acquisition was done on FACSCanto II flowcytometer. Lipid profile and C reactive protein tests have been done using chemistry analyzer and qualitative test respectively. Mann-Whitney U test were used to analyze Memory T cells and lipid profile analysis.

Result: The percentage of memory T cells (CD45RO+) expressed on CD3+ was significantly higher in PB patients than MB patients. Activated T cells (CD62L-) expressed on CD4+ were significantly higher in PB patients than in MB patients. MB patient's CD3+ effector T cells were significantly higher than the healthy controls. PB patients showed significantly higher CD3+, CD4+ and CD8+ effector memory cells than MB patients. Regarding the lipid profile it showed that, healthy control serum total cholesterol level was significantly higher than MB patients. On the contrary serum HDL level was significantly higher in healthy control than MB patients.

Conclusion: This study provides further evidence that there is a significant difference in lipid profile among leprosy spectrum. The percentage of both activated and memory T cells in MB patients are significantly lower than in PB patients and this confirms the unresponsiveness of T cells in MB patients regardless of the high bacterial load in these patients.

Recommendation: We recommend that monitoring and regulate the lipid homeostasis of leprosy patients may be useful. We also recommend further study on host immune responses in leprosy diseases in which lipid metabolism and inflammation intersect.

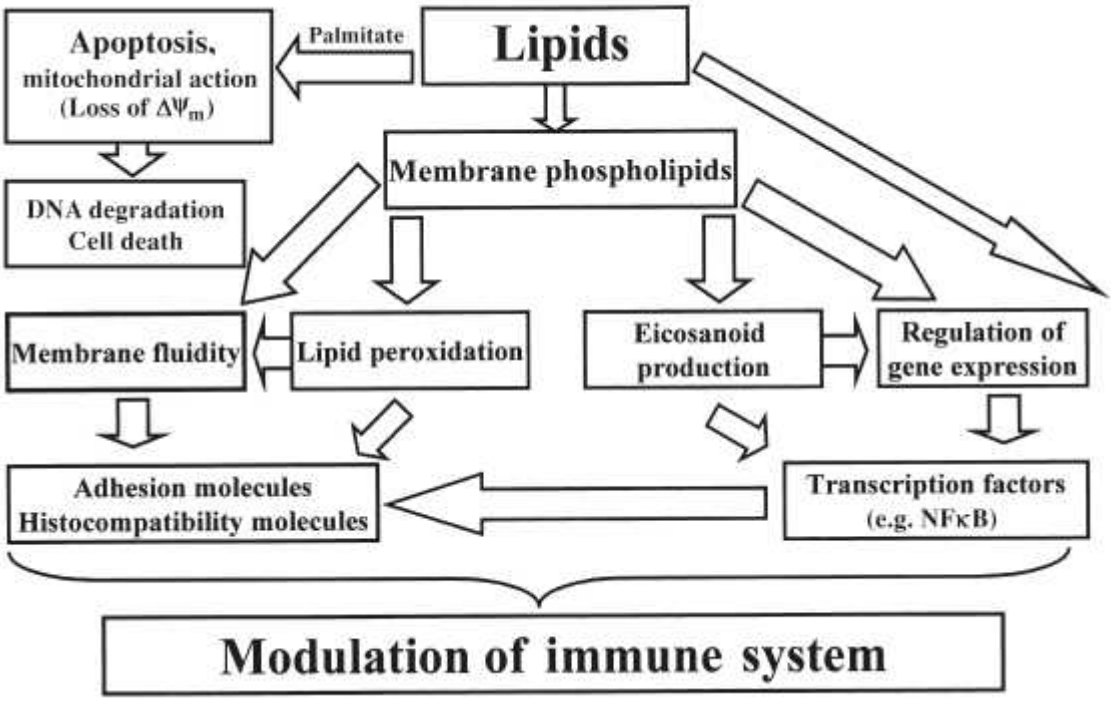
1. Introduction

1.1 Background

Leprosy or Hansen disease is a bacterial disease caused by *Mycobacterium leprae*, an obligate intracellular pathogen, which infects the skin and peripheral nerves. *M. leprae* invasion of Schwann cells leads to nerve damage, disability and deformity. The precise mechanism of transmission of Mycobacterium leprae is unknown. No highly effective vaccine has yet been developed, and extensive laboratory efforts have not yet produced any practical tools for early diagnosis of clinically unapparent disease(1). WHO 2013 report showed, globally 180, 618 leprosy patients were on record fortreatment. However, not all infected patients have the same clinical course(2). Ethiopia is one of the countries with high prevalence and incidence of leprosy. All clinical forms of leprosy exist in the country with high prevalence of leprosy reactions.

The course of the disease may be punctuated by spontaneous and/or recurring episodes of immunological imbalances that need immediate medical attention and immune suppressive treatment. Leprosy is characterized by five spectrums of clinical forms depending upon the host's immune response (3). At one end of the spectrum there is lepromatous leprosy (LL) which is associated with impaired cell-mediated immunity (CMI) and an uncontrolled replication of the *Mycobacteriumleprae* which results in high bacterial load (4). At the other end of spectrum there is tuberculoid leprosy (TT) which is associated with pronounced CMI and low numbers of *Mycobacterium leprae* in skin macrophages (4). In tuberculoid leprosy the infection is usually localized to either a skin patch or nerve trunk. The majority of patients, however, fall into a broad borderline category between these two polar forms; this is subdivided into borderline lepromatous (BL), mid-borderline (BB), and borderline tuberculoid (BT)(1).Additionally leprosy patients can develop acute reactional complications which are characterized as reversal reactions (RR) or erythema nodusum leprosum (ENL)(5).

The host responseto infection and inflammation is accompanied by several alterations in lipid metabolism and also changes in the hepatic synthesis of a number of acute phase proteins that play a crucial role in maintaining homeostasis during the course of infection and inflammation. The stimulation of acute phase protein synthesis and the changes in lipid metabolism during infection and inflammation are now thought to be mediated by cytokines(4). It has also been shown that dietary lipid manipulation may affect a great number of immune parameters, such as


Apoptosis,
mitochondrial action
(Loss of $\Delta\Psi_m$)
Palmitate
Lipids
Membrane phospholipids
DNA degradation
Cell death
Membrane fluidity
Lipid peroxidation
Eicosanoid
production
Regulation of
gene expression
Adhesion molecules
Histocompatibility molecules
Transcription factors
(e.g. NFκB)
Modulation of immune system

1.2 Statement of the problem

Leprosy is a granulomatous disease affecting the skin and peripheral nerves and ranks as the second most pathogenic mycobacterial infectious disease after tuberculosis (7),(8). Leprosycontinues to be a significant public health problem. It is not going to disappear anytime soon. Effective multidrug regimens are now used worldwide, and the infection in individuals is curable. However, although the reported number of registered cases worldwide has declined in the last two decades, the reported number of new cases registered each year has remained the same (at 500,000 to 700,000) over the same interval. Mathematical modeling of the potential decline in leprosy incidence and prevalence, using various premises regarding efficacy of treatment and prevention, suggests that the disease will remain a major public health problem for at least several decades.The precise mechanism of transmission of Mycobacterium leprae is unknown. No highly effective vaccine has yet been developed, and extensive laboratory efforts have not yet produced any practical tools for early diagnosis of clinically unapparent disease (1).

Multidrug therapy (MDT) cures the infection, but immunological reactions may occur and neuropathy may lead to disability and deformity even after the patient declared cured. It is important that the manifestations of the condition are recognized as early as possible so that early nerve damage can be identified and treated rapidly (8). In spite of nearly many decades of intensive research into the immunology of leprosy, the mechanism by which *M. leprae* is able to elicit the entire range of human cellular immune responses has still not been explained. Only recently has the probable mechanism of intracellular killing of *M. leprae* been identified. The regulation of cell-mediated immunity to *M. leprae* by cellular and cytokine interactions continues to be investigated(1).

The majority of individuals infected by M. leprae do not get sick, with only a small portion developing clinical manifestations. This makes the disease even more intriguing, and raises questions about the factors responsible for the greater susceptibility of somepeople. The balance between Th1 and Th2 responses was for a long time utilized to explain the clinical forms of the disease and the susceptibility to getting sick, since the spectrum of clinical manifestations is related to the immunological response pattern of the host. The cell-mediated immune response characterizes the PB form, with a predominance of Th1 cytokines, limitation of bacillary proliferation and relative resistance to the pathogen, while the lack of Th1 response and predominance of Th2 response characterizes the MB form, which has intense bacillary

multiplication. However, the development of the immune response, whichcontrols the growth of the pathogen and limits the tissue lesioncaused by an exacerbated response, is important for the resolution of the infection.

Several major studies of local immune responses in leprosy skin lesions have been published. These are difficult to compare because they have used different designs, different methods of quantification, and different conventions to express their results.A study by immunohistological staining reveals that, TT lesions exhibited mostly $CD4^+$ helper cells with a $CD4^+/CD8^+$ ratio of 1.9:1 (9). Although the $CD4^+/CD8^+$ ratio in normal peripheral blood is also 2:1, there appeared to be a preferential migration into, proliferation in or retention of selected cells in the various types of leprosy lesions in those cells of the T helper/memory phenotype outnumbered the naive phenotype 14-fold in TT lesions. T cytotoxic cells were numerous in TT lesions. These cells may play a role in mediating the macrophage localization, activation, and maturation that lead to restriction or elimination of the pathogen. Interestingly, $CD4^+$ cells were distributed throughout the lesion, whereas $CD8^+$ cells were stationed at the periphery in the TT lesion(10).

LL lesions, in contrast, displayed a $CD4^+/CD8^+$ ratio of 0.6:1, and unlike TT lesions, the $CD8^+$ T cells were distributed throughout the lesion rather than at the periphery. Using monoclonal antibodies which could distinguish T-cell subsets, it was found that the $CD4^+$ cells present were primarily of a naïve phenotype and the $CD8^+$ cells were predominantly of a suppressor subset; thus, they proposed that these $CD8^+$ suppressor cells may serve to down regulate macrophage activation and suppress cell-mediated immunity (1). The basic defect in lepromatous leprosy is the selective unresponsiveness of T cells to the antigens of *Mycobacterium leprae*. This defect may be partial or complete and does not appear to change with prolonged chemotherapy. A lack of T cell-derived lymphokines such as IFN-γ and IL-2 could account for the inability of macrophages and other cells to eliminate *M. leprae* in these patients(11).

The reversal reaction is associated with a marked rise in lymphocyte transformation to *M. leprae* antigens in vitro, and is therefore thought to be delayed type hypersensitivity (DTH) reaction against *M. leprae* antigens(12).

There are multiple known and undefined factors that modulate the range of susceptibility to clinical outcomes, including metabolic and immune functions. The pathogen *M. leprae* is unique in that its genome has undergone massive decay, particularly in catabolic pathways and energy

generating processes, and is therefore thought to be highly dependent on the host system for growth. In this context, where leprosy is a product of complex host pathogen relationships, there is a need for modern approaches to uncover underlying and/or novel biochemical signals that may be informative regarding those pathways that contribute to disease. It is believed that there are multiple factors (genetic, bacterial, nutritional and environmental), which may explain the differences in clinical manifestations of the disease(13).

The ability to synthesize different lipid moieties and their distribution through plasma to all the body tissues seems to be altered in leprosy. Some research workers have attempted to study the biochemical alterations including the study of lipid metabolism as a guide to early diagnosis. Furthermore, lipids may also play an important role as an etiological agent in various vascular abnormalities seen in leprosy. Thus the study of lipid metabolism in leprosy is of great significance as the lipid metabolism is the hub of pathology of leprosy and cholesterol dynamics in macrophages might harbor the secrets of cultivation of *M. leprae.* Moreover, very few studies are available in the literature about the various lipid fractions and their correlation with the type of leprosy or bacterial indices in individual patients (14).

It has been proposed that, in addition to changes in acute phase protein synthesis, changes in lipid and lipoprotein metabolism are also a part of the acute phase response. The stimulation of acute phase protein synthesis and the changes in lipid metabolism during infection are now thought to be mediated by cytokines which modulate the immune and inflammatory responses (3). The documented studies on the serum values of lipids in leprosy have shown contrary and conflicting observations. A study on Serum lipids and lipoproteins on paucibacillary and multibacillary leprosy showed a decrease in mean level of TG (15). In contrast, another study on serum of lepromatous leprosy who are in nonreactive stage showed an increase in serum TG (16).

C-reactive protein (CRP) and serum amyloid A (SAA) are two such proteins whose levels are often elevated 10- to 100-fold in humans during acute inflammatory episodes and are generally lower in chronic inflammatory conditions. A recent study showed the levels of CRP and SAA are markedly elevated in ENL patients as compared to non-reactional lepromatous patients and endemic controls, indicating that the synthesis of acute phase proteins is stimulated during ENL reactions (3). Other study done on untreated leprosy patients, showed that there CRP has limited significance in detecting ENL reactional patients(17).

1.3 Significance of the study

The significance of the study is to better interpret the clinical feature by linking them to the underlying pathology and immunology and also a potential for clinical monitoring disease progression, particularly during therapeutic intervention. Results from this study will also help facilitate early diagnosis; monitor the progression of the disease and to allow complications to be predicted.

2. Literature review

2.1 Global Epidemiology of Leprosy

The global prevalence of leprosy has declined from 5.2 million in the 1980 s to 200,000 in 2014. However, the new case detection rate remains high. Over the last 8 years, around 220,000-250,000 people have been diagnosed with leprosy each year (18). Despite a spectacular decrease, leprosy is still considered a public health problem in 32 countries, particularly in the African, Asian, and South American continents which cover 92% of all registered cases (19). High number of new cases (210,075) was reported in 2011 in spite of the multi-drug regimen and good public health practices (20). The South East Asian region accounts for about 66.21% of the global prevalence and 71% of all new cases at the end of first quarter of 2013(21). Brazil, India, Nepal, Myanmar, Madagascar, and Mozambique contribute almost 90% to the leprosy cases registered worldwide (22). For instance, the prevalence rate in 2011 reached 1.54 cases per 10,000 inhabitants in Brazil (23). Transmission of leprosy is sustained, as evidenced by the hundreds of thousands of new cases of leprosy that keep being detected globally every year. Contemporary estimates of the frequency of ENL among lepromatous patients ranges from 25% of new multibacillary patients in Brazil, to 26% of lepromatous patient in northeastern Thailand, to 5% in Ethiopia (24).

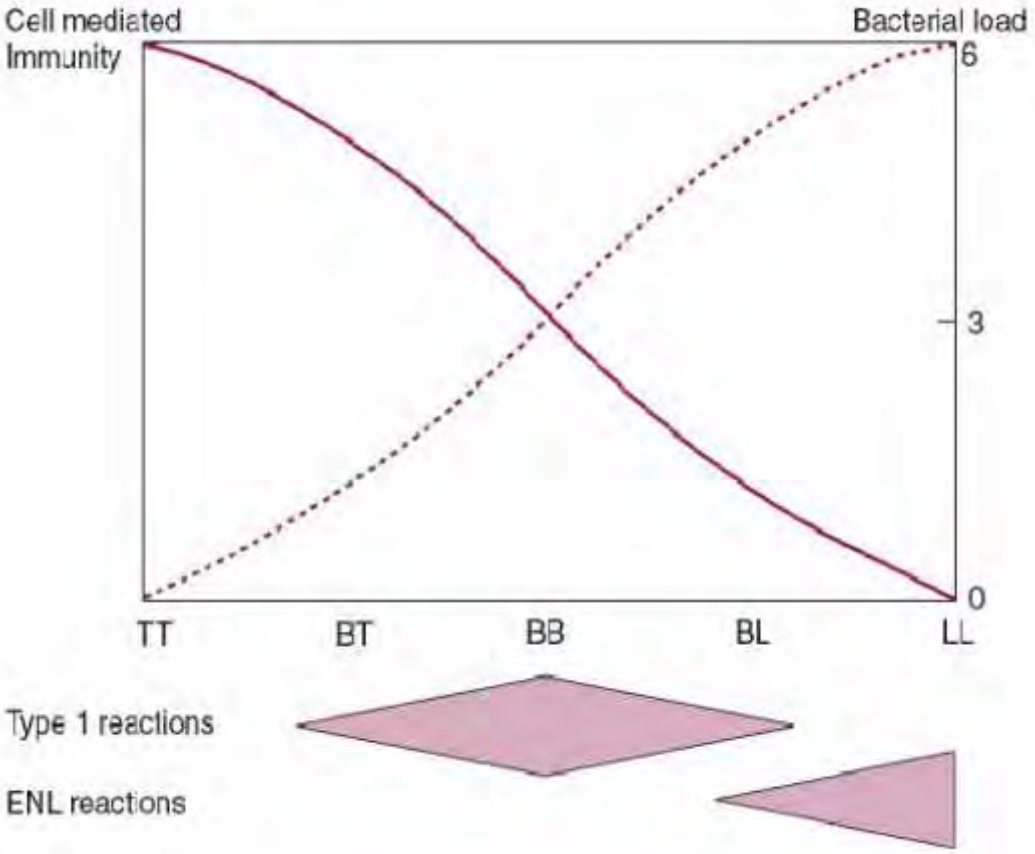
2.2 Epidemiology of Leprosy in Ethiopia

The 1992 WHO report showed that Ethiopia as one of the top 25 counties having the largest number of estimated leprosy cases (25). The 2014, WHO Weekly epidemiological record showed the new case detection trends in countries that reported more than 1000 new cases in the past 5 years, in which Ethiopia detected 4,374 of new cases in 2013. This report also showed a 4, 925 of Registered prevalence in Ethiopia (2). A retrospective study done on the burden of leprosy in children and adolescents in rural southern Ethiopia form1999 to 2011 showed that 298 patients with leprosy were registered for treatment. Multibacillary leprosy was the most common type of leprosy encountered in both age groups, (95.5% of children and in 84.1% of adolescents) among which 27.3% children and 28.6% adolescents had deformities of the hand, feet or eyes (WHO grade II). According the report, childhood leprosy continues to be a common problem in rural southern Ethiopia. Multibacillary disease and disabilities remain common in children (26).

2.3 Classification of leprosy

Leprosy presents a wide range of clinical and histopathological manifestations. This great diversity puzzled and frustrated clinicians and investigators until it was appreciated that this diversity was based on the ability of the host to develop a cellular immune response to *M. leprae* (1). The need for an internationally accepted classification system for leprosy was recognized long ago. The first system proposed at an international meeting was in Manila in 1931. This was followed by systems proposed in Cairo in 1938, Rio de Janeiro in 1946, Havana in 1948 and Madrid in 1953, followed by an Indian classification in 1955. These evolving classifications were based on clinical features with some support from histological and prognostic features and lepromin testing. They separated out the tuberculoid and lepromatous poles and recognized borderline, dimorphous or intermediate categories between the two poles (27).

The first full formulation of this concept was described by Skinsnes as an “immunopathological spectrum” in 1964. Soon thereafter, a practical classification scheme based on the same principles was proposed by Ridley and Jopling (28), enabling a degree of global uniformity in clinical practice that gave renewed momentum to research on this disease. The five-part Ridley-Jopling classification identifies, at one extreme, patient with a high degree of cell-mediated immunity and delayed hypersensitivity, presenting with a single, well-demarcated lesion with central hypopigmentation and hypoesthesia. Biopsies from these patients reveal well-developed granulomatous inflammation and rare acid-fast bacilli demonstrable in the tissues; this is termed as the polar tuberculoid (TT). At the other extreme, patients have no apparent resistance to *M. leprae*. These patients present with numerous, poorly demarcated, raised or nodular lesions on all parts of the body, biopsies of which reveal sheets of foamy macrophages in the dermis containing very large numbers of bacilli and microcolonies called globi. This non-resistant, highly infected form of the disease is termed as polar lepromatous (LL). The majority of patients, however, fall into a broad borderline category between these two polar forms; this is subdivided into borderline lepromatous (BL), mid-borderline (BB), and borderline tuberculoid (BT) (1).


Cell mediated
Immunity
Bacterial load
6
3
0
TT
BT
BB
BL
LL
Type 1 reactions
ENL reactions

tuberculoid granulomas were 1.7, compared with a ratio of 0.6 in lepromatous granulomas. CD4+ cells were distributed throughout the tuberculoid granuloma, with CD8+ cells confined to the mantle surrounding it. In lepromatous leprosy lesions, both CD4+ and CD8+ subsets were admixed throughout the biopsy specimen. Another observation was the ratio of CD4+4B4+ (helper/inducer) cells to CD4+2H4+ (suppressor/inducer) cells was 14:1 in tuberculoid lesions and 1.1:1 in lepromatous lesions. In contrast, the ratio of CD4+4B4+ cells to CD4+2H4' cells in peripheral blood was 1.2:1 in tuberculoid patients and 1.9:1 in lepromatous patients (9) .

Early studies in experimental animals showed that the vast majority of cells infiltrating delayed-type hypersensitivity lesions were non-sensitized, consisting primarily of the cells of the mononuclear phagocyte lineage. While endothelial cells can be induced to express class II antigens and can activate sensitized T cells in vitro, there is little evidence that lymphocytes are locally activated by specific antigen presented by vascular endothelium to enter the local tissue. From a wide variety of studies of lymphocyte trafficking, it would appear that activated T lymphocytes and possibly memory T cells have the ability to home selectively to inflammatory sites. Entry into the tissues may require prior antigen activation, but tissue entry appears to be independent of antigen recognition at the site(8).

Modlin and colleagues studied lymphocyte subsets in the tissues of fourteen patients with leprosy using monoclonal antibodies and a modified immunoperoxidase technique. Two immunohistological patterns were observed. In tuberculoid leprosy, helper-inducer cells were present among the aggregates of mononuclear phagocytes (epithelioid cells), but the suppresser-cytotoxic cells were predominantly in the lymphocytic mantle surrounding the epithelioid cell aggregates. In reversal reaction and lepromatous tissues, the helper-inducer and the suppresser-cytotoxic cells were both distributed among the mononuclear phagocytes (histiocytes). In tuberculoid specimens the Langerhans cells of the epidermis were increased in number as compared to lepromatous and normal tissues(10).

A report on the characteristics of cells in the cutaneous lesions and blood of 21 patients with lepromatous, tuberculoid, and intermediate forms of leprosy showed a large proportion of the infiltrates in lepromatous lesions consist of macrophages heavily parasitized with *Mycobacterium leprae*. The T cells in the lesions are devoid of OKT4/Leu 3a-positive ("helper") cells and consist almost exclusively of OKT8/Leu 2a-positive ("suppressor") populations. In contrast, the

tuberculoid infiltrates contain well-organized epithelioid and giant-cell granulomas and only remnants of bacilli, and the predominant T cell is from the OKT4/Leu 3a-positive subset. In both tuberculoid and lepromatous infiltrates, T cells and macrophages expressed HLA-DR antigen. No marked alteration in the distribution of blood T-cell phenotypes was noted. The study concludes that there is a marked difference between T-cell subsets in lepromatous and tuberculoid infiltrates, which may influence the microbicidal activity of macrophages in the lesions (30).

2.5 Lipid profile in leprosy

The host response to infection and inflammation is accompanied by changes in the hepatic synthesis of a number of acute phase proteins that play a crucial role in maintaining homeostasis during the course of infection and inflammation. C-reactive protein (CRP) and serum amyloid A (SAA) are two such proteins whose levels are often elevated 10- to 100-fold in humans during acute inflammatory episodes and are generally lower in chronic inflammatory conditions. A study have shown that the levels of CRP and SAA are markedly elevated in ENL patients as compared to non-reactional lepromatous patients and endemic controls ,indicating that the synthesis of acute phase proteins is stimulated during ENL reactions (3). The host response to bacterial, viral or parasitic infections is also accompanied by several alterations in lipid metabolism, such as increased serum triglyceride (TG) levels, enhanced hepatic lipid synthesis, and a decrease in lipoprotein lipase activity. Decreased serum cholesterol levels also have been reported during infection (3). The stimulation of acute phase protein synthesis and the changes in lipid metabolism during infection and inflammation are now thought to be mediated by cytokines (4).

A study by Riaz A.Memon et al. investigated alterations in serum lipids in lepromatous Leprosy patients with and without ENL Reactions and their relationship to Acute Phase Proteins. In the study they measured serum triglyceride, total cholesterol, and HDL- and LDL-cholesterol levels. The result showed that serum triglycerides were increased by 37% over controls in LL/BL patients whereas HDL-cholesterol levels were significantly decreased in LL/BL patients. They also revealed that serum triglyceride levels were significantly lower in ENL patients. Serum total cholesterol levels were decreased by 30% in ENL patients Similarly, LDL-cholesterol levels were significantly lower in ENL patients compared to LL/BL patients. Serum HDL-cholesterol levels, which are already lower in LL/BL patients compared to controls, further declined by 44% in ENL patients Thus, as compared to controls, a net decrease of 58% in HDL-cholesterol levels was

observed in ENL patients. They concluded that there are marked changes in serum lipid concentrations in patients with lepromatous disease and ENL reactions (3).

A study in eastern part of Nigeria described the abnormalities in serum lipids and liver function in seventy- three leprosy patients. None of the patient was in reactionary state at the time of study and thirty healthy control subjects were included. There were significant difference between total serum cholesterol of the different group of leprosy patient The mean total cholesterol of the active lepromatous leprosy patient differed significantly from that of inactive lepromatous group and control subjects. The highest triglyceride concentration were observed in active lepromatous leprosy, with a progressively diminishing plasma triglyceride concentrations across the leprosy disease spectrum in order Tg-LL>Tg-BL>Tg-TT. The study suggests that the total serum cholesterol and triglyceride concentrations are higher among lepromatous leprosy patients than in control subjects and in addition total serum triglyceride but not cholesterol concentration is higher in inactive lepromatous leprosy than in control subjects (31) .

In a case control study the concentrations of serum lipids and tumor necrosis factor (TNF) were measured in leprosy patients across the spectrum of the disease and in erythema nodosum leprosum (ENL). The study included 27 untreated leprosy patients with no known history of any reactional episode. The study group also included I2 ENL patients and 14 controls. The study result confirmed pervious study suggesting that patients with lepromatous disease have higher serum triglyceride and lower HDL-cholesterol levels and extends these findings across the spectrum of leprosy. While LL/BL patients had significantly higher serum triglyceride and lower HDL-cholesterol levels similar changes were not observed in BT/TT patients. Furthermore ENL patients have markedly lower serum total, LDL-, and HDL-cholesterol levels as compared to patients with stable lepromatous disease. The study documented the changes in HDL-cholesterol metabolism are a specific part of the host response to lepromatous leprosy and to the ENL reaction, and maybe mediated by increased TNF production (4).

While the above studies support the association between serum lipid and the type of leprosy, this study have found no correlation between the levels of different lipid fractions in the sera, the type of leprosy or the bacterial indices in the study groups. The study included fifty, fresh and untreated cases of leprosy and fifty controls. The study showed that the serum triglyceride level was lower than normal in TT, showed no alteration in BT or BB and was insignificantly increased in BL and LL patients. The total cholesterol was lower than normal in TT, showed no alteration in

BT or BB and was insignificantly increased in BL and LL patients. The total cholesterol was lower than normal in TT, whereas in BT, BB, BL and LL groups the levels were statistically decreased. The HDL cholesterol was significantly decreased in BT and LL patients. The LDL cholesterol in BT, BB, BL and LL groups the levels were statistically decreased. (14).

2.6 The Relationship between C - reactive protein Levels and Leprosy

Human CRP is the classical acute phase protein, the circulating concentration of which increases rapidly and extensively in response to most forms of tissue injury, infection and inflammation. Reich and Tolentino studied the relationship of C-reactive protein levels in lepromatous leprosy to erythema nodosum leprosum. Blood was drawn from 312 patients with leprosy. C-reactive protein (CRP) was evident at the 1+ or higher levels in 57 per cent of the leprosy patients. The study indicated that the elevated CRP levels in leprosy patients were related to the cumulative effect of the various manifestations of EN. It was suggested that CRP determinations could be used as a measure of the success of ENL therapy, both clinically and experimentally(32).

Seventy individuals were selected to study the correlation between TNF Production, increase of plasma C-reactive protein level and suppression of T lymphocyte during erythema nodosum leprosum. The result reveals that TNF and CRP were significantly more elevated in the scrum of patients with ENL, with a positive correlation of about 95% when compared with patients with non -reactive lepromatous leprosy (L) or tuberculoid leprosy (T) or with control individuals. Furthermore, in another series of experiments CRP had a specific and significant suppressive action on concanavalin A (ConA) induced lymphoproliferation in cultures from patients and controls, the reduction being more marked (75%) in patients with ENL(33).

A study was conducted in lepromatous leprosy patients to look for a change in SAA and C-reactive protein in relation to alteration to serum lipid. Both SAA and CRP levels were significantly higher in patients with ENL compared to patients with lepromatous disease and endemic controls; whereas the differences between LL/BL patients and endemic controls were not statistically significant. SAA and CRP have been shown to interact with lipoproteins, and a significant negative correlation was also observed between SAA and total or LDL-cholesterol concentrations; whereas there is no statistically significant correlation between CRP and total or LDL-cholesterol concentrations (3).

A study conducted in Brazil assessed the utility of measuring serum levels of C-reactive protein, anti-PGL-I antibody and neopterin in monitoring leprosy patients during multi-drug treatment and reactions. Twenty-five untreated leprosy patients, 15 multi-bacillary (MB) and 10 paucibacillary (PB), participated. The result showed that the CRP levels did not differ significantly between MB and PB patients. Moreover, no significant correlation was seen between the CRP levels and BI in the ENL patients, however, had significantly higher levels of CRP than NE patients (LL and BL without reactions) Furthermore, the CRP levels did not decline during MDT. The study suggests measuring serum CRP, although not useful in monitoring the patients, has limited significance in detecting ENL reactional patients (17).

Marian J. Ridley et al investigated C-reactive protein and apo-B containing lipoproteins in lesions of human leprosy 87 patients were studied by taking skin biopsy. Slit skin smears were taken from patients. All biopsies were examined with specific immunoperoxidase staining and Immunohistochemical staining with anti-CRP antibodies. There was little or no positive staining with anti-CRP antibodies in biopsies from lesions of patients with TT or BT leprosy. However, in tissue from patients with BB, BL and LL leprosy, anti-CRP antibodies stained bacilli within macrophages. The amount and intensity of staining increased across the spectrum from BB to the active LL group, coinciding with breakdown of bacilli as revealed by fragmented or granular acid fast staining. In ENL acute phase of the reaction anti-CRP there was strong staining of coarse aggregates located both intracellular within macrophages and extracellular within the reactive area. The study findings demonstrate remarkable persistence of CRP in association with *M. leprae* in vivo, and raise intriguing questions about the possible role of CRP in relation to the handling of leprosy bacilli (34).

3. Research hypotheses

1. Multi-bacillary and pauci-bacillary leprosy patients have similar patterns of T cell phenotypes and memory T cells

2. The lipid profiles in Multi-bacillary and pauci-bacillary leprosy patients is different

4. Objectives

4.1 General objective

To assess the immunological and lipid profile of patient among multi-bacillary and pauci-bacillary patients

4.2 Specific objectives

1. To describe the phenotypic characteristic of memory T cells in leprosy disease spectrum
2. To assess the relationship between leprosy patients classification and lipid profile
3. To assess the association between C reactive protein and leprosy disease classification

5. Material and methods

5.1 Study area description and study duration

This study was conducted at ALERT Center, Addis Ababa, Ethiopia. Samples were obtained from All African Leprosy, Tuberculosis and Rehabilitation Training Centre (ALERT), a specialized hospital which is located in the capital city of Ethiopia, Addis Ababa. ALERT hospital give service to leprosy patient in a special clinic at Red Medical Clinic (RMC), give examination to approximately 300 patients annually. ALERT hospital also practices these WHO recommend multidrug therapy for 6 months in patients with paucibacillary disease (up to five skin lesions) and for 12 months in patients with multibacillary disease (more than five skin lesions). Sample processing had taken place at Armauer Hansen Research Institute (AHRI) and ALERT clinical laboratory. The duration of the study was from May - July, 2015.

5.2 Study design and study groups

A cross- sectional study design was used. The study groups included:

Group one: included untreated MB leprosy cases

Group two: included untreated PB leprosy cases

5.3 Facilities available at ALERT AHRI laboratory

ALERT laboratory is an accredited central clinical laboratory serving several patients with different tests, Immunohematology, Serology, Chemistry and Microbiology. Mainly it gives service to ART, TB, Dermatology, and Surgical, medical and to other clinics. The laboratory also participates in different research programs. AHRI is a biomedical research institute in Ethiopia operating in basic and applied biomedical research in TB, leprosy, leshmaniasis, meningitis, HIV and STIs. FACSCanto II flowcytometer (8 colours), ELISA and ELIISPOT reader, Luminex, Real time PCR and histopathology lab are among the core facilities available in the institute. There are also experienced experts especially in flowcytometry. For the proposed research, we will use the ALERT laboratory facilities to test lipid profile and C reactive protein and use FACSCanto II flowcytometer at AHRI laboratory.

5.4 Source population

The source population included all patients who visited the dermatology clinic at ALERT referral hospital during the study period.

5.5 Study population

The study population included Multi-bacillary, pauci-bacillary patients and reactions patients who attended the hospital during the study period. Healthy control wererecruited from ALERT hospital staffs.

5.6 Sample size determination

A total of 51 samples which comprise 21 healthy controls and 30 leprosy patients (pauci-bacillary and Multibacillary) were included. A Judgment sampling method was used for this study.

5.7 Inclusion criteria

Patient who fulfilled the following criteria were eligible to participate in the study:

1. Age group : above 18 and below 60
2. Individuals who were willing to give consent and willing to participate in the study
3. Individual diagnosed as having leprosy
4. Absence of history of leprosy and TB were assessed for healthy controls.

5.8 Exclusion criteria

Any individual characterized by at least one of the following criteria was not eligible to participate in the study.

1. Pregnant women
2. Individuals with other immunological disease such as TB, HIV and other chronic diseases
3. Individual who were on treatment for leprosy

5.9 Sampling procedure

A Judgmental sampling method was used.

5.10 Study variables

5.10.1 Dependent variables

1. T-cell phenotype and memory T cells
2. Lipid profile
3. C-reactive protein Status

5.10.2 Independent variable

1. Socio-demographic characteristics
2. Leprosy classification
3. Bacteriological index (BI)

5.11 Demographic and clinical data collection

Data of each study patient and control group were collected in pre tested forms. From each group detailed socio-demographic data were collected at recruitment time. Clinical data was collected from all cases at the recruitment. The data collection sheet included demographic, clinical and diagnostic parameters set following the guideline. Detailed demographic and clinical data collection form is annexed.

5.12 Clinical sample collection and laboratory method

Eight ml of blood was collected from each patients and healthy controls using EDTA and SST tube. A 200µl of Whole blood was used for T cells surface staining, which was done within 2 hrs. T cells enumeration was done using flow cytometry with specific marker for each cell (Annex 3). Serum was obtained from the 4mL blood collected using serum separator tube (SST) and used for lipid and C-reactive protein determination. For measuring lipid profile (total cholesterol, triglyceride (TG), High density cholesterol (HDL) and low density cholesterol (LDL) A25 BIOSYSTEM chemistry analyzer was used. To evaluate the C reactive protein qualitative precipitation test was used.

5.13 Memory T cell surface staining

To evaluate the phenotype of T cell subsets from leprosy patients and healthy controls, a 200µl whole blood were stained by adding each 15µl antibodies (CD45RO, CD3, CD62L, CD4 and

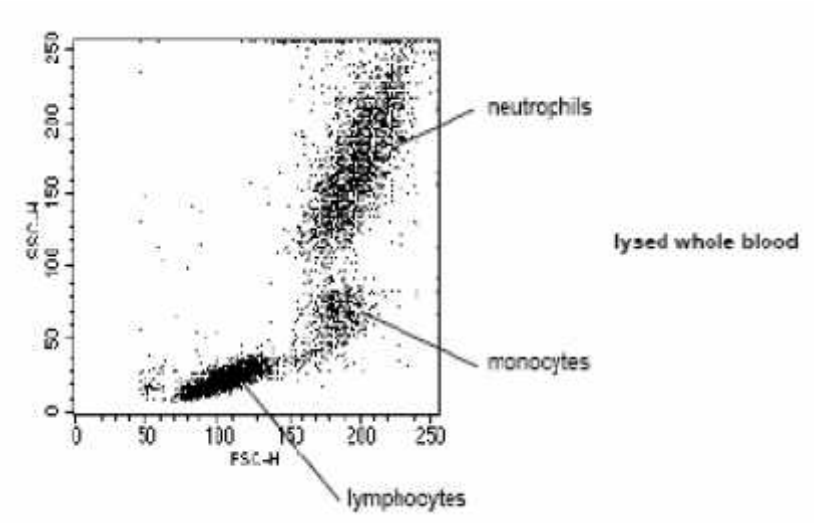
CD8). Incubating at 4^0 for 30 minute then followed by wash with FACS Buffer (1400rpm for 5 minute). Then the pellet was re-suspended in a buffer and followed by adding 1x lysing buffer. The cells were then incubated for 30 minute at room temperature, dark. After lysis cells were washed once by spinning at 1400rpm for 5 minutes. Finally the pellet was re-suspended in 400µl FACS buffer and made ready for acquiring(35).

Table 1 Markers and flourochromes used for flow cytometry, ALERT Hospital,Addis Ababa, May 2015

No	Marker	Fluorochrome	Company	Clone Host
1	Antihuman CD3	Percp-cy5.5	BD	Mouse
2	Antihuman CD4	FITC	BD	Mouse
3	Antihuman CD8	PE	BD	Mouse
4	Antihuman CD45RO	PE-Cy7	BD	Mouse
5	Antihuman CD62L	APC	BD	Mouse
6	live/dead Fixative Aqua Dead cell stain kit	-	Life Technologies	-
7	One Comp eBeads	-	eBioscience	-

5.13.1 Principle of Flowcytometry

Flow cytometry is a laser based technology that simultaneously measures and then analysis multiple physical characteristics of single cells as they flow in a fluid stream through a beam of light. Fluorescence-activated cell sorting (FACS) is a specialized type of flow cytometry which provides methods for sorting for mixtures of biological cells into groups based upon the specific light scattering and florescent characteristics of each cell. FACS is trademark and owned by Beckton, Dickinson Company(35). Once light signals have been converted to electronic pulses and converted to channel numbers, the data must be stored by the computer system. After a data file has been saved, cell populations can be displayed in several different formats. A single parameter can be displayed as a single parameter histogram, where the horizontal axis represents the parameter's signal value in channel numbers and vertical axis represents the number of events per channel number(35).


SSC-H
FSC-H
0
50
100
150
200
250
neutrophils
monocytes
lymphocytes
lysed whole blood

populations into naïve and memory T cells. CD45RO is used to delineate between naïve and memory T cells while CD62L is used to obtain activated T cells. The combination of CD45RO and CD62L divide the population into naïve, central memory, activated memory and terminally differentiated T cells (Table 2)(35).

Table 2 Memory T cells and their markers.

T cells	**Marker**	
	CD45RO	CD62L
Naïve T cells (NV)	**-**	**+**
Central Memory cells (31)	**+**	**+**
Activated Memory cells (AM)	**+**	**-**
Terminally differentiated Memory Cells (AC)	**-**	**-**

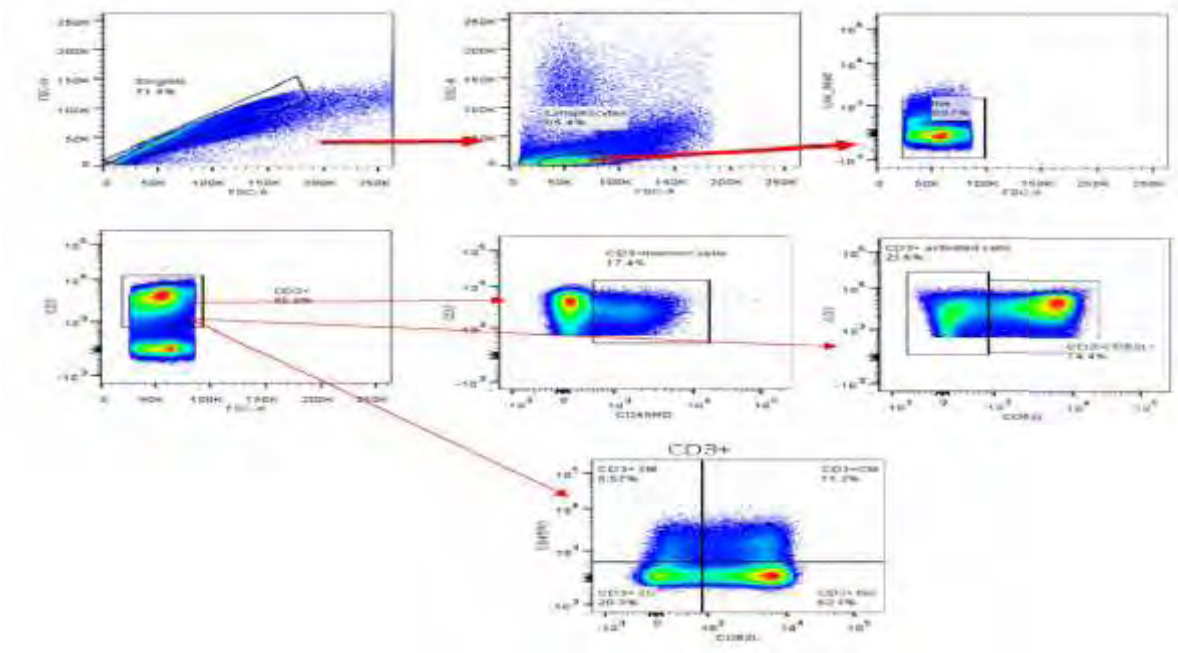


Figure 4 Gating memories and activated T cells for both patients and controls,ALERT Hospital,Addis Ababa, May 2015

5.14 Measurement of lipid profile

Nonhemolyzed serum or plasma, free from clots was analyzed with A25 Biosystem to measure lipid profile. Testing methods for total cholesterol uses cholesterol oxidase reactions along with cholesterol esterase and usually a peroxidase reaction for the “color” or final determination reaction, Triglycerides are composed of three fatty acids and a glycerol moiety, analyzing a serum or plasma sample for triglycerides typically involves four reactions(36).

5.14.1 Principle of turbidimetric clinical analyzer

The A25 analyzer is an automatic analyzer for random access In Vitro Diagnosis specially designed for performing biochemical and turbidimetric clinical analyses. The instrument is controlled on-line in real time from an external dedicated PC.

With the advent of homogeneous reagents, LDL is measured using the cholesterol reaction along with reagents that block the contribution of HDL and VLDL to the resulting answer. In the homogeneous LDL assay, detergents block the other two lipoprotein cholesterol products from forming colored chromogens. Only the LDL forms a colored chromogen that can be measured spectrophotometrically. The quantitation of HDL is using two methods; one method uses an antibody to apolipoprotein B-100 to bind LDL and VLDL in the sample. This leaves the HDL to react with the second reagent, which contains enzymes and substrate for cholesterol analysis. In a second method, a synthetic polyanion reagent binds the sites on VLDL and LDL particles, blocking their products from forming cholesterol colored products. The second reagent added has detergent, enzymes, and substrate that react with the HDL in the sample. Only the HDL particle cholesterol is allowed to form a colored product and be measured.

5.14.2Reading and interpretation

The analyzer performs patient-by-patient analyses and enables the continual introduction of samples. The results are shown immediately after each measurement(36).

Interpretation

Cholesterol

Up to 200 mg/dl – Desirable

>240 mg/dl – High

Triglyceride

Up to 130 mg/dl – Normal

200 – 499 mg/dl – High

HDL

Up to 35mg/dl – High risk

>60 mg/dl – Low risk

LDL

>190mg/dl – Very high

Up to 100mg/dl – Optimal

5.15Measurement of C reactive protein

The RapidTex CRP Test is based on the latex-agglutination method. The principle of this test is based on the immunological reaction between CRP as an antigen and the corresponding antibody coated on the surface of biologically inert latex particles. The test is done by adding one drop of reagent and one drop of undiluted patient serum onto the same circle and mixing both together with a paddle end. Reaction is indicated by agglutination(37).

5.15.1Reading and interpretation

The test can be read within 2 minute. Positive – visible agglutination. Negative – No visible agglutination.

5.16 Data storage system

All patient data was coded and kept confidential. Data was stored on password protected personal computer.

5.16 Statistical methods and data analysis

5.16.1 Data entry

All demographic, clinical and diagnostic data were properly coded and key was prepared for each code. All data were double entered into an Excel spreadsheet and verified using SPSS version 20 and was ready for analysis. Flowcytometry data were entered on excel spread sheet and transported to Graphpad ver.6 for analysis.

5.16.2 Data analysis

Memory T cells and lipid profile data analysis was done using Non-parametric tests (Mann-Whitney U). Explanatory variables were individually cross tabulated with the outcome variable and statistical significance was assessed as mentioned above. Proportion of CRP positive among

leprosy disease spectrum was compared by Chi-square test. Analysis of variance was used to compare hematological parameters. Descriptive analysis which includes mean, standard deviation, frequency, and standard error of the mean, proportions, percentages as well as descriptive graphs and tables were used with respect to the given demographic, clinical and diagnostic variables. Flow cytometry data was generated using FlowJo v-10 and analyzed using Graph pad version 6. All other data were analyzed by SPSS V 20.

6. Quality assurance

6.1 Quality control for flowcytometry

6.1.1 Setting fluorescence Compensation

Fluorescence compensation is the process of excluding the spectral overlap when two or more fluorescents are used in multicolor experiments. For this experiment proper compensation controls were included, a negative control (unstained cells) and beads were used for fluorescence compensation. And we used a single-stained OneComb eBeads (affymetrix, eBioscience) for all fluorescence compensation except for the live dead stain. For the viability dye cells were used for fluorescence compensation.

6.2 Quality control for measurement of lipid profile

For this experiment (Lipid profile) a minimum of two level (concentration) control were done and also the value obtained were checked against the defined ranges. LJ chart were used to plot the values and were governed by westgard quality control rules.

6.3 Quality control for measurement of C reactive protein

For this experiment commercial prepared negative and positive control were done before testing.

7. Ethical consideration

Ethical approval was obtained from departmental of ethics committee of medical laboratory of the Addis Ababa University, College of Health Science and also from ALERT/ AHRI Ethical Review Committee. Consent was obtained from all participants prior sample collection.

- **Consent.** The purpose of the study and why they had been chosen were explained to each participant. And also an information sheet was given to keep and be asked to sign consent form.
- **Autonomy**. Patients were told that they are free to choose not to participate
- **Harm.** Sample was collected only by trained, authorized and experienced medical personnel. Potential risk of blood collection such as haematoma formation was, therefore minimized.
- **Confidentiality.** The information that was collect from this research project was kept confidential and was stored in a file, which had no name on it, but a code number assigned to it.

8. Results

8.1 Demographic and clinical characteristics of the study subject

A total of 51 study subjects (30 leprosy patient and 21 health controls) were involved. 17 PB and 13 MB patients were enrolled to the study. The proportion of males in PB and MB were 11 (64.7%) and 11 (84.6%) respectively. The mean age of the study subject was 37.3 years, the majority (33.3 %) of being in the age group 25-34 years. About 56.7% of leprosy patients had a mean bacteriological index of 0 and 43.3% had also bacteriological index of 1-6 (Table 3). Twelve female and nine male healthy controlsparticipated in the study. The proportion of males in healthy control was 9 (42.9%). The mean age of the study subject was 33.45years, the majority (57.1 %) of being in the age group 25-34 years.

A one-way analysis of variance(ANOVA) was conducted to compare the RBC count, WBC count, hemoglobin concentration and platelet count for PB patients, MB patients and Healthy controls (Table 4).The mean RBC count of PB patients was 4.98 ± 0.46, of MB patients was 5.13 ± 0.36 and healthy controls was 5.08 ± 0.59, the mean WBC count of PB patients was 5.76 ± 2.58, of MB patients was 5.92 ±1.49 and healthy controls was 5.61 ± 1.74, the mean platelet count of PB patients was 262.59 ± 77.6, of MB patients was 277.2± 49.2 and healthy controls was 268.7 ±57.7 and the mean hemoglobin concentration of PB patients was 14.88 ± 1.53, of MB patients was 15.38 ± 1.44 and healthy controls was 15.90 ± 1.65.The result showed no significant difference between the observed RBC count, WBC count, hemoglobin concentration and platelet count of PB patients, MB patients and healthy controls.

A Pearson chi square model was made to assess the association between C reactive protein and leprosy classification. CRP positive were detected in 13 (43.3%) study subjects: 6 (35.3%) of the 17 PB patients and 7 (53.8%) of 13 MB patients (Table 5).However, no statistically significant association was observed.

Table 3 Demographic and clinical characteristics of study subjectsof study subjects, ALERT Hospital Redmedical Clinic Unit , Addis Ababa, May 2015.

Variables	Group of individual (n)		
	PB (n=17)	MB (n=13)	Healthy control (n=21)

Age group (%)	15-34	6 (35.3.8)	8(61.6)	14 (66.6)
	35-44	4 (23.5)	3 (23.1)	4 (19.0)
	≥45	7 (41.1)	2 (15.4)	3 (14.3)
Sex (%)	M	11 (64.7)	11 (84.6)	9 (42.9)
	F	6 (35.3)	2 (15.4)	12 (57.1)
BI (Mean ± SE)		0	3.54 ± 0.45 (Range 1-6)	0

Table 4RBC, WBC, Hb concentration and platelet count in PB, MB patients and healthy controls, ALERT Hospital Redmedical Clinic Unit , Addis Ababa, May 2015.

Parameters	**PB**	**MB**	**Healthy controls**	**F**	**P**
RBC (95 % CI)	4.75 to 5.22	4.91 to 5.35	4.80 to 5.35	0.35	0.70
WBC (95 % CI)	4.43 to 7.09	5.02 to 6.83	4.82 to 6.41	0.10	0.90
PLT (95 % CI)	222.6 to 302.4	247.4 to 307.0	242.4 to 295.0	0.20	0.82
Hgb (95 % CI)	14.1 to 15.7	14.5 to 16.3	15.2 to 16.7	1.98	0.14

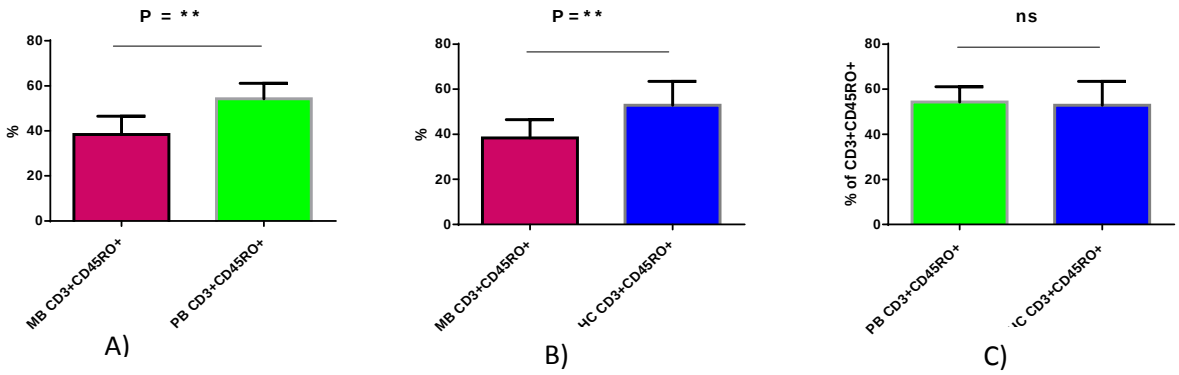
Table 5 C reactive protein status in PB and MB patients, ALERT Hospital Redmedical Clinic Unit , Addis Ababa, May 2015

Clinical status	CRP status		X^2	P
	Positive (%)	Negative (%)	1.033	0.31
PB	6 (35.3)	11 (64.7)		
MB	7 (53.8)	6 (46.2)		

8.2 Immunological profile

8.2.1 Memory T cells in leprosy patients and controls

To evaluate the phenotype of T cell subsets from MB, PB patients and healthy controls, a whole blood were stained with T_{NC} (CD45RO-/CD62L+), T_{EC} (CD45RO-/CD62L-), T_{CM} (CD45RO+/CD62L+) and T_{EM} (CD45RO+/CD62L-) lymphocyte specific mAbs. The frequencies obtained from each T cell subpopulation are shown in Fig 1. The percentage of memory T cells (CD45RO+) expressed on CD3+ was significantly higher in PB patients than MB patients (p= 0.0009). It was also found that the percentage of memory T cells (CD45RO+) expressed on CD3+ was significantly lower in MB patients than healthy controls (p= 0.0303). PB patients showed significantly higher CD4+ ((p = 0.0288) and CD8+ (P= 0.0004) memory T cells (CD45RO+) than MB patients. CD8+ memory T cells were significantly lower MB patients (p = 0.0009) compared to health controls (Figure 6).



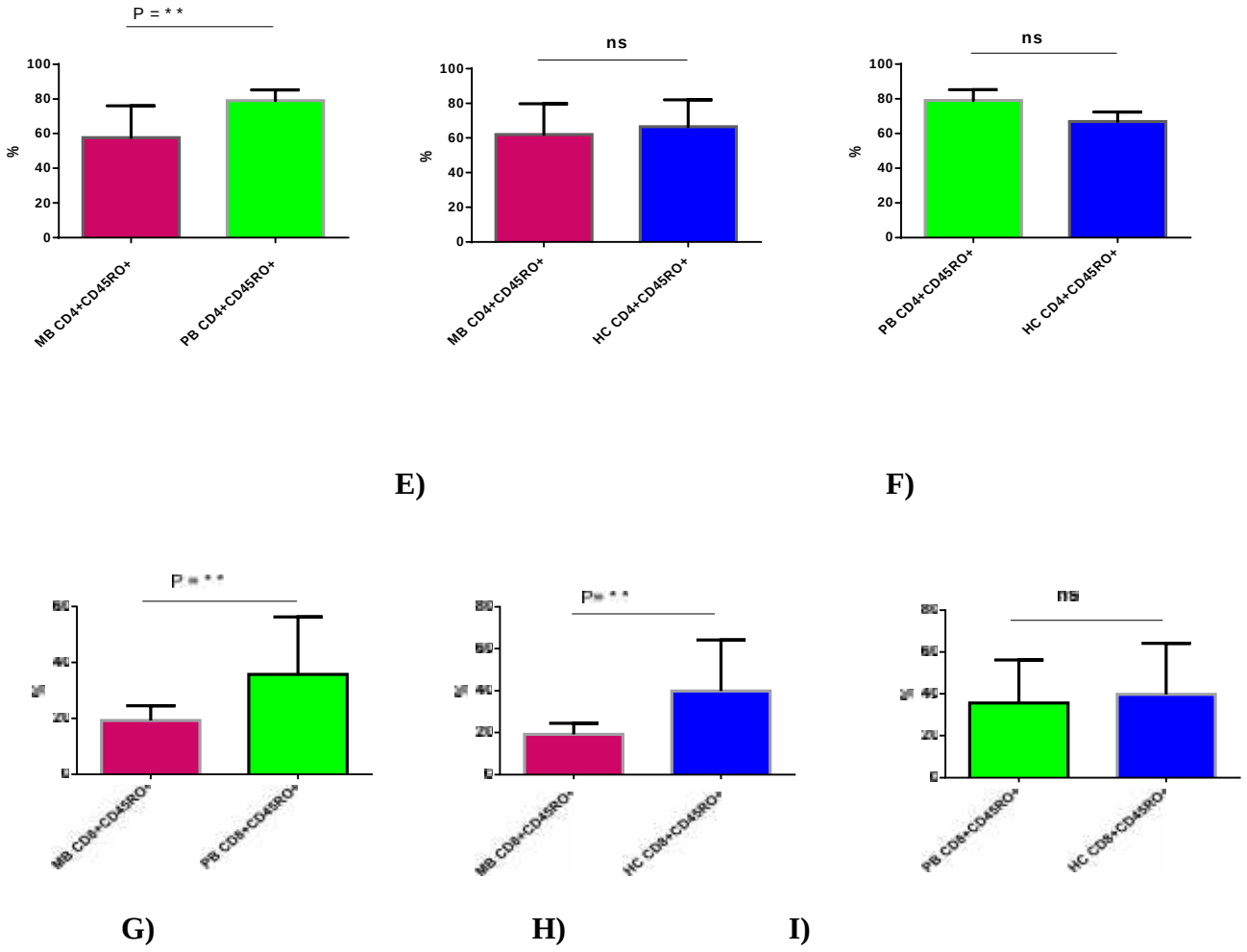
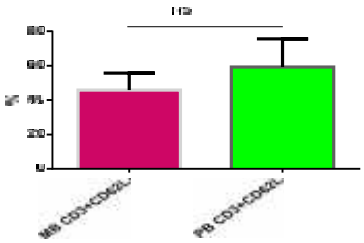


Figure 5 Memory T cellsof leprosy patient and healthy control, ALERT Hospital,Addis Ababa, May 2015

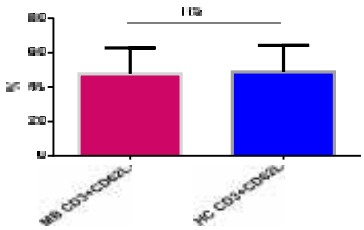
A) Percentage of memory T cells expression on CD3+ in MB and PB patients, B). Percentage of memory T cells expression on CD3+ in MB patients and HC, C) Percentage of memory T cells expression on CD3+ in PB patients and HC, D) Percentage of memory T cells expression on CD4+ in MB and PB patients, E). Percentage of memory T cells expression on CD4+ in MB patients and HC, F). Percentage of memory T cells expression on CD4+ in PB patients and HC. G) Percentage of memory T cells expression on CD8+ in MB and PB patients, H). Percentage of memory T cells expression on CD8+ in MB patients and HC, I) Percentage of memory T cells expression on CD8+ in PB patients and HC

8.2.2Activated CD3+, CD4+ and CD8+ T cells in leprosy patients and controls

The percentage of activated T cells ($CD62L^-$) expressed on CD3 showed no significant difference between MB, PB patient and healthy controls. Activated CD4 T cells were significantly higher (P= 0.001) in PB patients compared to MB (Figure 7).



A)



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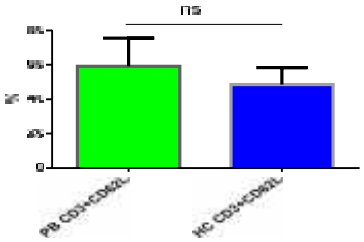

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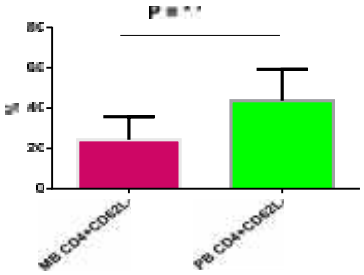

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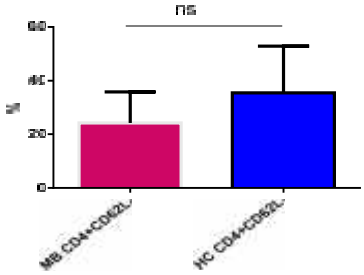

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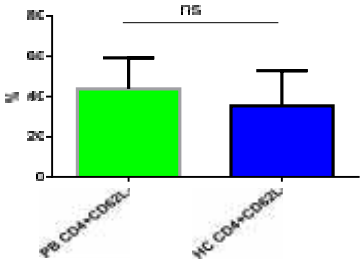

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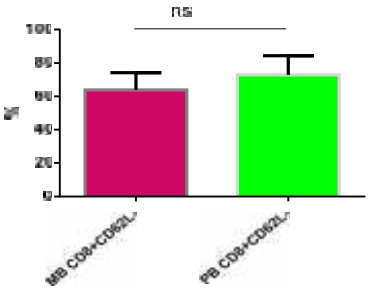

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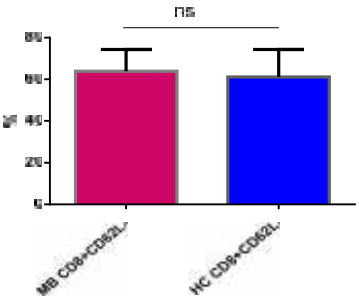

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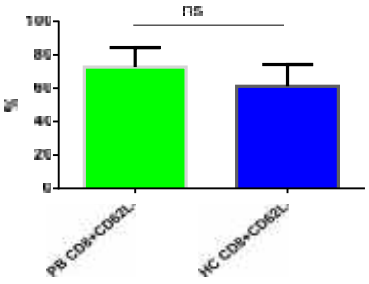


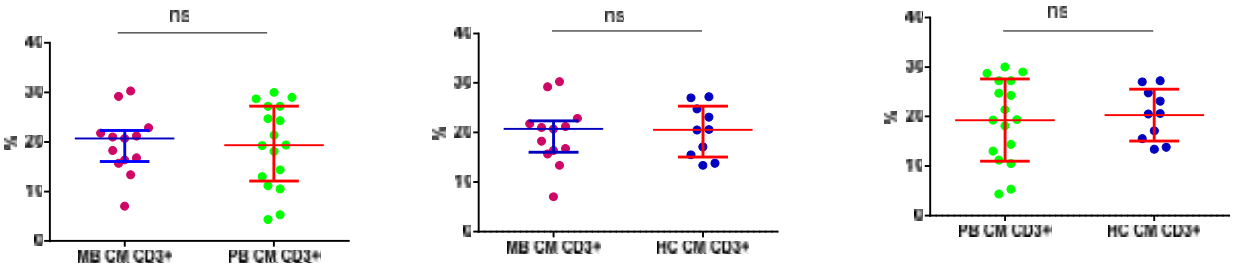
I)

Figure6 - Activated T cellof leprosy patients and healthy controls,ALERT Hospital, Addis Ababa, May 2015

A) Percentage of activated T cells expression on CD3+ in MB and PB patients, B). Percentage of activated T cells expression on CD3+ in MB patients and HC, C). Percentage of activated T cells expression on CD3+ in PB patients and HC. D) Percentage of activated T cells expression on CD4+ in MB and PB patients, E). Percentage of activated T cells expression on CD4+ in MB patients and HC, F). Percentage of activated T cells expression on CD4+ in PB patients and HC. G) Percentage of activated T cells expression on CD8+ in MB and PB patients, H) Percentage of activated T cells expression on CD8+ in MB patients and HC, I) Percentage of activated T cells expression on CD8+ in PB patients and HC.

8.2.3 Central memory CD3+, CD4+ and CD8+ T cells in leprosy and controls

Interestingly we observed no significant difference in the percentage of central memory CD3+ and CD4+ T cells between MB, PB and healthy controls. Similarly the central memory CD8+ has no significant difference between PB and MB patients (Figure 8).



A) **B)** **C)**

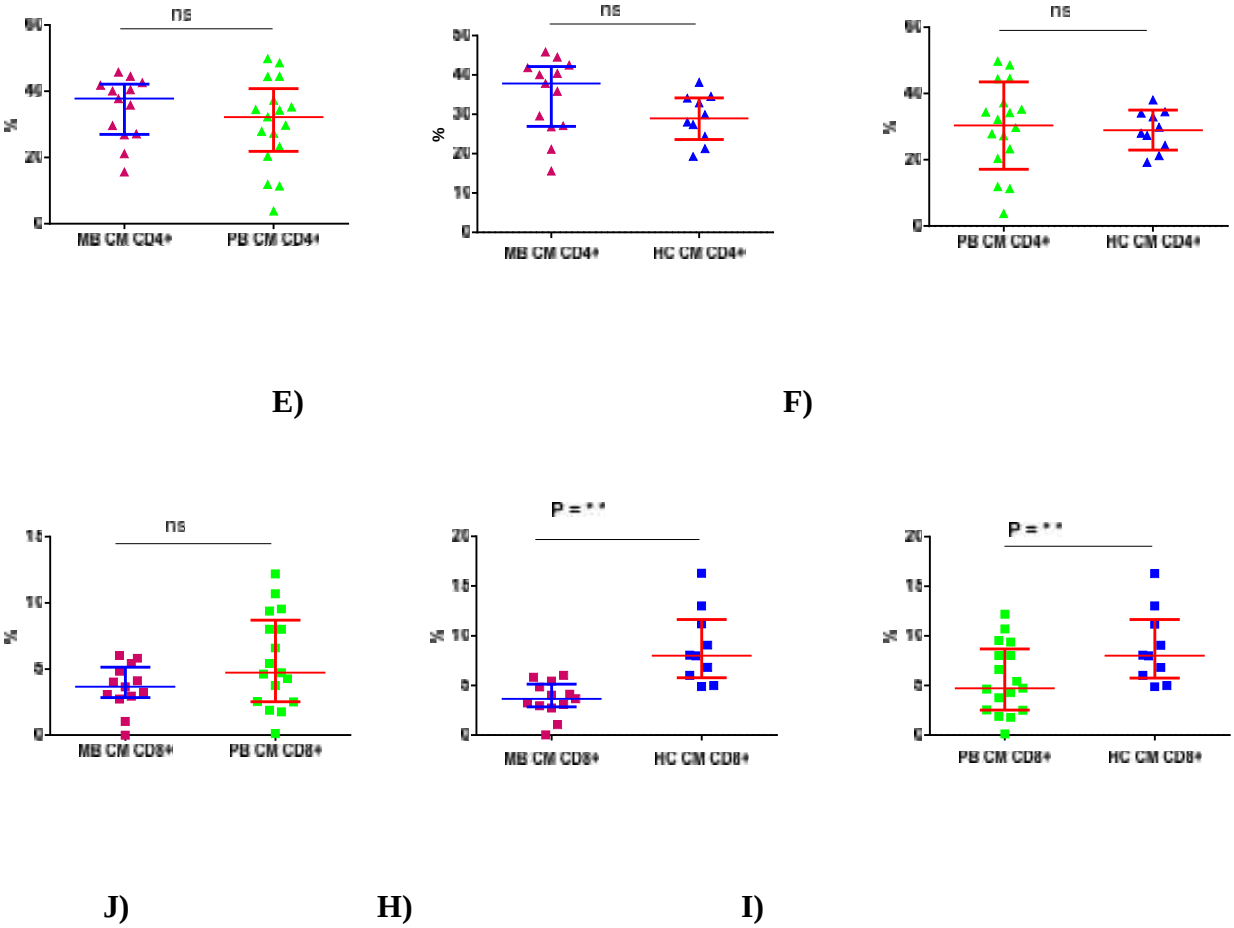
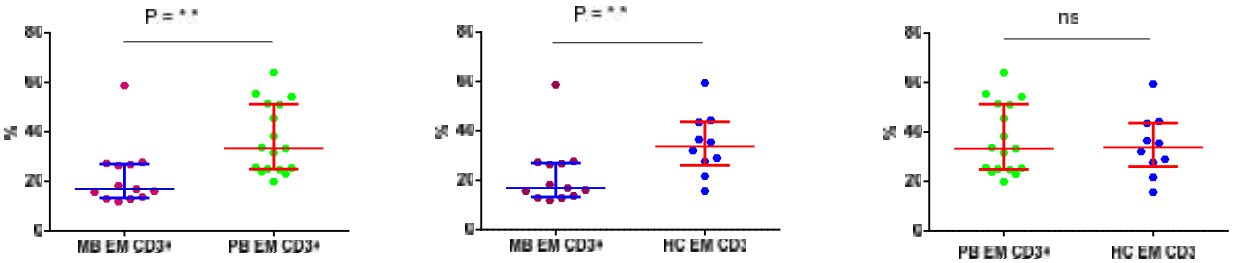


Figure 7-Central memory Tcellof leprosy patients and healthy controls, ALERT Hospital,Addis Ababa, May 2015

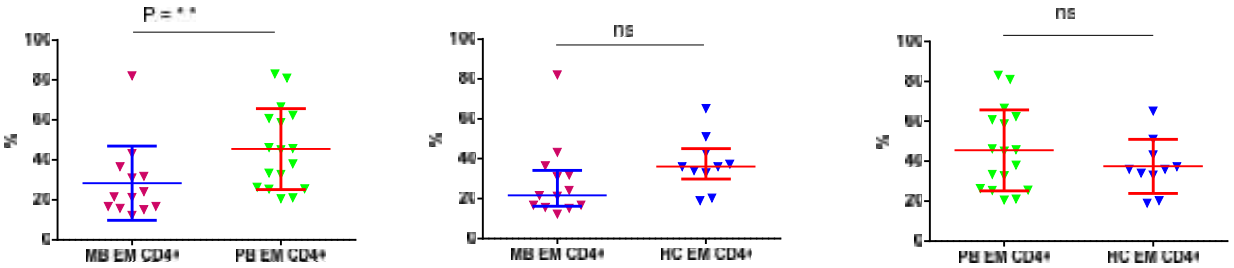
A)Percentage of Central memory T cells expression on CD3+ in MB and PB patients,B) Percentage of Central memory T cells expression on CD3+ in MB patients and HC, C). Percentage of Central memory T cells expression on CD3+ in PB patients and HC,D). Percentage of Central memory T cells expression on CD4+ in MB and PB patients, E). Percentage of Central memory T cells expression on CD4+ in MB patients and HC, F). Percentage of Central memory T cells expression on CD4+ in PB patients and HC, J). Percentage of Central memory T cells expression on CD8+ in MB and PB patients, H) Percentage of Central memory T cells expression on CD8+ in MB patients and HC, I). Percentage of Central memory T cells expression on CD8+ in PB patients and HC.

8.2.4 Effector CD3+, CD4+ and CD8+ T cells in leprosy and controls

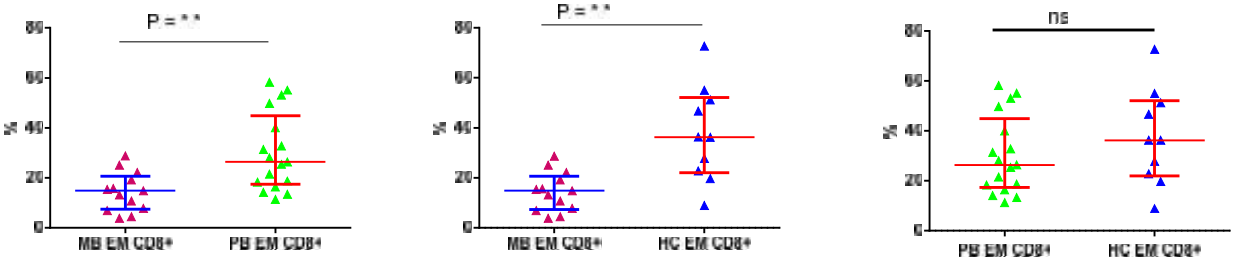
We did not find significant differences in the frequency of CD3+ effector T cellsbetween PB and MB patients. CD4+ effector (EC) cells in PB patients were significantly higher than in MB patients (p= 0.0272). When we compared PB and MB patients with respect to CD8+ effector T cells, we found no significant differences. However MB patients CD8+ effector cells were significantly higher than healthy controls (p < 0.0001). Similarly PB patients CD8+ effector cells were significantly higher than healthy controls (p= 0.0048) (Figure 9).



A) **B)** **C)**



D) E) F)



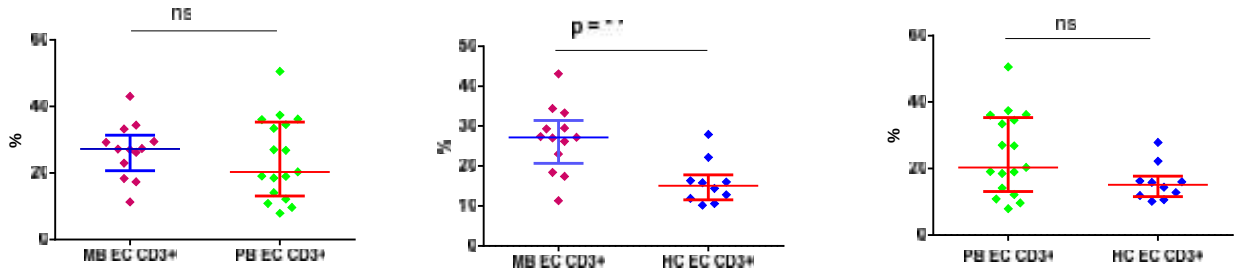
G) **H)** **I)**

Figure 8 - Effector T cell of leprosy patients and healthy control, ALERT Hospital,Addis Ababa, May 2015

A) Percentage of effector T cells expression on CD3+ in MB and PB patients, B). Percentage of effector T cells expression on CD3+ in MB patients and HC, C). Percentage of effector T cells expression on CD3+ in PB patients and HC.D). Percentage of effector T cells expression on CD4+ in MB and PB patients, E). Percentage of effector T cells expression on CD4+ in MB patients and HC, F). Percentage of effector T cells expression on CD4+ in PB patients and HC .J). Percentage of effector T cells expression on CD8+ in MB and PB patients, H) Percentage of effector T cells expression on CD8+ in MB patients and HC, I). Percentage of effector T cells expression on CD8+ in PB patients and HC**.**

8.2.5 Effector memory CD3+, CD4+ and CD8+ T cells in leprosy and controls

PB patients showed significantly higher CD3+, CD4+ and CD8+ effector memory cells than MB patients (p = 0.0045, 0.0069 and 0.0024 respectively). There was no significant difference between healthy controls and PB patients CD3+, CD4+ and CD8+ effector memory cells. Similarly, there was no significant difference between MB patients and healthy control with respect to CD4+ effector memory cells (Figure 10).



A) **B)** **C)**

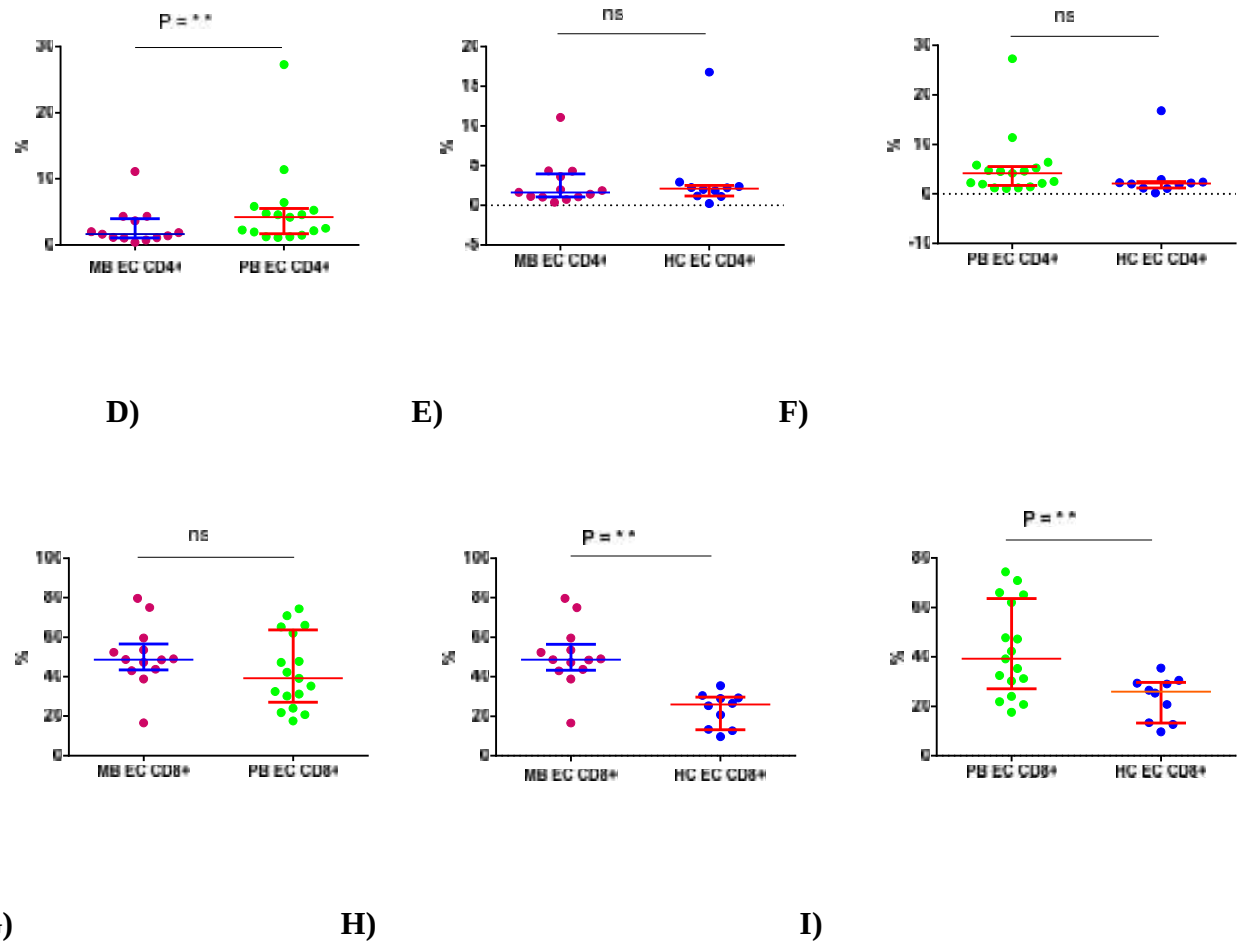
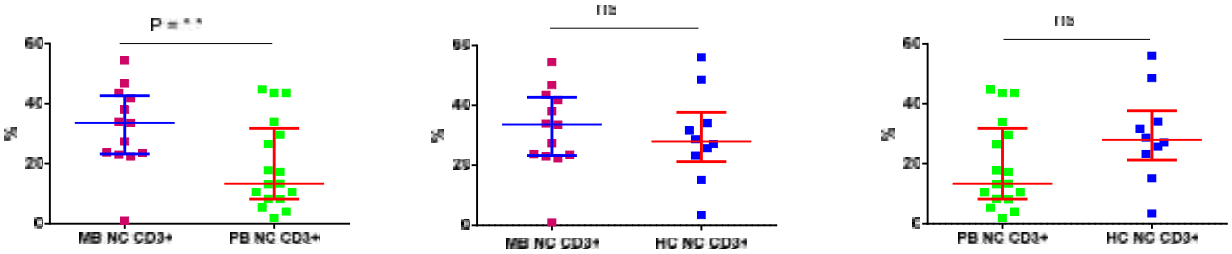


Figure 9 - Effector memory T cell of leprosy patient and healthy controls, ALERT Hospital,Addis Ababa, May 2015

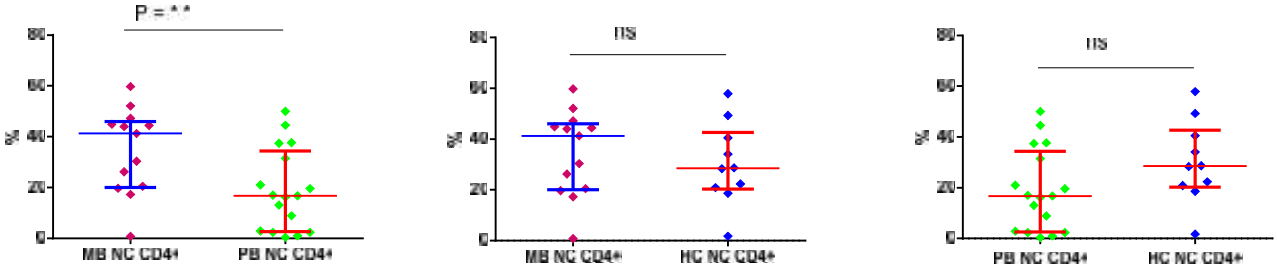
A) Percentage of effector memory T cells expression on CD3+ in MB and PB patients, B). Percentage of effector memory T cells expression on CD3+ in MB patients and HC, C). Percentage of effector memory T cells expression on CD3+ in PB patients and HC, D). Percentage of effector memory T cells expression on CD4+ in MB and PB patients, E). Percentage of effector memory T cells expression on CD4+ in MB patients and HC, F). Percentage of effector memory T cells expression on CD4+ in PB patients and HC. G) Percentage of effector memory T cells expression on CD8+ in MB and PB patients, H) Percentage of effector memory T cells expression on CD8+ in MB patients and HC, I). Percentage of effector memory T cells expression on CD8+ in PB patients and HC.

8.2.6Naive CD3+, CD4+ and CD8+ T cells in leprosy and healthy controls

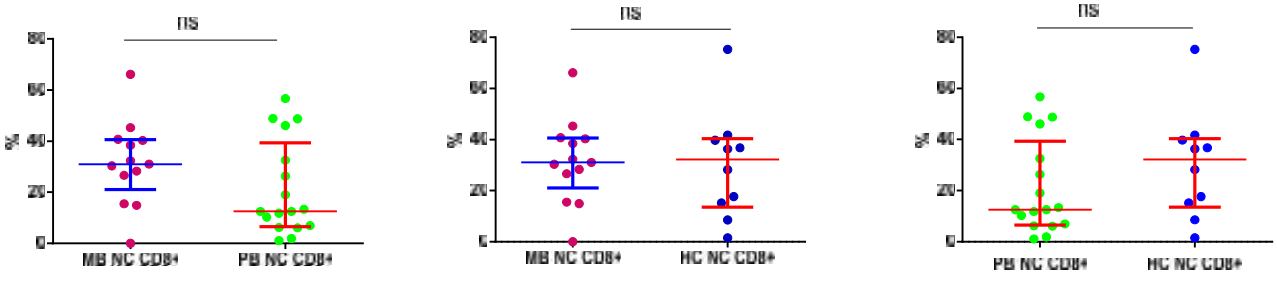
Naïve CD3+ and CD4+ T cells in MB patients were significantly higher than PB patients (p = 0.0399 and p = 0.0154 respectively). But naïve CD8+ T cells showed no significant difference between PB and MB patients (Figure 11).



A) B) **C)**



D) **E)** **F)**



G) **H)** **I)**

Figure 10 - Naïve Tcell of leprosy patients and healthy controls, ALERT Hospital, Addis Ababa, May 2015

A) Percentage of naive T cells expression on CD3+ in MB and PB patients, B) Percentage of naive T cells expression on CD3+ in MB patients and HC, C) Percentage of naive T cells expression on CD3+ in PB patients and HC, D) Percentage of naive T cells expression on CD4+ in MB and PB patients, E) Percentage of naive T cells expression on CD4+ in MB patients and HC, F) Percentage of naive T cells expression on CD4+ in PB patients and HC, G) Percentage of naive T cells expression on CD8+ in MB and PB patients, H) Percentage of naive T cells expression on CD8+ in MB patients and HC, I) Percentage of naive T cells expression on CD8+ in PB patients and HC**.**

8.3 Lipid profile

8.3.1 Total cholesterol and Triglyceride profile

The serum triglyceride level showed no significant differences in MB, PB patients and healthy controls. The mean serum triglyceride of PB patients was 134.6 ± 17.87, of MB patients was 138.6 ± 16.47 and of healthy controls was 165.5 ± 26.56. Likewise no significant difference was observed in serum total cholesterol level between PB patients and healthy controls. The mean serum cholesterol of PB patients was 163.3 ± 8.807. The serum total cholesterol level was significantly lower in MB patients compared to the health controls (P = 0.0346) (Figure 3). The mean serum cholesterol level of MB patients was 142.9 ± 10.79 and in healthy controls was 186.9 ± 14.13

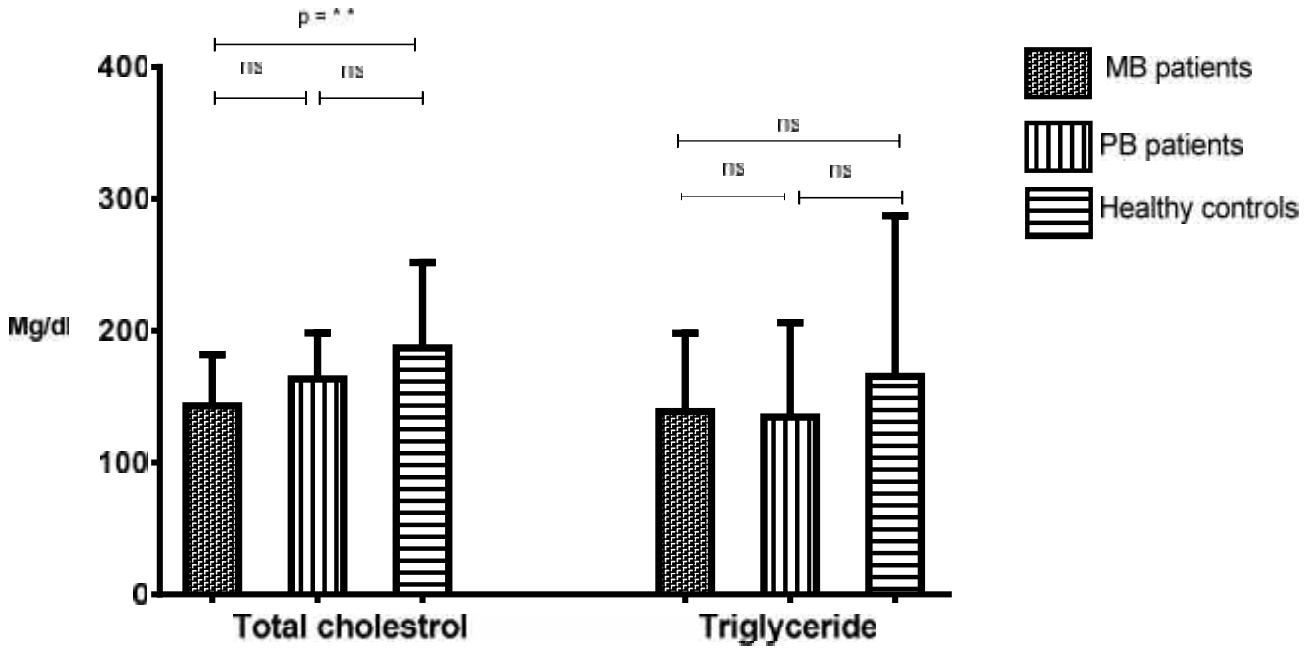


Figure 11 Total cholesterol and Triglyceride levels in study subjects, ALERT Hospital,Addis Ababa, May 2015.

8.3.2 Low density lipoprotein and High density lipoprotein profile

Both the serum HDL and LDL level were not significantly different between PB and MB patients. But we observed MB and PB patients serum LDL level was significantly higher than healthy control ($p = 0.0015$ and $p = 0.0016$). The mean serum LDL level of PB patients was 81.69 ± 10.16, of MB patients was 77.62 ± 8.082 and healthy controls was 43.95 ± 5.768 .On the other hand, serum HDL level was significantly lower in MB patients than controls ($p = 0.0006$). The level of serum LDL and HDL was not significantly different in PB and Healthy controls (Figure 5). The mean serum HDL level of PB patients was 40.44 ± 3.884, of MB patients was 81.69 ± 10.16 and a healthy control was 47.86 ± 2.570.

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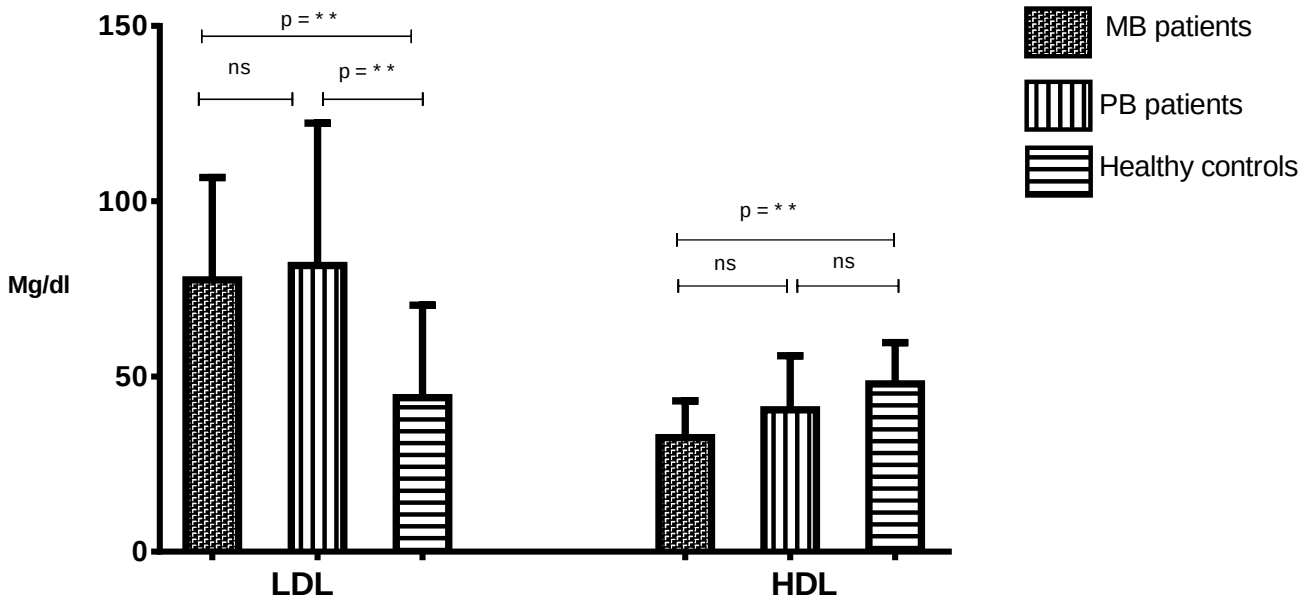

Figure 12 Low density lipoprotein and High density lipoprotein profile in study subject, ALERT Hospital,Addis Ababa, May 2015

9 Discussion

Although studies have been done on the immunology of leprosy patients, we couldn't find any study involving CD3+, CD4+ and CD8+ memory cells in leprosy patients. The adaptive immune response plays a critical role in infection control through generation of immunological memory, which composes the basis of protection against previously encountered antigens. Memory T cells encompass CD4 and CD8 T cells, which rapidly trigger effector functions and kill infected cells and secrete inflammatory cytokines (29).

Since most of the mature circulating T lymphocytes can be divided into naïve and memory cells, based on their antigenic response, one of our goals was to observe if there is a similar pattern of T cell phenotypes and memory T cell within the leprosy classification and healthy control. We observed memory T cells (CD45RO+) expressed on CD3+, CD4+ and CD8+ was significantly higher in PB patients than MB patients indicating that PB patients cell mediated immunity is competent to protect the host unlike in MB patients. Central memory T cells are antigen-experienced T cells that lack immediate effector function, but can mediate rapid recall responses. These cells are a long-lived memory population that circulates through the secondary lymphatic organs and blood (29).

In the current study we didn't find any significant difference of CM between PB and MB patients which could indicate that in both cases some T cells primed to memory T cells upon exposure to *M. leprae* infection. The absence of significant difference of CM between leprosy cases and health controls can be explained by the fact that the CM we measured is not specific to *M.leprae* infection. Central memory is a long lasting memory T cells in response to past infection(31). We measured the phenotypes of CM in the unstimulated blood of leprosy patients and health controls. Hence the CM obtained in healthy controls could be due to past infection to any diseases in these controls.

PB patients showed significantly higher CD3+, CD4+ and CD8+ effector memory cells than MB patients which indicates the responsiveness of cell mediated immunity in PB patients. Naïve CD3+ and CD4+ T cells were significantly lower in MB patients which could be explained by the fact that MB patients are diffident in cell mediated immune response.

Disturbances of lipid and lipoprotein metabolism are commonly observed in a variety of bacterial, viral and parasitic infections in experimental animal models as well as humans (3). Intracellular pathogens survive by accessing host metabolic pathways while at the same time evading the host immune response, which for mycobacteria depends on the use of host-derived lipids. Metabolism of host-derived fatty acids is required for the synthesis of mycobacterial lipids including virulence factors. Therefore, host lipids are used both for growth and virulence (13).

In the present study we have examined the immunological and lipid profile among leprosy disease spectrum and found no significant differences in serum triglyceride level between MB, PB patients and healthy controls. This result is comparable to a study which has also shown no alteration in serum triglyceride in TT, BT or BB and insignificant increase in BL and LL patients (14). Another study reported a significant increase in serum triglyceride in LL/BL patients (3). Change in serum lipid in leprosy may be multi-factorial and this observational difference could be due to activity, duration of illness, liver function status and dietary habitare among other factors.

Our study has revealed MB patients total cholesterol level was significantly lower than healthy controls. This result is analogous to changes in lipid metabolism in humans where infection/inflammation decreases serum cholesterol as a result of decreases in HDL cholesterol (38).

A study by Gupta et al. also indicated the level of serum cholesterol in MB patients was significantly low compared with PB patients, which is consistent with our result (25).

One of the mechanisms by which oxidized lipids are removed from the peripheral tissues is via the function of HDL. In addition to reverse cholesterol transport, HDL hydrolyzes oxidized phospholipids, in part through associated enzymes such as platelet-activating factor acetyl hydrolase and paraoxanase-1(13). Different studies showed that during infection and inflammation, there is a marked decrease in serum levels of HDL and apo-A(38). Our study revealed a significant reduction in serum HDL level in MB patients compared to healthy control ($p = 0.0006$). A study by Menton, *et al* reported that in MB patients HDL-cholesterol levels was lower compared to in PB which is consistent with our current finding (4).

Different studies have shown the possible hematological alteration in leprosy patients. In the current study we didn't find any significant difference inRBC count, WBC count, Hb concentration and platelet count between PB patients, MB patients and healthy controls. A study by Halim N, *et al* reveals a marked decrease in Hb concentration in patients on dapson and a marked lymphocytosis in patient's pre and post therapy. The finding was suggestive of dapson induced hemolysis(39). Our find difference could be due to our study participant, our study groups not being on treatment.Another study has shown elevation of the platelet count as a part of untreated, symptomatic ENL and revealed IL-6as an important mediator ofreactive thrombocytosis. thrombocytosis is of importance as a phenomenon to be understood infurther elucidating the pathogenesis ofENL(40).Our study only included 3 ENL patients.

Serum CRP levels are known to be raised in leprosy, possibly to a greater extent in lepromatousthan in the tuberculoid form andmarkedly elevated in ENLpatients as compared to non-reactional lepromatous patients(34). ENLpatients may presenta defense mechanism at the cell level characterized by the action of CRP which, athigh serum concentrations, may reduce theactivity of suppressor/cytotoxic T lymphocytes by as much as 75% without changingPHA-induced lymphoproliferation. Thiswould cause a relative increase in T-helper-cell activity with consequent improvementof the immunologic response(33).

In our study CRP values were done for all PB and MB patients.All ENLcases were tested positive for C reactive protein.Howeverwe found no association between leprosy classification and C reactive protein. This could be due to small sample size, owing to the lack ofpower of the statistical analysis.

A study done by Silva EA*et al* showed the serum levels of CRP did not differ significantly between theMB and PB patients.However, ENL patients had higher serum CRP levels than non-reactional MB patients. A major limitation of the measurement of serum CRP in leprosy patients is the lack of diseasespecificity, as it is indicative of generalinflammation and likely to be elevated in all immune mediateddiseases.Hence, the significance of serum CRP measurementneeded to be

interpreted with caution. Measurement of CRP levels is likely to be valuable in monitoring the treatment of acute reactional episodes in serial samples,rather than as diagnostic biomarkers(17).

10 Conclusion and recommendation

Conclusion

1. In conclusion, our findings indicate that, there is no significant difference in serum triglyceride level between MB, PB patients and healthy controls. However MB patients total cholesterol level was significantly lower than healthy controls. Similarly MB patients serum HDL level showed significant reduction compared to healthy controls.

2. Memory T cells (CD45RO+) expressed on CD3+, CD4+ and CD8+ are significantly higher in PB patients than MB patients. The percentage of central memory CD8+ T cells in healthy controls was significantly higher than both PB and MB patients. However PB patients CD8+ effector cells were significantly higher than healthy controls. And also PB patients showed significantly higher CD3+, CD4+ and CD8+ effector memory cells than MB patients. Healthy controls CD3+ and CD8+ effector memory cells were significantly higher than MB patients.Naïve CD3+ and CD4+ T cells in MB patients showed to be significantly higher than PB patients.

Recommendation

1. We recommend that monitoring and regulate the lipid homeostasis of a leprosy patients may be useful.

2. We also recommend further study on host immune responses in leprosy diseases in which lipid metabolism and inflammation intersect.

3. We recommend routine determination of Hb concentration, WBC count, RBC count and platelet count for leprosy patient prior to dapson therapy and subsequently.

11 Strength and limitation

Strength

1. Patients recruitment and sample processing took place in well-established and accredited laboratories
2. Several process controls were used during the laboratory analysis which leads to high quality data.

Limitations

1. We used only leprosy patients those visited ALERT hospital which could be a potential limitation to use the current result to all leprosy patients as some values such as the lipid profiles are tied to the socio-demographic characteristics of patients in addition to their disease status
2. Small sample size: since immunological studies are very expensive, our sample size is not large enough to give conclusion to a wide population
3. Patient to control ratio is not one to one: due to shortage of logistic.
4. shortage of literature

12 Reference

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ANNEX 1 Information sheet English version

‘Participant Information sheet’

1. **Study title:**

Immunological and Lipid profile across Leprosy disease spectrum

2. Invitation paragraph:

You have been invited to take part in this research study. Before you decide whether to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Ask question if there is anything that is not clear or if you would like more information.

3. Introduction of the disease

Leprosy, or Hansen’s disease, is a mycobacterial disease caused by *Mycobacterium leprae* which manifestations, course and prognosis are strictly associated to the patient’s immune system. Leprosy is a serious infectious disease, for both the patient (because of disabilities and social consequences) and the community. Early diagnosis and treatment reduces disabilities and transmission.

4. The purpose of the study

The study involves investigating the Immunological and Lipid profile across Leprosy disease spectrum. The study aim is to understand more about the disease and early diagnosis.

5. Why you have been chosen?

You are invited to participate in this study as a leprosy patient. We want to know the immunological and Lipid profile across Leprosy disease spectrum. In this study a total of 50 patients, 10 TT patients, 10 BT patients, 10 BB patients 10 BL patents and 10 LL patients will participate.

6. Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign consent form. You are free to withdraw at any time, without giving a reason. A decision not to take part or to withdraw at any time, will not affect the standard of care you receive.

7. What will happen to me if I take part?

Your role in the study:

If you agree to participate, the doctor in charge to treat your case (leprosy) will ask you some questions which you are expected to answer. You will be asked these questions as a normal treatment procedure even if you will not take part in this study.

If you take part in the research you will be asked to give 4ml blood which is not part of the routine test and will be collected by inserting a small needle into a vein in your arm. At the same time you will be given anti leprosy treatment free of charge according to the national treatment guideline for leprosy irrespective of this study by your doctor.

8. What is the study procedure

If you take part in the research the demographic data assembled by your physician will be used and also you will be asked to give blood which is not part of the routine test. This sample will be used for the study.

9. What are the possible benefits of taking part and incentives?

You will not be provided any incentive to take part in this research. However, you will be given 50 ETB for transportation and time lost during participation in the project. And also anything found in the study based on your laboratory results will be communicated to you and your physician. In addition, your participation is likely to help us in understanding the disease process of leprosy which may benefit future patients by developing new and better treatments.

10. What are the possible disadvantages and risks of taking this part?

There is no major risk in participating in this research, but the minor bleeding that may occur during blood collection will be avoided, as the procedure is carried out by trained experienced health professionals on the standard good clinical practice.

11. What happens when the research study stops?

On completing the study no further participation is required.

12. Will my taking part in the study is kept confidential?

The information that we collect from this research project will be kept confidential. Information about you that will be collected from the study will be stored in a file, which will not have your name on it, but a code number assigned to it. Which number belongs to which name will be kept separately in a password protected data management file and it will not be revealed to anyone except the principal investigator and your treating physician. Your personal information will not be disclosed even during the reporting of the findings. Reports will be written and disclosed anonymously.

13. What will happen to any samples I give?

As already described, during the laboratory analysis we will use your given code not your name for your sample. The samples are immediately processed and analyzed. If there is any abnormal result, it will immediately be communicated to you and your Doctor, so as your Doctor will take the appropriate action. The data collected will be written and published in peer reviewed scientific journals.

14. If there is any abnormal result?

If there is any abnormal result, it will immediately be communicated through your treating physician , so as your Doctor will take the appropriate action.

15. What will happen to the results of the study?

Data from this study will be analyzed and published in scientific journals but your identity will not be revealed. Data will also be presented at seminars at national meetings. No information containing your name will be disclosed.

16. Who is organizing and funding the project

The cost of this research project is covered by AHRI.

17. Who has reviewed the study?

This study was given a favorable ethical opinion by the AHRI/ALERT Research Ethics committee and by the Addis Ababa university Research Ethics committee.

18. How to give my consent

If you have interest to take part in this research, the PI or the delegated person will be available at the leprosy clinic and will provide you the consent form which you can sign if you agree to participate.

You will be given a copy of the information sheet and a signed consent form to keep

Thank you in advance for considering taking part in this study

Study coordinator and Principal investigator

Tihitna Nega MSC fellow

Mobile: 0911843846

ALERT/Ethiopia

AHRI/ALERT Ethical Committee:

Tel No: 0113 481285

ANNEX 2 Information sheet Amharic version

የመረጃቅጽ

1. **<u>የጥናቱመጠሪያ</u>**

የስጋደዌበሽታከሥውነትተከላካይህዋስእናከቅባትመጠንጋርያለውንግንኙነት

2. **<u>በጥናቱእንድሳተፋስለመጋበዝ</u>**

በዚህጥናትላይእንዲሳተፋእንጋብዘዎታለንነገርግንበጥናቱከመሳተፍዎበፊትየጥናቱአላማናአስፈላጊነትንበቅድሚያመረዳትያስ ፈልገዎታል፡፡እባክዎጊዜወስደውየሚከተለውንመረጃያንብቡ፡፡ማንኛዉምጥያቄወይምግልፅያልሆነነገርካለመጠየቅይችላሉ፡፡

3. **<u>የበሽታውምንነት</u>**

የስጋደዌበሽታማይኮባክቴሪየምሊፕሬበሚባልባክቴሪያየሚመጣሲሆንለበሽታውመከሰት፤ለበሽታውአካሄድእንዲሁምየሚያ መጣውጉዳትከታማሚውየበሽታየመከላከልአቅምጋርበጥብቅይገናኛል፡፡ለበሽታውበፍጥነትህክምናናመድኃኒትማግኘትበሽታ ውበአካልላይየሚያመጣውጉዳትእንዲሁምተላላፊነቱንይቀንሰዋል፡፡

4. **<u>የጥናቱዓላማ</u>**

እኔአሁንየማጠናዉየስጋደዌበሽታከሰውነትቅባትናከተከላካይሕዋስጋርያለዉንግንኙነትነዉ፡፡የዚህምጥናትዉጤትስለበሽታ ውየተሻለእውቀትእንዲገኝእንዲሁምበሽታውንበጊዜለማግኘትናለመከላከልበሚደለገዉጥረትጉልህድርሻይኖረዋል፡፡

5. **<u>እርሶለምንበዚህጥናትእንዲሳተፉተመረጡ?</u>**

እርሶበዚህጥናትላይእንዲሳተፉየተመረጡበትምክንያትየሥጋደዌህመምተኖበመሆንዎናበጥናቱሊካተቱየሚችሉትየሥጋደዌ ህመምተኞችብቻበመሆናቸውነው፡፡በዚህጥናት 50 የሚሆንየሥጋደዌህመምተኖይሳተፋሉ

6. **<u>በዚህጥናትላይለመሳተፍየግድያስፈልጋል?</u>**

በጥናቱላይለመሳተፍየግድአያስፈልግም፤በፍላጎትላይብቻየተመሠረተነው፡፡በጥናቱላይለመሳተፍከወሰኑይህመረጃናመስማ ማትዎንየሚገልጽቅጽይሰጠዎታል፡፡መረጃውንካነበቡናየሚጠይቁትጥያቄካለምበመጠየቅበሚገባከተረዱበኃላመስማማትዎ ንይገልጻሉ፡፡ከጥናቱበፈለጉትጊዜናሰዓትያለምንምቅድመሁኔቴማቋረጥይችላሉ፡፡እራስዎንከጥናቱበማግለልዎምክንያትለህ መምዎየህክምናእርዳታከማግኘትአያግድም፡፡እንደማንኛውምታካሚአስፈላጊውንየህክምናእርዳታያገኛሉ፡፡

7. **<u>በጥናቱላይከተሳተፍኩከእኔምንይፈለጋል?</u>**

በጥናቱላይለመሳተፍከተስማሙእርስዎንለማከምየተመደበውህኪምስለበሽታዎአንዳንድእርስዎእንዲመልሱየሚጠበቁጥያቄ ዎችይጠይቅዎታል፡፡ይህጥያቄበመደበኛየሕክምናጊዜየሚጠይቅበመሆኑየዚህጥናት-ብቻጥያቄአይደለም፡፡በጥናቱላይለመሳ ተፍከወሰኑከመደበኛውምርመራበተለየ 4
ሚሊሊትርደምከክንድዎላይእንዲሰጡይጠየቃሉ፡፡እዚህጥናትላይባይሳተፉምቢሳተፉምበህጉመሰረትመድህኒትዎንበነፃይ ወስዳሉ፡፡

8. **<u>ከእርስዎምንይጠበቃል?</u>**

በጥናቱለመሳተፍከተስማሙዶክተርዎእርስዎንበመጠየቅየወሰደውማስታወሻለጥናቱይውላልእንዲሁምከላይየተገለፀውንከ መደበኛውምርመራበተለየደምእንዲሰጡይጠበቃል፡፡ይህየደምናሙናለጥናትምርምሩይውላል፡፡

9. <u>በጥናቱላይቢሳተፉጥቅማጥቅምአገኛለሁኝን?</u>

በዚህጥናትላይበመሳተፍዎየተለየጥቅምበግልአያገኙም፡፡ነገርግንየትራንስፖርትወጭዎይሸፈንልዎታል(50 ብር) በተጨማሪምበጥናቱወቅትየተገኘውንየላቦራቶሪውጤትለእርስዎናለዶክተርዎይገለጻል፡፡እንዲሁምየእርስዎበጥናቱመሳተፍ ስለበሽታውያለውንጥሩግንዛቤእንዲኖረንናበሽታውንለመቆጣጠርጠቃሚመረጃበመስጠትይጠቅመናል፡፡

10. <u>በጥናቱላይበመሳተፌየሚደርስብኝጉዳትአለ?</u>

ጥናቱላይበመሳተፍዎየሚደርስብዎትልቅጉዳትየለም፤ነገርግንየደምናሙናበሚወሰድበትወቅትሊፈጠርየሚችለውንአነስተኛ የደምመፍሰስለማስወገድልምድባላቸውናስልጠናበተሰጣቸውባለሙያዎችይከናወናል፡፡

11. <u>ጥናቱሲያልቅከእኔምንይጠበቃል?</u>

ምንምአይጠበቅም፡፡

12. <u>በዚህጥናትመሳተፍበሚስጥርይያዛልን?</u>

አዎንለዚህጥናትየሚሰበሰበውናሙናእናየናሙናውጤትበሚስጥርይያዛል፡፡ስለእርስዎየሚገልጽማንኛውምነገርበናሙናም ሆነበውጤቱላይአይጻፍም፡፡ውጤትሲገለጽስምአልባይሆናል፡፡ለእያንዳንዱናሙናልዩመለያቁጥርወይምምልክትይሰጠዋል፡ :የትኛውቁጥርየማንእንደሆነዋናተመራማሪውብቻያውቃል፡፡ስለናሙናዎውጤትከዋናተመራማሪውበተጨማሪእርሶንየሚ ከታተለውህኪምምሊያውቀውይችላል፡፡

13. <u>እኔየምለግሰውናሙናምንይሆናል?</u>

የርስዎናሙናየተለያቁጥርይሰጠዋል፡፡በናሙናውላይየርስዎስምአይጻፍም፡፡አብዛኛውናሙናወዲውኑስራላይይውላል፡፡በጥ ናትየሚገኘውመረጃበህትመትመልክለጤናባለሙያናለሳይንትስቶችይደርሳል፡፡ውጤትበጅምላበሚገለጽበትጊዜየማንንም ውጤትአይወክልም፡፡

14. <u>ያልተጠበቀየላቦራቶሪዉጤትካለ?</u>

በጥናቱላይየተገኘማንኛውምያልተጠበቀውጤትካለለዶክተርዎእንዲሁምለእርስመ,ዎይገለፃል፤ዶክተርዎሚገባውንህክምና ያረግልዎታል፡፡

15. <u>የጥናቱውጤት?</u>

የጥናቱውጤትበሳይንሳዊመፅሔትላይይታተማልእንዲሁምየጥናቱውጤትበተለያዩስብሰባዎችላይይቀርባል፡፡ነገርግንየእ ርስዎማንነትበምንመልኩአይገለፅም፡፡

16. <u>የጥናቱንወጪየሚደግፈው?</u>

የጥናቱወጪየሚደገፈውበ AHRI

17. <u>ፈቃደኝነቴንለመግለፅ?</u>

ጥናቱላይየመሳተፍፍላጎትካለዎትከሚታዩበትክፍልከጥናቱዋናአስተባባሪወይምከተወከለውሰውየስምምነትቅፅበመውሰ ድፊርማዎትንበማሰፈርስምምንዎትንማረጋገጥይችላሉ፡፡

18. <u>ይህጥናትተቀባይነትንአግኝቷል?</u>

ይህጥናትበአህሪ/አለርትየስነ-ምግባርኮሚቴናበአዲስአበባዩንቨርስቲየስነ-ምግባርኮሚቴተገምግሞተቀባይነትንአገኝቶፀድቋል፡፡

<u>ተጨማሪመረጃከፈለጉየሚከተሉትንባለሚያዎችማነጋገርይችላሉ</u>

1. የጥናቱአስተባባሪናዋናተመራማሪትህትናጋ: ስልክቁ. 0911843846

ANNEX 3 Consent form English version

Please read this form and sign it once the above named or their designated representative has explained fully the aims and the procedures of the study to you.

1. I voluntarily agree to take part in this study.
2. I confirm that I have been given a full explanation by the above named and that I have read and understood the information sheet given to me which is attached.

3. I understand that the investigators will take 4 ml of blood sample.

4. I have given the opportunity to ask questions and discuss the study with the investigator or their deputies on all aspects of the study and I have understood the advice and information given as a result.

5. I authorize the investigator to disclose the results of my participation in the study, but not my name.

6. I understand that I am free to withdraw from the study at any time

7. I understand that information recorded during the study will be kept in a secure database.

Name:_____Signature:_____Date:_____

The participant is unable to read and write". As a witness, I confirm that all the information about the study was given and the participant consented to taking part voluntarily

_____ _____ _____

Name of Impartial Signature Date

I confirm that I have fully explained the purpose of the study and what is involved to:

…….

I have given the above named copy of this form together with the information sheet.

Signature: ……. Name: ……..Contact Address: Tihitna Nega **Tel:** 0911843846

ANNEX 4 Consent form Amharic Version

ተሳታፊየሚፈርሙትየስምምነትቅጽ

የጥናቱንአላማናሂደትበዝርዝርከተረዱበኃላየሚከተለውንቅጽበጥንቃቄይፈርማሉ፡፡

1. የጥናቱንተሳታፊእንድሆንበሙሉፈቃድወስኛለሁ

2. ከዚህጋርየተያያዘውንየመግለጫቅጽበትክክልአንብቤተረድቻለሁ፡፡በእኔላይምስለሚደረግማንኛውምጥናትተገንዝቤአለ ሁ፡፡በተጨማሪምአስፈለቃጊውንገለጻናማብራሪያከላይበተጠቀሱትሰውተደርጎልኛል፡፡

3. አጥኚዎቹከከንድዎላይ 4 ሚሊሊትርየሚሆንየደምናሙናእንደሚወስዱበሚገባተረድቻሉ።።

4. ጥያቄየመጠየቅመብትየመወያየትእድልከላይከተጠቀሱትአጥኚዎችወይምከነሱተወካይጋርተሰጥቶኝበጥናቱላይበቂምክ ርናውይይትአድርጌያለሁ።።

5. በተመራማሪዎቹየጥናቱንውጤትይፋእንዲያደርጉእፈቅዳለሁ።።ነገርግንስምመጠቀስየለበትም።።

6. ተመራማሪዎቹበጤናዬላይያለንችግርእንዲነግሩኝፈቅጀላቸኃለሁ።።

7. በማንኛውምጊዜከጥናቱእራሴንማግለልእንደምችልአውቄያለሁ።።

8. ከእኔየሚሰበሰበውማንኛውምመረጃበጥንቃቄናሚስጥራዊነቱበተጠበቀቦታእንደሚቀመጥአውቄያለሁ።።

ስም_____ ፊርማ _____ ቀን _____

ይህበጥናቱየሚሳተፈውሰውማንበብናመፃፍስለማይችልከላይየተዘረዘሩትመረጃዎችለተሳታፊውየተሰጡናተሳታፊውምለመሳተፍ መስማማቱንገለልተኛታዛቢበመሆንአረጋግጣለሁ።።

የገለልተኛታዛቢስም _____ ፊርማ _____ ቀን _____

ስለጥናቱዝርዝርመረጃሰለመስጠቴአረጋግጣለሁ

……..…

የመተማመኛቅጹንከስምምነትቅጽጋርአያይዤሰጥቻለሁ

ፊርማ…… ስም……..

ANNEX 5 Clinical data collection sheets

Alert No…… Study number…….

Entry criteria: Patient history; Patient examination; Consent form

4 ml blood sample for (Flowcytometry, Lipid profile, C-reactive protein)

Clinical data required:

Full clinical investigation report on first day

Date the patient enrolled in the study dd / mm /yy E.C.

PI in charge ‘’ ----------.

Data Collection Form

Patient ID _____ Study code _____ Tell. No _____

PATIENT DETAILS						
Date	**__ /__ /__**	**Gender**		**Date of birth**	**__/__/__**	**Age ____**
LEPROSY DETAILS						
Date of diagnosis of leprosy	**__/__/__**		**TT** ☐	**BT** ☐	**BB** ☐	**BL** ☐	**LL** ☐

Bacterial index		**Mean BI**	**____**	**High BI**	**____**		
Previous MDT	**NO**	**YES**					
Presented with reaction at leprosy diagnosis	**NO** ☐	**YES** ☐	**TYPE 1** ☐	**ENL** ☐	**NEURITIS** ☐		
Other illnesses		**TB**	**Diabetes**	**HIV**	**Other (specify)**		
EXAMINATION							
Lipid profile	**Cholesterol**		**Triglyceride**	**LDL**		**HDL**	
CBC	**RBC**		**WBC**	**Hgb**		**PLT**	
CRP							

ANNEX 6 Procedure for staining

Protocol 1

1. Take 200 ul blood to the labeled tubes
2. Add Antibodies (each 15ul/tube)

   CD45RO: PE-cy7

   CD3: Percp-cy5.5

   CD62: APC

   CD4: FITC

   CD8: PE

3. Incubate at 4^0 for 30 minute
4. Wash with FACS Buffer (1400rpm for 5 minute)
5. Decant and resuspend

6. Add lysing buffer (2ml to each tube) – 1x lysing buffer
7. Incubate for 30 minute at room temperature, dark
8. Centrifuge at 1400rpm for 5 minute
9. Decant and resuspend
10. Wash with FACS Buffer (1400rpm for 5 minute)
11. Decant and resuspend
12. Add 400ul FACS Buffer.

- ✓ All washes are with FACS Buffer (1ml)
  Start Flow cytometry staining from here

ANNEX 7Measurement of C - reactive protein Latex Test

Protocol 2

CRP is considered to be a sensitive indicator of acute stages of inflammation. Changes in the serum level of CRP with time from the same patient can be used as an index of recovery.

The RapidTex CRP Test is based on the latex-agglutination method introduced by Singer et al in 1957. The principle of this test is based on the immunological reaction between CRP as an antigen and the corresponding antibody coated on the surface of biologically inert latex particles.

Procedure

1. Bring all reagents, controls and samples to room temperature.
2. Shake the CRP latex reagent gently before use. Deliver one drop of reagent to the test circle. Using the disposable pipettes, add one drop of the undiluted patient sample onto the same circle and mix both together with the paddle end of the pipette.
3. Positive and negative controls should be run with each series of test serums in the same way as in Step 2.

4. Rotate slide back and forth for 2 minutes and read result under an indirect oblique light source.

ANNEX 8 Measurement of Lipid profile test

Protocol 3

Lipid profile includes measures of triacylglycerol and cholesterol in the form of lipoprotein-cholesterol molecules, low density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C). The results of testing for these lipids provide measures of risk for coronary artery disease

Procedure

1. Switch on the instrument Biosystem. Startup software package on your PC by clicking on the Administration icon. Biosystem requires a warm up time of at least 15 minutes after power up to allow the temperature control to warm up to the required temperature. Do not start any sample testing before these 15 minutes are completed

2. Load rotor

- ✓ Never touch the reading window of the rotor and press the “New rotor button”.

3. Check all the liquids including the waste tank. Place the prepared reagents into their position in the rack.

4. Set up patients work list by clicking “New sample” icon. In the new sample dialog box you can define test profile, ID, sex, age and name of the patient to prevent mistakes and then confirm with “OK”.

5. Select the parameters of the patient to be tested by clicking of the required profile.

6. Load the patient sample in to the rack according to the layout pre-defined.

7. Close the machine door

8. Click the start button

9. Click the “Current Result” button from the action menu bar in order to see results of patient