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DEPARTMENT OF MEDICAL LABORATORY SCIENCES

THE ASSOCIATION OF COPY NUMBER VARIATION OF FCγRIIIB GENE WITH THE RISK OF ENL IN LEPROMATOUS LEPROSY PATIENTS FROM SELECTED SITES IN ETHIOPIA

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This is to certify that the thesis prepared byDareskedar Tsehay, entitled: "***The Association Of Copy Number Variation Of FcγRIIIB Gene With The Risk Of ENL in Lepromatous Leprosy Patients From Selected Sites In Ethiopia"*** and submitted in partial fulfillment of the requirements for Master of Science degree in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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LIST OF ABBREVIATIONS

AAERC-AHRI/ ALERT Ethics Review Committee

ADCC- Antibody Dependent Cell-mediated Cytotoxicity

AHRI-Armauer Hansen Research Institute.

ALERT- All Africa Leprosy and Tuberculosis Rehabilitation and Training Center

ALM- American Leprosy Mission

BB- Mid borderline leprosy

BI- Bacterial Index

BL- Borderline Lepromatous leprosy

BT- Borderline Tuberculoid Leprosy

C1q- Complement 1q

CHS-College of Health Science

CNV- Copy Number Variation

Ct- Cycle Threshold

DNA- Deoxyribo Nucleic Acid

ENL- Erythema Nodosum Leprosum

ENLIST – Erythema Nodosum Leprosum International Study group

Fab- Fragment Antigen Binding

Fc- Fragment Crystalizable

FcγR- Fc Gamma Receptor

FcγRIIIB- Fc Gamma Receptor three B

FcγRIIA- Fc Gamma Receptor two A

FoSTeS- Fork Stalling and Template Switching

G2D-Grade 2 Disability

IC-Immune Complex

ITAM- Immunoreceptor Tyrosine-based Activation Motif

ITIM- Immunoreceptor Tyrosine-based Inhibitory Motif

LL-Lepromatous Leprosy

LSHTM- London School of Hygiene and Tropical Medicine

MDT- Multi Drug Therapy

MLS- Medical Laboratory Science

NADPH- Nicotinamide Adenine Dinucleotide Phosphate

NAHR- Non-Allelic Homologous Recombination

NET- Neutrophil Extracellular Trap

NHEJ- Non-Homologous End-Joining

NRERC-National Research Ethics Review Committee

OPD- Out Patient Department

PCR-Polymerse Chain Reaction

PI- Principal Investigator

PMN- Poly Morpho Nuclear Neutrophils

RA-Rheumatoid Arthritis

SIM- Sudan Interior Mission

SLE-Systemic Lupus Erytheromatus

TT- Tuberculoid Leprosy

ABSTRACT

Introduction: Erythema nodosum leprosum (ENL) is a Type 2 leprosy reaction which causes significant morbidity and mortality. It is characterized by systemic involvement. Despite its devastating nature, the underlying immunologic mechanisms of ENL have not been fully understood.Many researche findings have shown an association between low copy numbr of Fcgamma receptorIIIB (FcγIIIB) and immune complex associated diseases. We hypothesize that the development of ENL in lepromatous leprosy patients is associated to a low copy number variation (CNV) of FcγIIIB.

Objective: To investigate the association of the CNV of FcγRIIIBgene with the risk of ENL

Methods: A case-control study was conducted on total of100 patients from selected sites in Ethiopia; ENL patients (n=50) as cases and lepromatous leprosy (LL) patients with no history of ENL (n=50) as controls from April 2017 to June 2018. Clinical information was collected using ENL International Study group form. Blood sample (2.5ml) was collected on PAXgene tube And DNA was extracted using PreAnalytix PAX gene kit, followed by a quantitative Real Time-Polymerase Chain Reaction to assess genetic association of ENL with CNV of FCγIIIB gene. Clinical data was double entered in REDCap- Research Electronic Data Captureand analyzed using Graphpad prism version 7 and STATA version 11. P value less than 0.05 was considered statistically significant.

Result:The relative copy number of FCγIIIB was, median fold change (FC) =1.16 with 95%CI: (1.18-2.32)in cases as compared to controlsbut, the result was not statistically significant (p=0.05). There was also no statistically significant association between relative copy number and the clinical stages of ENL (p=0.07).

Conclusion: This study showed that there was no statistically significant difference inthe relative copy number of FCγIIIB in ENL patients as compared to controls. Further studies should be done to address the polymorphism of FCγIIIB andexpression of FCγIIIB on neutrophils should be measured using flow cytometry.

Key words: Leprosy, ENL, Fc gamma receptors, CNV, FCγRIIIB

1 INTRODUCTION

1.1. Background

Leprosy is an infectious disease caused by an obligate intracellular acid-fast bacillus *Mycobacterium leprae(M.leprae)*, that infects skin and peripheral nerves(1). Leprosy is a debilitating disease but can be treated effectively with multi drug therapy (MDT)(2). Leprosy presents a wide spectrum of clinical manifestations, ranging from polar tuberculoid (TT) cases with mild self-healing lesions to polar lepromatous leprosy (LL) showing chronic, progressive and destructive disease. In between the polar forms, the clinically and histologically less characteristic and immunologically unstable forms of borderline lepromatous (BL),mid borderline (BB) and borderline tuberculoid (BT) are found(3).

An estimated 214, 783 new cases and 12,437 newgrade 2 disabilities (G2Ds) were reported globally in 2016. Twenty two global priority countries in leprosy burden, including Ethiopia accounted for 95.03% of new leprosy cases. In Africa, 19,384 new cases were reported, Ethiopia reported 3692 new cases in 2016 (4).

It is estimated that 20% to 25% of patients with leprosy undergo inflammatory episodes; type 1 reactions (reversal reactions) and erythema nodosum leprosum(ENL) which is accompanied by systemic manifestations and tissue injury (5). ENL is an immune complex mediated reaction, which manifests as crops of numerous evanescent, erythematous, tender nodules and plaques over the extremities, trunk, face and other parts of the body. It has various morphological patterns; nodular, vesicular, pustular, bullous and necrotic with the nodular pattern being the most common. Due to immune complex mediated damage iritis, arthritis, lymphadenitis, orchitis and neuritis are common and treatment is with corticosteroid(6).

The histology of ENL lesions classically shows an intense perivascular infiltrate of neutrophils throughout the dermis and subcutis. In acute ENL lesions, neutrophils predominate in skin biopsies performed within 72hrs of occurrence (7). Leuckocytes of the immune system expess Fc receptors with which they interact with the Fc region of immunoglobulin G (IgG) FcγRIIIB isa receptor almost exclusively expressed on neutrophils, the signature cell of acute ENL(8).

FcγRIIA is also a receptor largely expressed on human neutrophils (9). However, expression of FcγRIIIB by neutrophils is 10-fold higher than the FcγRIIA (10)

1.2. Statement of the problem

Leprosy is one of the most common causes of non-traumatic peripheral neuropathy worldwide(8). Nerve damage is a clinical hallmark of leprosy and a major source of patient morbidity(9). The damage to peripheral nerves results in sensory and motor impairment with characteristic deformities and disabilities(1). Besides its measurable medical consequences, leprosy hampers the freedoms and capabilities of individuals and affected communities; and often excludes individuals from social life due to the often associated stigma. Ninety percent (90%) of beggars in cities of Africa are cured leprosy patients with irreversible disability (10).

Leprosy is further complicated by immunological reactions; reversal reaction and ENL reaction (11, 12). ENL is common in lepromatous leprosy patients; it causes systemic illness and affects multiple organs (13, 14). Available data indicates that ENL incidence ranges between 0.7 - 4.6% of all Multi Baciliary (MB) cases (15) and the prevalence shows geographical variation (15, 16). A study done in India shows households affected by ENL face significant economic burden and are at risk of being pushed further into poverty (17).

TNF-alpha is a pivotal molecule involved in the inflammatory manifestations of ENL and in mediating tissue damage(18).Corticosteroids are used to treat ENL and are often required for extended periods of time(14).Hypertension, steroid induced diabetes, cataracts, osteoporosis, weight gain, infection andsuppressed adrenal gland hormone production were reported to be induced by prolonged treatment with prednisolone(19).

In Ethiopia, the prevalence of ENL is 5.3%(20).In a five year retrospective study done in Ethiopia, eight patients with ENL died compared to patients with reversal reaction, which indicates the significant impact of ENL on morbidity and mortality of leprosy patients (21).

Despite its devastating nature, the underlying immunologic mechanisms of ENL have not been fully understood. Granular deposits of immunoglobulin and complement were found in the dermis of lesions from patients with ENL but not in LL patients supporting the long standing

dogma that ENL was caused by IC deposition. The exact mechanism of failure in IC clearance has not been fully understood. Therefore, this study was conducted to understand the immunopathogenesis of ENL specifically focusing on the role of neutrophilsFcγ receptor in the pathogenesis of ENL.

1.3. Significance of the study

Considering the unknown pathogenesis of ENL, the result of this study tried to show the association of FcγRIIIB with ENL. The result of this research may serve as important guide for future studies on the pathogenesis of ENL. The result of this study could provide supportive data for responsible stake holders to plan intervention and prevention activities on ENL.

2. LITERATURE REVIEW

2.1. Leprosy

In 2016, 214, 783 new leprosy cases were reported globally where 12, 437 were with Grade 2 Disabilities (G2D) and reported children cases with G2D were 281.Twenty two leprosy endemic countries, including Ethiopia account for 95.03% of new leprosy cases (4).

In Africa, 19,384 new cases were reported in 2016, of them 3692 were in Ethiopia. Multi baciliary (MB) cases account for 89.1% of the new cases; new case among children were 360, new G2D419 and new child cases with G2D 39 (4).

The Global Leprosy Strategy 2016–2020: "Accelerating towards a leprosy-free world "was released in April 2016. In endorsing the global strategy, 3 key targets have been agreed by all national programs: zero G2D among children diagnosed with leprosy; the reduction of new leprosy cases with G2D to <1 case per million populations; and zero countries with legislation allowing discrimination on the basis of leprosy(22).

The principal mode of transmission of *Mycobacterium leprae* is by aerosol spread of nasal secretions and uptake through nasal or respiratory mucosa. The incubation period of the disease ranges from 3 to 10 years (8)

Ridley and Jopling classified leprosy into tuberculoid leprosy, borderline tuberculoid, mid borderline, borderline lepromatous and lepromatous leprosy based on histopathological and clinical features(Figure 1). The borderline forms are immunologically unstable. WHO also classified leprosy into multi baciliary and paucibaciliary based on the number of lesions for the sake of treatment (23).

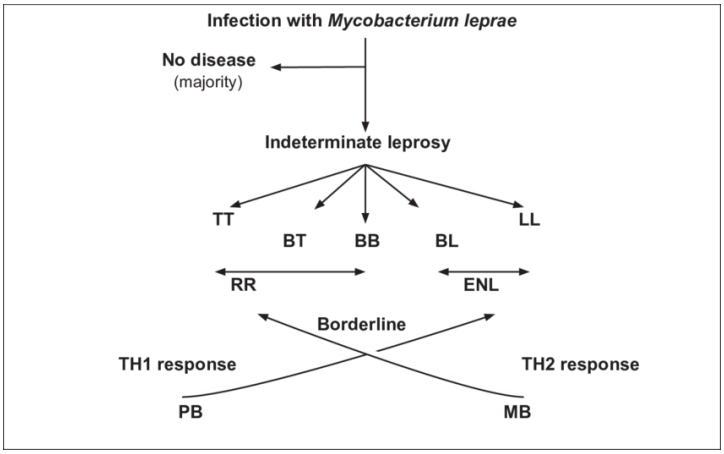


Figure 1: Ridley and Jopling classification of leprosy (source-Guidelines for the Control of Leprosy in the Northern Territory 2010)

2.2. Erythema nodosum leprosum (ENL)

There are two types of leprosy reactions type 1 (reversal) reaction and type 2 reaction (ENL)(23). ENL is associated with skin lesions (Figure 2a), neuritis, arthritis, dactylitis, eye inflammation, osteitis, orchitis, lymphadenitis and nephritis (24). Patients with ENL have an increase in the Th2 subtype, which stimulates the formation of plasma cells and production of immunoglobulin. Increased evidence has so far implicated TNF-alpha as a pivotal molecule involved in the inflammatory manifestations of ENL and in mediating tissue damage. In patients undergoing ENL increased IL-1β levels in their sera was also reported (18).

Granular deposits of immunoglobulin and complement were found in the dermis of lesions from patients with ENL but not in LL patients. In some cases the deposits apparently also contained soluble mycobacterial antigens. It is believed that ENL results from the deposition of immune complex (IC) in and around venules of the connective tissue septa of subcutaneous fat(25). Increased deposition of C1q was reported in skin lesions of patients with ENL, suggesting that complement is involved in systemic and skin lesions (26).

There are four hypersensitivity reactions; Type I: Immediate Hypersensitivity (Anaphylactic Reaction) these allergic reactions are systemic or localized, as in allergic dermatitis (e.g., hives, wheal and erythema reactions, Type II: Cytotoxic Reaction (Antibody-dependent), Type III: Immune Complex Reactions and Type IV: Cell-Mediated (Delayed Hypersensitivity)(27).

Histopathological features in fully developed lesions suggest a type III (antigen-antibody) hypersensitivity reaction mechanism; whereas other authors demonstrated a type IV delayed hypersensitivity reaction (Cell-mediated immunity) may also play an important role in the pathogenesis of ENL (28).

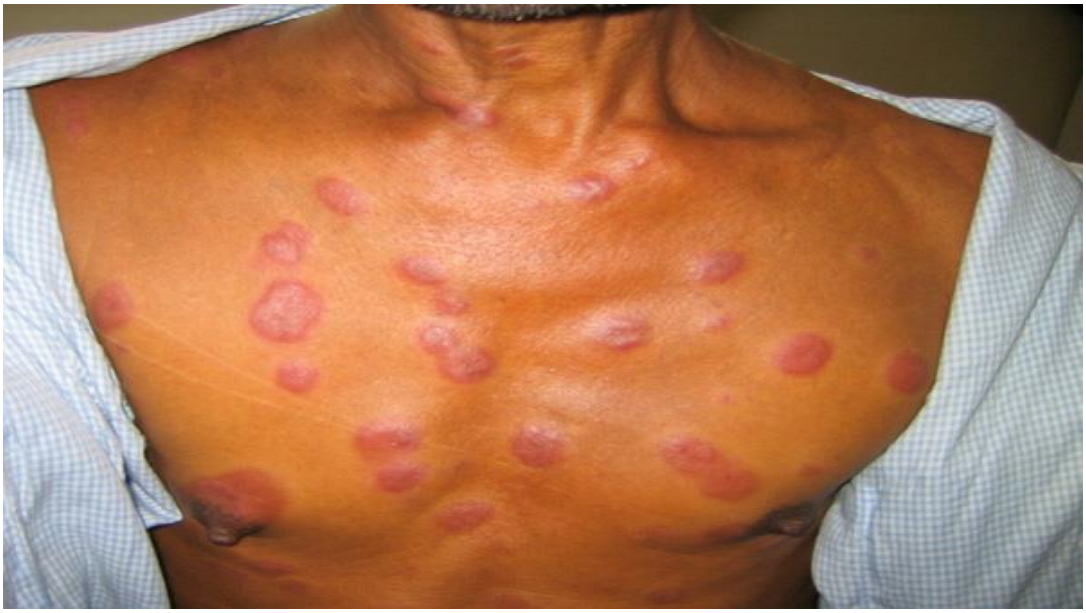


Figure 2a: Patient with ENL (source- Kahawita IP, Lockwood DN., 2008)

2.3. Neutrophils in ENL

Neutrophils are the first line of innate immune defense against infectious diseases (29). Neutrophil recruitment is pivotal to host defense against microbial infection, but also contributes to the immunopathology of disease (30). They are involved in phagocytosis, nicotinamide adenine dinucleotide phosphate (NADPH) oxidative burst, toxic granule dependent microbial killing, and neutrophil extracellular trap (NET) formation (31).

Neutrophil infiltration is considered to be the histological hallmark of ENL (Figure 2b). However, not all ENL lesions are characterized by the presence of neutrophils (32). A study of skin biopsies of ENL lesions within 72 h of onset showed a predominance of neutrophils (7). A retrospective study of 64 Indian leprosy patients (22 RR and 42 ENL) also confirmed the

presence of neutrophils within the granuloma in all ENL cases. It was also shown that neutrophils obtained from lepromatous leprosy patients with ENL showed accelerated ex vivo apoptosis compared with cells of non reactional lepromatous patients or normal individuals. In addition, Lipo Poly Saccharide (LPS), *M. leprae* and LipoArabinoManan (LAM-ML) were able to trigger cytokine production by PMN *in vitro*, namely IL-8 and TNF-alpha (33).

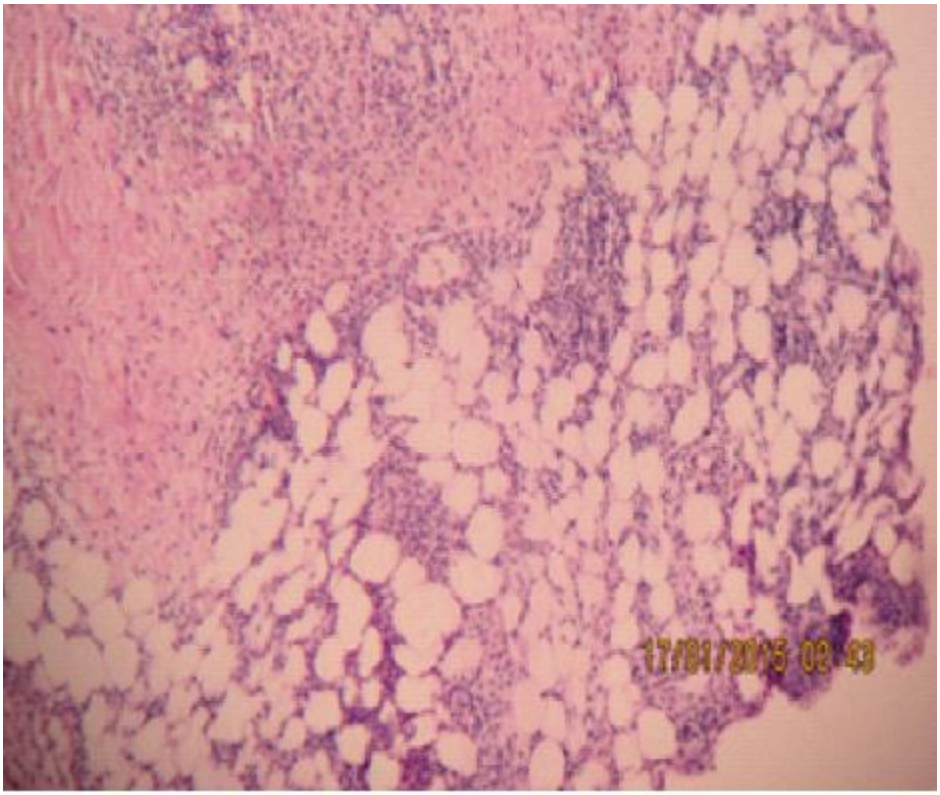


Figure 2b:H&E staining x40 showing PMN infiltration (ENL) (Source: Negera et al, 2017)

2.4. Fc gamma receptors

Fc receptors are a class of cell surface receptors that bind to the Fc portion of antibodies to form immune complexes and recruit the complement and effector system to defend the body against pathogens (34).They constitute critical elements for activating or down modulating immune responses and combines humoral and cell-mediated immunity (35).

Immune cell signaling can be initiated by binding of Abs to FcRs. After activation, FcRs trigger effector functions such as Antibody-dependent cellular cytotoxicity (ADCC), release of oxygen radicals, endocytosis, phagocytosis, degranulation, Ag presentation, clearance of circulating IC and antibody bound cells, and release of inflammatory mediators (36).

Human $Fc_{\gamma}Rs$, which interact with the Fc region of IgG are expressed by leukocytes and divided into four classes designated FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16) and neonatal Fc receptor. In addition, non-classical receptors for IgG include two FcR-like receptors, FcRL4/CD307d and FcRL5/CD307e that are homologous to FcγRI. $Fc_{\gamma}RI$ is the high affinity receptor for IgG, mainly restricted to monocytes/macrophages and dendritic cells (5, 6).

Functionally, there are two different classes of Fc receptors: the activation and the inhibitory receptors, which transmit their signals via immunoreceptor tyrosine-based activation (ITAM) or inhibitory motifs (ITIM), respectively. There is one single-chain inhibitory receptor, FcγRIIB. The paired expression of activating and inhibitory molecules on the same cell is the key for the generation of a balanced immune response (34, 37).

Neutrophil Fc receptors play an important role in recognition of the Fc portion of IgG prior to the ingestion of opsonized pathogen (34). Neutrophils co-express ITAM containing FcγRIIA and GlaycoPhosphotidyl Inositol (GPI) anchored FcγRIIIB, which is exclusively expressed on neutrophils. The density of FcγRIIIB expressed on neutrophils is 4 to 5 times higher than that of FcγRIIIA(38) .

The physiological role of FcγRIIIB remains enigmatic. *In vitro*, cross-linking of FcγRIIIB in human neutrophils induces Ca^{2+}mobilization, promotes actin assembly to prime FcγRIIA effector responses recruits FcγRIIA to lipid rafts to promote ITAM-based signaling and induces degranulation, but is unable to signal a respiratory burst and phagocytosis (39). FcγRIIIB plays a major role in the secretion of toxic products in response to ICs, but little or no role in the phagocytosis and killing of serum opsonized bacteria (40).

Studies have shown that IC mediated phagocytic and tumor-cell ADCC functions of human neutrophils are critically dependent on the signal delivered by FcγRIIA. FcγRIIIB alone is unable to deliver signal for phagocytosis, indicating that FcγRIIA is required for phagocytosis in normal neutrophils. Functional studies on neutrophils also suggest that the polypeptide anchored

FcγRIIA is a potent trigger molecule for cytotoxicity, whereas GPI-anchored FcγRIIIB is not (41).

The low-affinity Fc_{γ} receptor gene family is clustered on chromosome 1q23 and are highly polymorphic.The three polymorphic forms of Fc_{γ}RIIIB have been characterized and shown to alter neutrophil function. The polymorphic forms are known as neutrophil antigen (NA)1, NA2 and SH, exerting different levels of phagocytic function(42).

Fc_{γ}RIIIb-NA1 and -NA2 nucleotide sequences differ at five positions, resulting in four amino acid differences. The SH variant differs from NA2 at only one additional position, resulting in an A→Damino acid change that may affect the tertiary structure of the protein. Functionally, neutrophils from Fc_{γ}RIIIb-NA1 individuals bind and phagocytize IgG-opsonized bacteria and IgG-sensitized erythrocytes more efficiently than do those from NA2 individuals.(43, 44)

Recent work in a cohort of healthy volunteers used genotyping to analyze how the different haplotypes of neutrophil Fc_{γ}Rs functionally interact and showed the impact of Fc_{γ}RIIIB variants on neutrophil binding affinity to IgG, which could not be explained by differences in Fc_{γ}R surface expression.(43)

During normal physiological conditions, IC, antibody-coated microbial cells and antigen-antibody complexes, will be removed from circulation as a physiological response to infection. However, the lack of clearance of IC owing to the lack of complement activation or aberrant FcγR binding results in IC-mediated diseases (41).It is commonly accepted that low affinity FcγRs are critically involved in a wide range of antibody-complex diseases (45).

2.5. Copy number variation (CNV)

A copy number variation (CNV) has been defined as a DNA segment that is 1 kb or larger and present at variable copy number in comparison with a reference genome with the usual copy number of N = 2. It is simply different numbers of the same DNA sequence across different individuals and includes not only simple deletion and duplications but also more complex multiallelic variation, with copy numbers ranging from 0 to 14(46).It may arise from deletions, insertions, duplications and complex multi-site variants leading to quantitative variation in

expression (Figure 4) (47, 48). Copy number variation (CNV) has been shown to be common in regions of the genome coding for immune-related genes (49). Three genes, namely, FcγRIIA,FcγRIIC, and FcγRIIIB, show copy number variations (CNVs) (50).

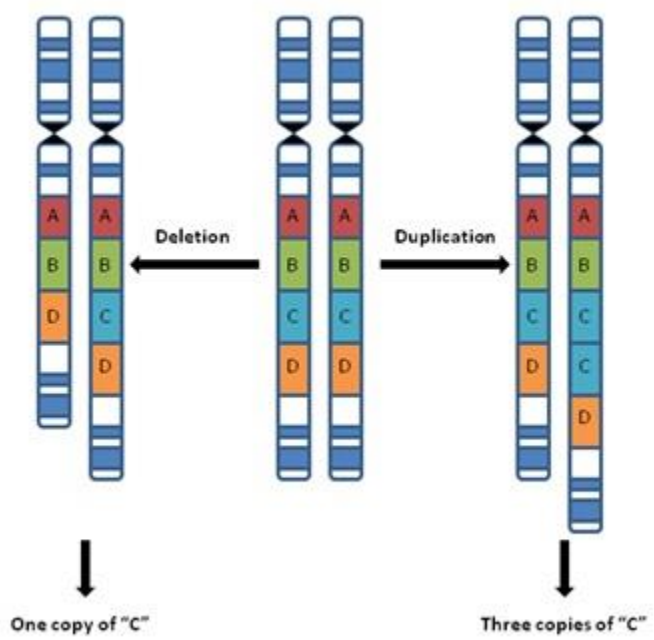


Figure 3: Copy number variation (source- Clancy, S. 2008)

Copy-number variants (CNVs) reshape gene structure, modulate gene expression, and contribute to significant phenotypic variation. Most genes present in CNVs show no evidence of increased or diminished transcription, and the fraction of such dosage-insensitive CNVs is greater in heterozygotes. More than 70% of the dosage-sensitive CNVs are recessive with undetectable effects on transcription in heterozygotes. A deficiency of singletons in recessive dosage-sensitive CNVs supports the hypothesis that most CNVs are subject to negative selection(51).

Non-allelic homologous recombination (NAHR), non-homologous end-joining (NHEJ), retro transposition, and fork stalling and template switching (FoSTeS) have been implicated in genomic rearrangements and the formation of CNVs(52).

Recent work has demonstrated an unexpected prevalence of copy number variation in the human genome, and has highlighted the part this variation may play in predisposition to common phenotypes(53).

2.6. Copy number variation (CNV) of FcγRIIIB

The frequency of individuals with only one copy of FcγRIIIB in Caucasians is estimated to be 15-20%, two copies are found in 60% and more than two copies are 10-20%. Similar copy number distribution has been recorded in African and Caucasian populations (54).

Individuals with reduced numbers of copies of the FcγRIIIB gene have an increased risk of developing systemic lupus erythematous (SLE) and SLE-associated glomerulonephritis. Low copy number of the gene FcγRIIIB was also associated with other IC-mediated conditions like rheumatoid arthritis (55), microscopic polyangiitis and wegener's granulomatosis (56).

A meta-analysis of 28 comparative studies showed a significant association between low FcγRIIIB copy number and autoimmune diseases (57).

In a study done in Kenya, 833 Kenyans were genotyped for FcγRIIIB CNV and 18% of the study participant had <2 copy number frequency of FcγRIIIB (42). A study done on 77 healthy Ethiopians also showed that all subjects had at least one copy of the FcγRIIIB gene (58).

Taking this into consideration, the characteristics of ENL and previous evidences of associations between FcγRIIIB and certain diseases, we found it necessary to investigate if there is an association between copy number of FcγRIIIB gene and ENL; no studies so far have addressed this.

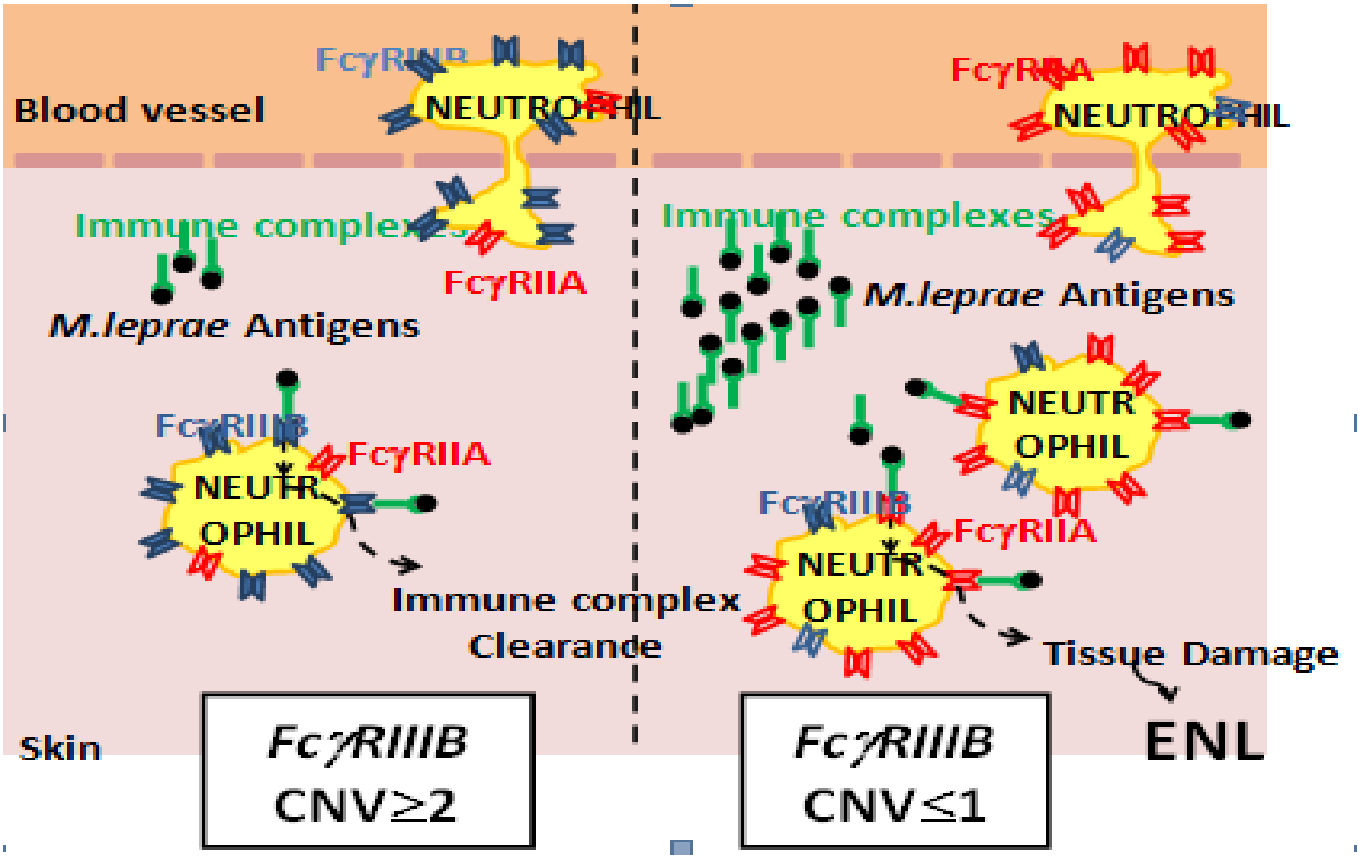


Figure 4: Copynumber variation of FcγRIIIB

Left: Copy number in FcγRIIIB gene is ≥2 because of normal diploid gene number or gene duplication, the predominant FcγR on the neutrophils is FcγRIIIB which interacts with formed IC in leprosy and leads to internalization and clearance of IC.

Right: When copy number in FcγRIIIB gene is ≤1 because of gene deletion, the expression of FcγRIIIB on neutrophils is decreased. This leads to an increased FcγRIIA expression on neutrophils due to compensation. FcγRIIA has a smaller capacity to interact and clear IC, which accumulate and deposit in tissues during leprosy, but concurrently leads to neutrophil extracellular traps (NET) formation and tissue damage.

2.7. Null hypothesis

The development of ENL in lepromatous leprosy patients is not associated with FcγRIIIBcopy number variation.

3. Objectives

3.1.1. General objective

- To investigate the association of the CNV of FcγRIIIB gene with the risk of ENLin lepromatous leprosy patients from selected sites in Ethiopia

3.1.2. Specific objectives

- To compare the CNV of FcγRIIIB gene in patients with history of ENL and with those who never had ENL
- To describe the clinical features of ENL and association with CNV of FcγRIIIB gene

4. MATERIALS AND METHODS

4.1. Study area and period

The study sites were ALERT (All Africa Leprosy and Tuberculosis Rehabilitation and Training Centre) hospital, Addis Ababa, Shashemene referral hospital (Kuyera), and Enemay and Shebel Berenta Woredas, East Gojjam Zone, Ethiopia. Samples were collected from all study sites, and transported to Armauer Hansen Research Institute (AHRI) for laboratory work, which was conducted from April 2017 up to June 2018.

ALERT, the former Princess Zenebework Hospital was initially established as a leprosy hospital by Dr. Thomas Lambie in July 1933. It has been serving as a referral hospital for leprosy and a leprosy training center for the whole Africa. The Red medical clinic at ALERT is dedicated for leprosy diagnosis, treatment and reaction management; more than 300 new leprosy cases visit the clinic every year(59).

Shashemene Referral Hospital, the former Kuyera leprosarium is among the pioneers in treatment of leprosy patients. It is located in Oromia region, West Arsi Zone 240 km away from the capital city Addis Ababa and 12km from Shashemene town. It was established in 1951 by the Sudan Interior Mission (SIM) and American Leprosy Mission (ALM) together with the Ethiopian Ministry of Public Health (MOPH). Currently it is serving as a referral hospital for people around Shashemene(59).

East Gojjam zone is located in Amhara region 300 km North West to the capital Addis Ababa. In 1960's high number of leprosy patients were reported. According to the Ministry of Health report 78.9% (15 out of 19) of the woredas in the zone are identified as hot spot areas for leprosy. For this study, in collaboration with the Bichena Health Center and ex-leprosy patient associations, tracing of ex-leprosy and active patients was done in Enemay and Shebel Berenta woredas.

AHRI is a biomedical research institute located in ALERT campus. It is involved in Mycobacterial, Bacterial & viral, Malaria & Neglected tropical diseases, Biotechnology & Bioinformatics and Non- communicable diseases researches. It was established in 1970 G.C. by the Swedish and Norwegian save the children seconded by the Ethiopian Federal Ministry of

Health. Recently, in Feb 2016, it was re-established as one research arm of the Federal Ministry of Health (FMOH) with expanded mandates.

4.2. Study design

A case-control study was conducted at ALERT hospital Addis Ababa, Shashemene referral hospital Kuyera, Enemay and Shebel berenta woredas, East Gojjam zone, Ethiopia.

4.3. Source population

All leprosy patients who visited ALERT and Shashemene referral hospitals and Ex-leprosy patients residing in Enemay and Shebel Berenta woredas during the study period were the source of population for this study.

4.4. Study population

Cases -Lepromatous leprosy patients aged 18 and above with ENLin ALERT and Shashemene referral hospitals and Ex-leprosy patients residing in Enemay and Shebel Berenta woredas during the study period were the study population.

Controls- lepromatous leprosy patients aged 18 and above with no history of ENL in ALERT and Shashemene referral hospitals and Ex-leprosy patients residing in Enemay and Shebel Berenta woredas during the study period were the study population.

4.5. Inclusion and exclusion criteria

4.5.1. Inclusion criteria

Cases -Lepromatous leprosy patients aged 18 and above with ENL in ALERT and Shashemene referral hospitals and Ex-leprosy patients residing in Enemay and Shebel Berenta were the study population.

Controls- lepromatous leprosy patients aged 18 and above with no history of ENL in ALERT and Shashemene referral hospitals and Ex-leprosy patients residing in Enemay and Shebel Berenta were the study population.

4.5.2. Exclusion criteria

For both cases and controls, patients from vulnerable groups (pregnant women, prisoners, individuals with mental disability, unconscious patients), with tuberculosis or other infectious diseases and who didn't give consent to participate in the study were excluded from this study.

4.6. Study variables

4.6.1. Dependent variable

Copy Number Variation of FcγRIIIB gene among ENL and non-ENL groups

4.6.2. Independent variables

Age

Sex

Treatment history with MDT

Treatment history with prednisolone

Neutrophil count

Stage of ENL

4.7. Sample size determination

The sample size was calculated using the online sample size estimator (OSSE) from the Bioinformatics Institute (BII) in Singapore (http://osse.bii.a-star.edu.sg/calculation1.php). It is estimated that genotyping 50 ENL cases and 50 controls will give 80% power at a level of significance of 5% to detect an Odds Ratio >1.98.

4.8. Sampling method

Consecutive sampling technique was used. Study participants were recruited at Red Medical Clinic (a clinic dedicated to leprosy diagnosis and treatment) and Sick Out Patient Department (where ex-leprosy patients and their families get treatment) at ALERT Hospital. In addition participants who came to the dermatology OPD and leprosy ward of Shashemene Referal Hospital (Kuyera) were recruited to the study.

In East Gojjam Zone leprosy registration books were reviewed going back to 1960's from the record found in Bichena Health center. This was done to include the right control groups (LL patients who completed their treatment 20-30 years back and didn't develop ENL) in the study. Data was cross checked with their medical card to make sure their eligibility for the study. Participants who fulfilled the inclusion criteria were traced through the leprosy associations in Enemay and Shebel Berenta Woredas by health extension workers.

4.9. Data collection procedure

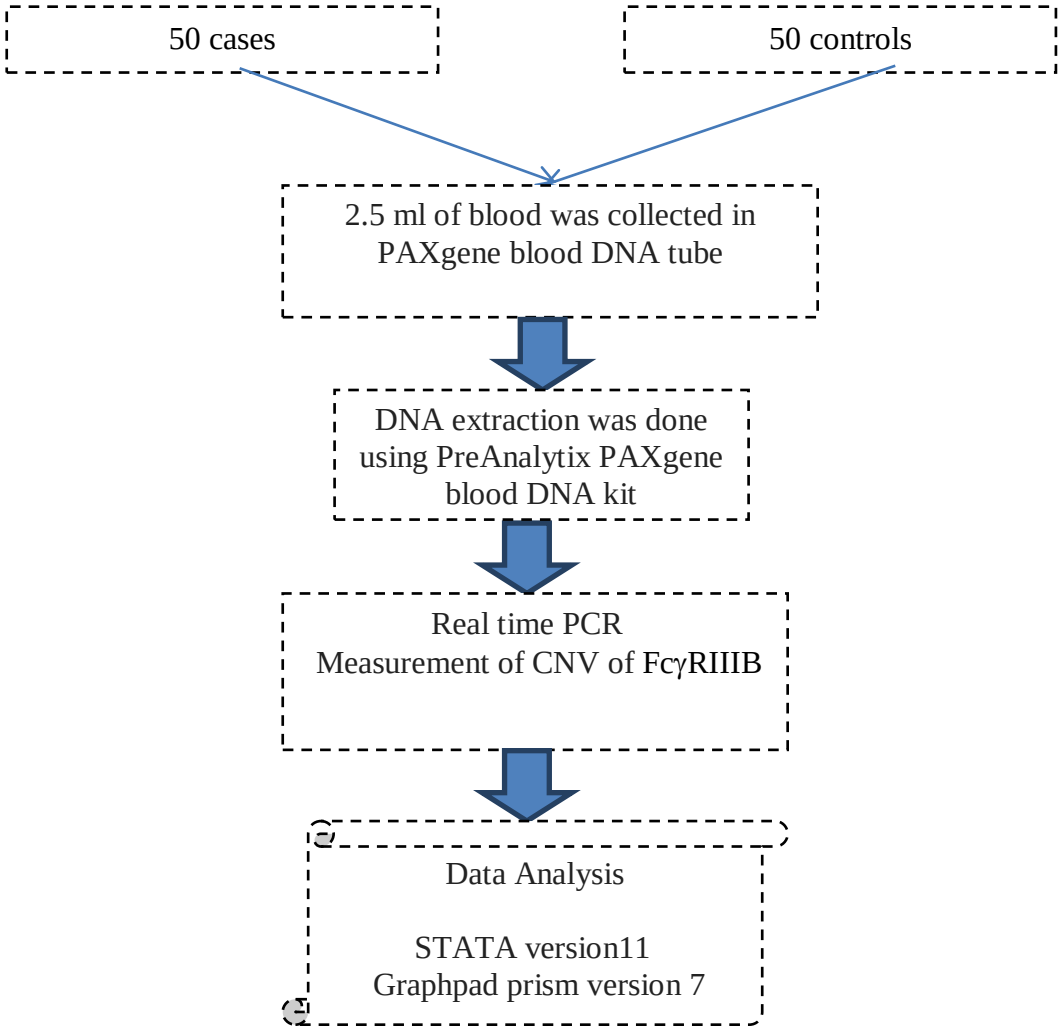
Information sheet and consentform were prepared in English and translated to Amharic (Annex II). Consenting was done by experienced health officers in the two hospitals and by health extension workers in Enemay and Shebel Berenta woredas.

The information sheet was read to the participants. They were told that they have the right to refuse and withdraw anytime from the study. Information about participants that were collected during the study was stored in a file, which didn't have the participant's name, but has a code number. The data was not revealed to anyone except the principal investigator and responsible individuals in the study.

A written consent was obtained from each participant after they agree to participate in the study. Participants signed on the consent and those who were unable to sign, gave a thumb print in the presence of an impartial witness that signed on the witness part of the consent. Participants were compensated for lunch and transport was given to participants who came from outside of Addis Ababa.

Modified ENL International Study Group (ENLIST) form was used to obtain clinical information about participant (Annex III). Each participant was asked about their current symptoms by the sample collector. In addition from the participants' information, hospital card was also collected to get background information about their medical history. Cross checking of each patient's card with the information filled by the data collector was done by the principal investigator before the laboratory work was initiated. This was done to make sure whether participants included in our study were eligible or not.

4.10. Work Flow



4.11. Specimen collection and storage

After the participants gave consent, specimen was collected by experienced nurses. From each participant 2.5 ml whole blood was collected using PAXgene® blood DNA tube (PreAnalytix, GmbH, Germany).The PAXgene blood DNA tube contains an additive which anti-coagulates the blood and preserves genomic DNA.

The sample was kept at room temperature for 2 hours; enough time for the anticoagulants and stabilizers to mix with the whole blood and then stored at -20ºc until DNA extraction.

4.12. DNA Extraction

Genomic DNA was extracted using PAXgene Blood DNA Kit(PreAnalytix, GmbH, Switzerland) following the manufacturer's protocol. Three basic steps were followed in DNA extraction. The first step was lysis using lysing buffers to disrupt the cell membrane to get the nucleus where the DNA is found. The second step was precipitation using isopropanol and the last step elution (Annex IV) (60).

4.13. Measurement of relative CNV of FcγRIIIB using Real Time-PCR

4.13.1. Optimization using density gradient

Optimization for both melting temperature and primer concentration was done on Bio-Rad T 100 thermal cycler. Density gradient method was used on thermal cycler. The PCR was amplified at different primer concentrations. The temperature was set at 8 different temperatures to get the best optimization condition easily. Result was compared by running the amplified products on agarose gel.

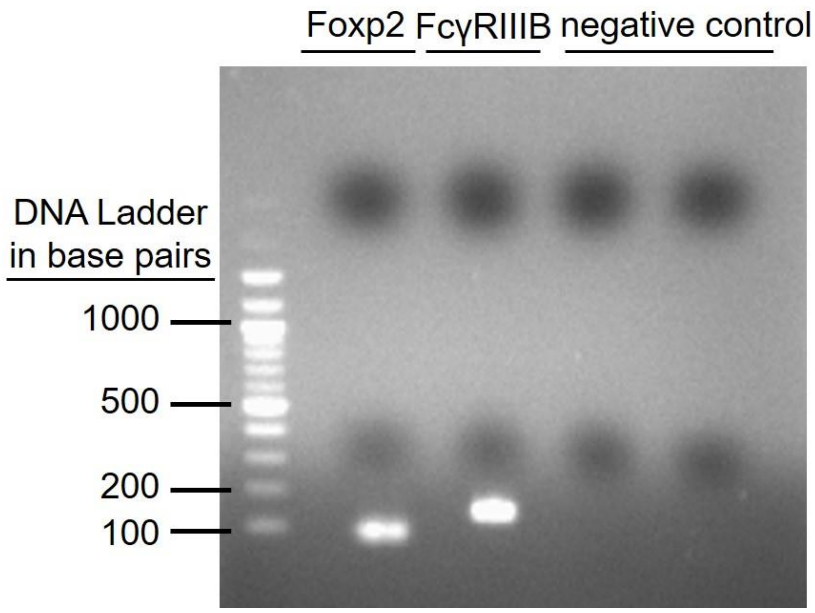


Figure 5: Gel electrophoresis of PCR product using 100 bp ladder.

4.14. Quantitative Real Time PCR

Standard Operating Procedure (SOP) was adopted by the PI. (Annex V).PCR was done using Rotor-Gene 3000 programmable thermal cycler (Corbett Life Science (Qiagen), Crawley, UK). PerfeCTaqPCRToughMix(Quantabio, USA) was used. Primers and probes (Sigma Aldrich, USA) were also used (Table 1).

Table 1: **Primer and probe sequences for qPCR**

No.	Gene		Amplico size
1.	FcγRIIIB FP	AGTTTGAGATGCCTTGGGTTC	125 bp
	FcγRIIIB RP	CCATCTTGGCTTGTCCTGGTA	
	FcγRIIIB probe	5'-HEX-CCACAGCTATAGATGTGGTGAGGGG-BHQ1-3'	
2.	FOXP2 FP	TCACTACTAACAATTCCTCCTCGACTAC	69 bp
	FOXP2 RP	GATGAGTTATTGGTGGTGATGCTT	
	FOXP2 probe	5'-FAM-TCCTCCAACACTTCC-BHQ3-3'	

All the PCR reaction mixtures were prepared using Corbett Robotics (Corbett Research, Australia) and run in duplicates including non-template controls; to test contamination of reagents. The total reaction volume was 10 µl.Primers were used at a final concentration of 400nm and probes at 200nm the DNA was diluted to a final concentration of 10 ng/ µl (Table 2)

Table 2: Volumes of reagents for Master mix preparation.

Reagent name	Amount in microliters
Master mix	5µl
FCGR3B forward primer	0.4µl
FCGR3B reverse primer	0.4µl
FCGR3B probe	0.2µl
FOXP2 forward primer	0.4µl
FOXP2 reverse primer	0.4µl
FOXP2 probe	0.2µl

Water	2µl
Template DNA/ water	1µl

The PCR condition: initial denaturation at 95^{o}C for 15 minutes followed by 40 cycles of denaturation at 95^{o}C for 10 seconds, annealing at 50^{o}C for 45 second and extension at 72^{o}C for 30 seconds. The sample was run in duplicates. Data/fluorescence was acquired at the end of the extension phase. The acquisition was made on FAM/SYBR channel for FOXP2 and on JOE channel forFcγRIIIB.

We used FOXP2 as a reference gene. FOXP2 was selected because it doesn't show CNV it is found in two copies(56). Non template control was used as a negative control. The threshold cycle (CT), the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily point, was set at the exponential phase of amplification and used as the quantitative endpoint of qPCR as described earlier (61).

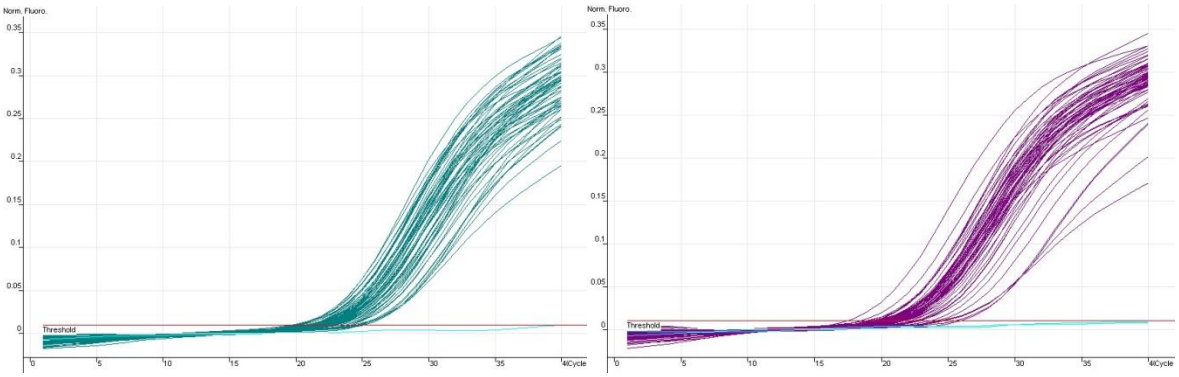


Figure 6a: Raw data showing the qPCR run for FcGR3B and NTC

Figure 7b: Raw data showing the qPCR run for FOXP2 and NTC

Calculating relative CNV

In this study we used a reference gene that is found in two copies. The control groups were considered as calibrator groups and the case groups were considered target groups. We used Livak method ($2^{-\Delta\Delta CT}$)

CNV was calculated using ΔΔCT

ΔCt = Ct of FCGRIIIB − Ct of FOXP2 of non-ENL (controls) then we took the average delat Ct of the controls and subtract from each of the delta Ct of the cases and controls.

ΔΔCT= ΔCt of cases- ΔCt of controls

$2^{-\Delta\Delta CT}$ was calculated to measure the fold change (FC)(61).

The final result of this method is presented as the fold change of target relative CNV in a target sample relative to a control sample, normalized to a reference gene. Since the relative gene expression is usually set to 1 for control samples, the $\Delta\Delta CT$ is equal to 0, resulting in 2^0, which is one. According to this concept, the mean fold change greater than one indicates increased CNV and fold change less than one indicates CNV less than 2 (62). P value less than 0.05 is considered statistically significant.

4.15. Quality Assurance

Pre Analytical

Experienced health officer in research was selected for sample collection. For samples collected from field sites,health extension workers were trained on sample collection. ENLIST form was used for collection of clinical and demographic information. Clinical data was double entered on Redcap software. Trainings and on bench supervisions were given for the PI ,standard operating procedure was adopted for qPCR.

Analytical methods

The purity and concentration of DNA was measured using Nano drop(Thermo scientific, Epsom UK). Gel electrophoresis was done to check whether the amplified product was our target of interest or not. In each PCR run non template controls were used as negative control. Standard curve was generated after serial dilution and amplification to see the efficiency of the reaction.

Post- Analytical

After the completion of the lab work the samplesand reagents were stored at at -20^{o}C. In addition the data generated from this research was stored in AHRI data base.

4.16. Data analysis and interpretation

Clinical information was double entered to Redcap software. Data cleaning was also done. Analysis for the clinical information was done using STATA version 11. Results are presented in Table, graphs and pie charts.

Laboratory results were analyzed using Graph pad prism V7.The normality of distribution of the data was done using D'Agostino & Pearson normality test. Since our data was not normally distributed we used median and non-parametric tests for analysis. The statistical difference for the relative copy number(expressed as FC) was analyzed using Wilcoxon Signed Rank Test. Relative copy number among the three stages of ENL was analyzed using Kruskal Wallis test. P value less than 0.05 was considered statistically significant. The result was presented in graphs.

4.17. Ethical considerations

Ethical approval was obtained from the Department Research and Ethics Review Committee of Medical Laboratory Sciences, CHS (MLS/411/17), National Research Ethics Review Committee (NRERC) (310/53/2018) and AHRI ALERT Ethics Review Committee (AAERC) (P004/15). Support letters were obtained from ALERT Hospital and East Gojjam zone.(Annex 1) The principal investigator was also trained on Research Ethics and Good Clinical Practice/Good Clinical Laboratory Practice (GCP/GCLP).

4.18. Dissemination of results

The findings of this study will be submitted to Addis Ababa University School of Allied Health Science Department of Medical Laboratory Science, Armauer Hansen Research Institute and London School of Hygiene and Tropical Medicine. An attempt will be made to present the findings in different conferences and workshops and will be sent to publication on peer reviewed scientific journals.

4.19. Operational definitions

ENL– When a patient has tender erythematous nodules with systemic involvement

Grade 2 Disability (G2D) –A patient with an irreversible disability

Acute ENL- For a single episode lasting less than 24 weeks

Chronic ENL - If occurring for 24 weeks or more during which a patient has required ENL treatment either continuously or where any treatment free period had been 27 days or less.

Recurrent ENL - If a patient experienced a second or subsequent episode of ENL occurring 28 days or more after stopping treatment for ENL

Nerve function impairment (NFI) - Is defined as any reduction in sensory or motor function.

Neuritis - inflammation of the peripheral nerve trunks. It may or may not be accompanied by clinically detectable NFI.

5. RESULTS

5.1. Demographic and clinical results

In this study 100 individuals were enrolled, 50 participants with ENL history (cases) and 50 participants with history of LL but never had ENL (Controls). The male to female ratio was 1: 1.2 with median age 42 (range 18- 87) for cases and 1.7: 1 for controls with median age 63 (range 19-81). Among the cases; 12 % were new ENL cases, 14 % recurrent and 74% were chronic ENL patients. The mean Bacterial Index (BI) at initial diagnosis was 3.7.

Nerve function impairment (NFI) was seen in 52% of patients where new nerve function impairment accounted for (16%) and old nerve function impairment (36 %) in patients with ENL.Conjunctivitis was seen in 18% of patients and cataract in 8% of patients with ENL. Not all patients have eye infection (Table 3).

During the study period, 36.7 % of the patients were on prednisolone treatment. Prednisolone (76.67 %) and clofazamine (45%) were used previously in the treatment of ENL reaction(Figure 7). There were patients who took more than one treatment; two treatments (33.33%), three treatments (3.33%) and four treatment (1.67%).

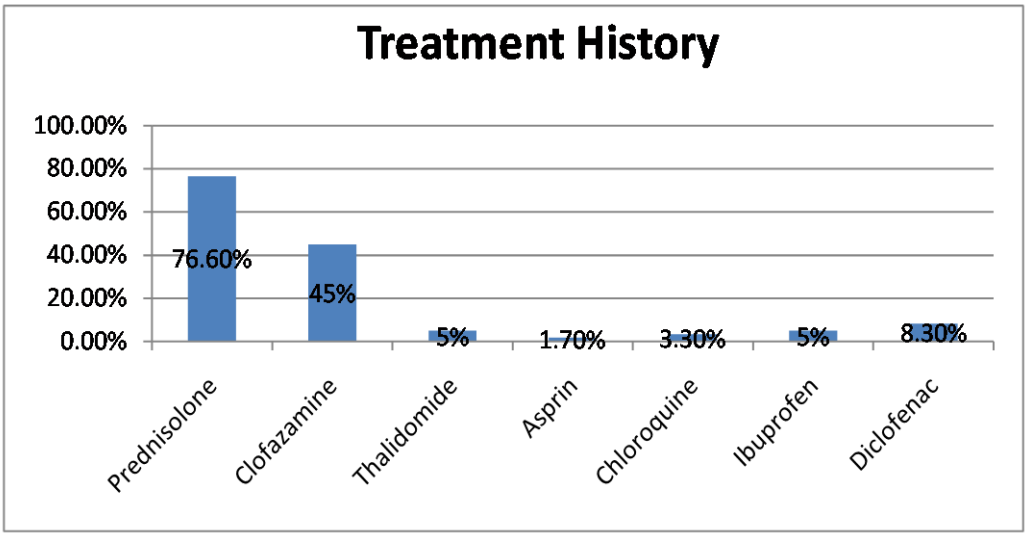


Figure 8: Treatment history of ENL among study participants in selected study sites, Ethiopia.

Table 3: Demographic and clinical results

Variables		ENL (n=50) n (%)	LL (n=50) n (%)
Sex	Male	21(42)	35(70)
	Female	29 (58)	15(30)
Median age in years (range) group		42 (18-87)	63 (19-81)
ENL type	New	6(12)	-
	Recurrent	7(14)	-
	Chronic	37(74)	-
Nerve symptoms	Pain	2(4)	1(2)
	Weakness	17(34)	15(30)
	Reduced sensation	31(61)	22 (44)
	Paraesthesia	13(26)	3(6)
	New NFI	8(16)	4(8)
	Old NFI	18(36)	11(22)
	G2D	5(10)	5(10)
Eye infection	Conjunctivitis	9(18)	7(14)
	Cataract	4(8)	2(4)
	Sceleritis	3(6)	1(2)
	Trichiasis	1(2)	1(2)
	Visual acuity	4(8)	2(4)
Madurosis		8(16)	6(12)

During the first diagnosis, 30.34 %of patients were presented with reaction. Most of the patients (41.66%) developed reaction while they were on treatment and a quarter of the patients developed the reaction after completion of treatment.

Pain symptom was observed in; joints (25%), digits (21.7%), nerves (18%), eyes (13.3%), skin (8.3%) and bone (8.3%).Among the study participants 41% of them took prednisolone for more than 4 years (Figure 8).

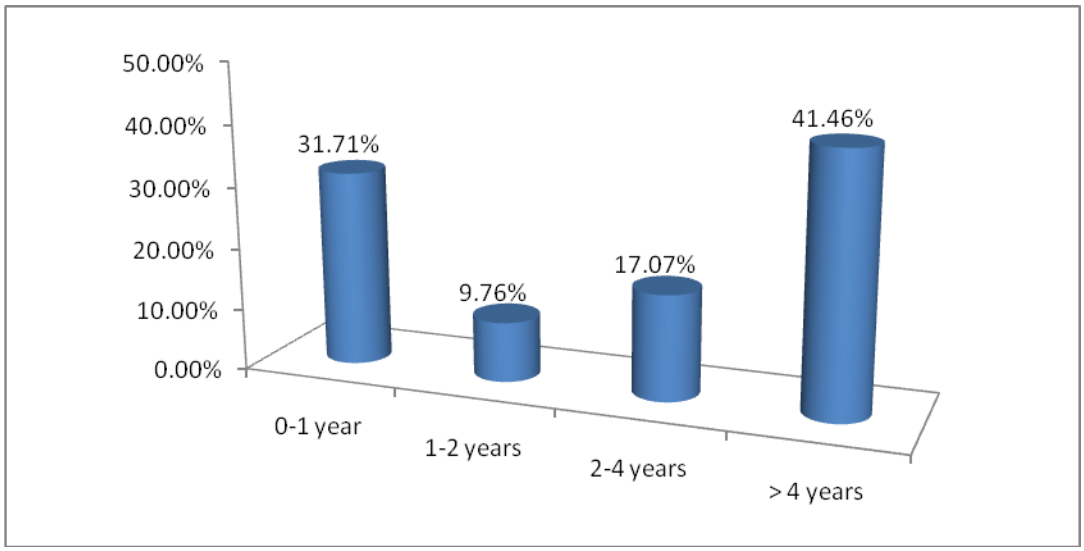


Figure 9 Duration of treatment with prednisoloneamong study participants in selected study sites, Ethiopia.

Insomnia was common in a quarter of patients. Other symptoms include; Malaise (18.3%), Nasal stiffness (16.7%), Anorexia (16.7%), Epistaxis (11.7%) etc. Pain symptoms were common in joints (25%), digits (21.7%), nerves (18.3%), eyes (13.3%), bone (8.3%), skin (8.3%) and muscle (3.3%).

5.2. CNV of FcγRIIIB

The difference in the average delta Ct between cases and controls was not statistically significant with p=0.72 (Table 3)

Table3: The Ct value between cases and controls

	ENL (Cases)	LL(Controls)	P value
Average Ct	**22.46 ± 0.27**	**22.33 ± 0.25**	**0.72**
ΔCT	**-4.84**	**-5.04**	**0.9**
ΔΔCT	**0.13± 0.27**		
Fold change (Median)	**1.16**		

The difference in the delta Ct was not statistically different among cases and controls with p=o.9 (figure 9)

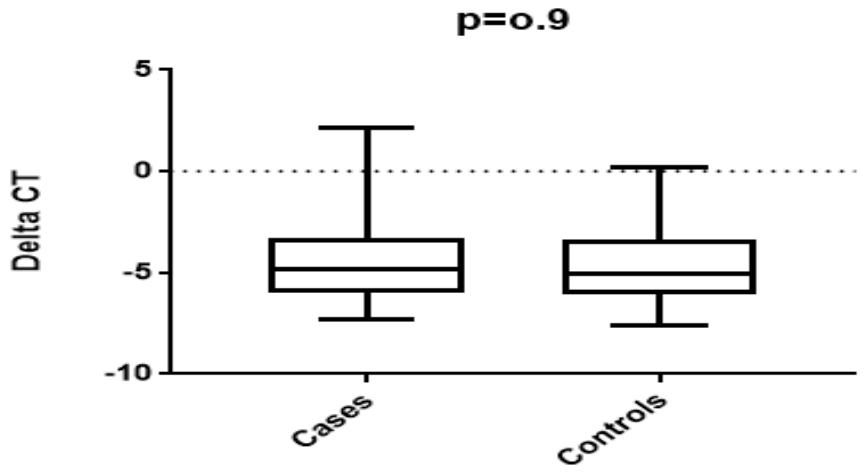


Figure 9: Difference in delta Ct between cases and controls

Our result showedmedian FC=1.16 with 95%CI: (1.18-2.32). There was no statistically significant difference between cases and controls (p= 0.06) (Figure 10).

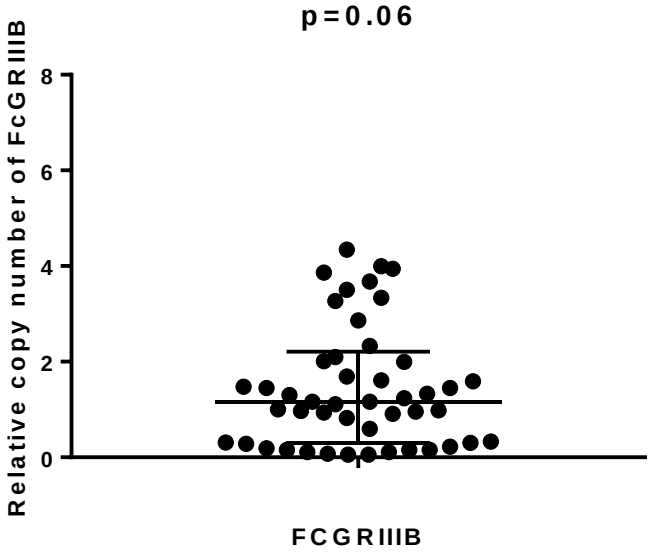


Figure10: The relative copy number of FCGRIIIB/FOXP2among study participants in selected study sites, Ethiopia.

Relative CN in the three groups of ENL; new, recurrent and chronic were compared.There was no statistically significant difference in the three groups (p= 0.07) (Figure 11).

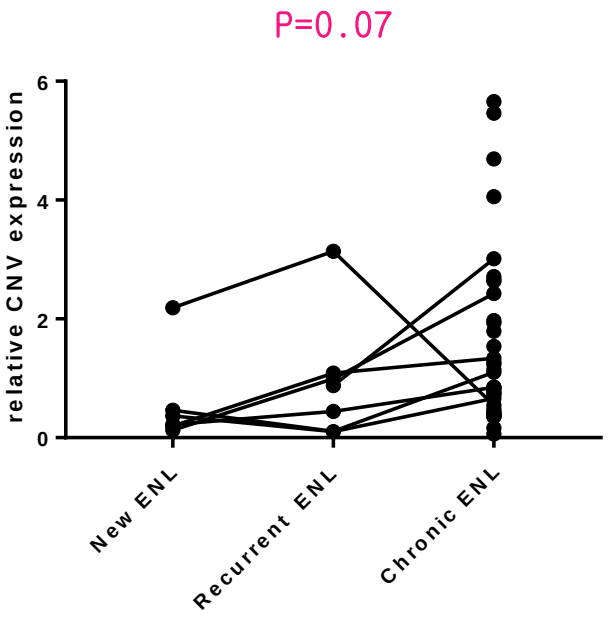


Figure11: Relative CNV new, recurrent and chronic patientsamong study participantsin selected study sites, Ethiopia.

6. DISCUSSION

In our study the male to female ratio of 1: 1.2 for ENL patients was different from other study done at ALERT which found a ratio of 1.7: 1(14). The difference might be due to; many study sites included in our study which were not included in other studies. In addition our study included all stages of ENL patients new, recurrent and chronic which is different from the above mentioned study on new ENL patients. A15 year retrospective study in India showed consistent finding with our result(63).

This study showed 74% of the participants were chronic ENL patients. This result is in line with a study done at ALERT. The extended duration of ENL has both social and medical implications for patients. Chronic ENL needs long time treatment with corticosteroid to control the reaction which itself pose a life threatening problem for patients. A study done at ALERT showed eight ENL patients died due to the complications of the immunosuppressive drug they were taking(21).In addition, ENL poses a socio-economic hardship for the patients as well as the affected family. A study done in India showed both treatment costs and decreased productivity due to leprosy affect the income of the family. The household cost of ENL was 28% of monthly income and 11% of households faced catastrophic health expenditure(17).

The long duration of treatment with prednisolone observed in this study, which is more than 4 years is an alarming data. WHO recommended the total duration of a standard course of ENL treatment with corticosteroids (prednisolone)to be 12 weeks. WHO also recommends the dosage should be tapered every two weeks and treatment should be done under supervision. This finding neither showed supervision nor tapering. Although treatment with corticosteroid has been shown to improve the patient situation, it does not always control the reaction(64).Prednisolone have been shown to cause complications such as Hypertension, steroid induced diabetes, cataracts, osteoporosis, weight gain, infection andsuppressed adrenal gland hormone production (65).

Our finding of; Mean BI of 3.7, is in line with a study done in Ethiopia and India(20, 66). Higher BI is considered as a risk factor for ENL. The result on percentage of patients who developed the reaction at diagnosis (30.34%) was similar to other studies(21, 65). The finding of this study on patients, who developed the reaction on treatment (41.66%) and after completion of treatment

(25%), is in line with a study done at ALERT(21). It has been known that a patient can develop ENL before initiation of treatment, after initiation of treatment and after completion of treatment.

The G2D in ENL patients was found to be 10 %. This was in line with a 10 year national report done in Ethiopia(67).

One third of ENL patients who developed the reaction on treatment developed the reaction within 9-12 months after the initiation of treatment. This finding was different from the one done in India which showed most of the patients developed the reaction within the first three months. This can be attributed to the geographical variation in ENL occurrence. In India the prevalence is 20-35% which is higher than 5% in Ethiopia(66).

In this study, we evaluated CNV ofFcγRIIIB between cases and controls. Our result showed the relative copy number of FcγRIIIB was found to be,median FC=1.16 and statistically there was no significant association (P=0.06). Although we know of no other publication in FCGRIIIB and ENL, our result is in line with some of the findings in autoimmune diseases.(68)

A study done in Chinese population showed, though the relative copy number variation of the FcγRIIIB gene varied in both lupus nephritis patients and healthy control subjects, no statistically significant correlation was found between the relative copy number variation and LN incidence. The difference was still not statistically significant when the data were stratified by gender, pathological phenotype (p=0.511) and chronicity Index (0.401)(69). Another study done on Behcet's disease (BD), an immune-mediated systemic vasculitis also showed no association between high (p=0.50) or low (p=0.16) CNV of the FcγRIIIB gene and Behcet's disease or its clinical features in Iranian population (70). Comparable result was also obtained in Anti-glomerular basement membrane antibody disease (anti-GBM disease). No statistically significant difference was found between anti-GBM patients and healthy controls(71). It was also shown that low CNV of FcγRIIIB is not associated with organ specific autoimmunity (56).

Although the above mentioned findings show no statistically significant associations, there are dozens of researches which showed a significant association between low CNV of FcγRIIIB with different diseases. Systemic lupus erythematous was found to be significantly associated with low CNV of FcγRIIIB gene. In addition it was shown that the low copy number variation also

affected the expression of this receptor on neutrophils (49). Low copy number of the FcγRIIIBgene was also associated with other IC-mediated conditions like rheumatoid arthritis, microscopic polyangiitis and wegener'sgranulomatosis (55).

The result of our study is different from the above studies. This difference might be due to different reasons. One possible reason is the immunopathogenesis of ENL and other autoimmune diseases is different. Most of the associations with low CN of FcγRIIIBwere observed in autoimmune diseases. ENL occur in leprosy patients which is caused by *M.leprae* a bacterial pathogen whereas autoimmune diseases occur when the body's immune system attacks and destroys healthy body tissue by mistake. The difference could be attributed to this.

CNV have shown different results in different populations. The same disease has been shown to give a different result in different population. Lupus nephritis was associated with low CN of FcγRIIIB in many populations, however, it was not associated with Chinese population (69); low FcγRIIIB which is associated with SLE was also found to be not significantly associated in Hong Kong SLE patients(49). Although there are limited researches done on people of African descent as compared to other populations, they are not enough to compare the association difference in different populations.

In Ethiopia we didn't come across studies on low CNV of FcγRIIIB and disease. The best available data found is on the polymorphisms of Fc gamma receptors in Ethiopian population. In the study it was shown Ethiopians had higher frequencies of the SH-FcγRIIIb (P=0.001) and FcγRIIIb-Na2 (P=0.046) alleles as compared to Norwegians. The study showed the variation of different polymorphisms both within and between ethnic groups may influence differences in the incidence rates of infectious and autoimmune diseases(58). We didn't come across publication on the FcγRs genetic polymorphisms in relation to ENL.

Finally the methods we used were different. In our study quantitative real time PCR was used. There are many methods for the measurement of CNV with increased precision and sensitivity such as; Droplet Digital PCR (ddPCR), Array Comparative Genomic Hybridization (aCGH) and Multiplex Ligationdependent Probe Amplification (MLPA).The methodological difference and the use of one single method might be another reason for the difference in result.

7. Strengths and Limitations of the study

Strength of the study

- The right control groups were included in the study
- Many study sites were included in the study which increases the representativeness of the result.

Limitations of the study

- There were no healthy controls.
- We were Unable to use ddPCR for CNV measurement.
- Other kinds of samples were not collected; plasma and whole blood for related *invitro* analysis which could have generated more relevant information.
- The study of one single gene
- Use of one single method that is qPCR

8. CONCLUSION

We have shown for the first time that the difference in the relative copy number of FCγIIIB in ENL patientsas compared to controls was not statistically significant.The difference was alsonot significantly associated in the three stages of ENL.

9. RECOMMENDATIONS

Based on the findings of the present study the following recommendations could be made:

- Expression of FcγRIIIB on neutrophils should be assessed using flowcytometry.
- A large scale study comprising different study sites and different samples using multiple laboratory approaches should be done.
- More genes and their expressions as proteins should be studied like FcγRIIA
- Polymorphism of FcγRIIIBwhich shows functional difference despite the normal copy number in neutrophils should be studied.

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Annex I: Ethical clearance and support letters for the study

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Addis Ababa UNIVERSITY
Collage of Health Sciences
School of Allied Health sciences
Department of Medical Laboratory Sciences

P.O. Box 1176, Addis Ababa PHONE (251) 112-755170 FAX: (251) 112-754669 e-mail: SMLT@ethionet.et

Date: 30 / 03 / 2017

Ref.No. MCS/482/17

Departmental Research and Ethics Review Committee (DRERC) decision

Meeting No: 004/2017

Protocol number: DRERC/269/17/MLS

Protocol title: " The role of neutrophil Fc_γ receptors in the pathogenesis of Erythema Nodosum Leprosum."

Principal investigator: Dareskedar Tsehay Sewasew
Institute: AAU-MF CLS

Elements reviewed (AAUMF 01) [√] Attached [ ] Not attached

Review of revised application [ ] Yes [√] No

Date of previous review: _____

Decision of the meeting: [√] **Approved** [ ] Approved with recommendation

[ ] Approved on Condition (Major revision) [ ] Disapproved

Obligation of the PI-

1. Should comply with the standard international and national scientific and ethical guidelines
2. All the amendments and changes made in protocol and consent form needs DRERC approval
3. The PI should report DRERC within 10 days of the event.
4. End of the study, including manuscripts and thesis works should be reported to the DRERC

Departmental Research and Ethics Review Committee (DRERC) Approval period: from March 30, **2017 to March 29, 2018.**

Follow up report expected in

3 months_____ 6 months__√__ 9 months_____ one year_____

Chairperson, DRERC: Kassu Desta

Signature: _____

Date: 24/03/2017

School head: Dr. Mistre Wolde

Signature: _____

Date: 23/3/17

	AHRI/ALERT Ethics Review Committee	Date: July 28, 2017 No:

ANNEX 4
Form AF-16-015.1

AAERC Approval Letter

Protocol number: PO04/15

Investigators: Dr. Abraham Aseffa, Anastasia Pobrcarpou, Dr. Diana NI Lockwood

Protocol Title: The role of Neutrophil Fcγ receptors in the pathogenesis of Erythema Nodosum Leprosum."

Study Site(s): Addis Ababa, Kuyera, Northern Shoa

Application Type: Initial ☐ Amendment ■ Renewal ■

Review Procedure: Full Board ☐ Expedited ☐ Secretariat ■

Review Date: July 27, 2017

Final Decision: ■ Approved Approval Date: July 28, 2017

Approval period: July 28, 2017 to July 27, 2018

I. Elements approved- The Request for amendment, to add a new co-investigator (Dareskedar Tsehay) and new study sites (Kuyera and Northern Shoa) and the request for extension of the ethical approval has been approved.

II. Obligations of the Principal Investigator-
1. Should comply with standard international & national scientific and ethical guidelines.
2. All amendments and changes made in protocol and consent form need AAERC approval.
3. End of the study, including manuscripts and thesis works should be reported to the AAERC.
4. Should submit support letter from Regional Health Bureau prior to the start of the study.

III. Does the protocol need to be reviewed by the National ERC (NRERC)? Yes ☐ No ■

Follow up report expected in:

3 Months ____ 6 Months ✓ 9 Months ____ One year____

Name:	Dr Martha Zewdie	Dr. Geremew Tasachegne	Dr. Taye Tolera
Signature:			
Date:	28/7/17		2 Aug. 2017
	AAERC Secretary	AAERC Chairperson	AHRI Director General

Tsige Gebre Mariam (Dr.)
Director General
Armauer Hansen Research Institute

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The Federal Democratic Republic of Ethiopia
Ministry of Science and Technology

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ቁጥር Ref.No. 3.10/53/2018
ቀን Date. March 30, 2018

To: Armauer Hansen Research Institute (AHRI)
Addis Ababa, Ethiopia

Subject: Acceptance Letter of Amendment Request

Dear Sir/Madam /Mr./Mrs./Dr.

We are writing this letter in reference to your amendment request letter Ref No: AH1453/0012/18 Date January 31, 2018

After having in depth review of your request on "**THE ROLE OF NEUTROPHIL Fcγ RECEPTORS IN THE PATHOGENESIS OF ERYTHEMA NODOSUM LEPROSUM**" the National Research Ethics Review Committee has accepted your amendment request for one year from **(March 30, 2018- March 29, 2019).** This is, therefore, to notify that the ethical approval is renewed and your group can proceed in accordance to the latest approved documents. Please ensure that you submit a biannual report and an annual renewal application 30 days prior to expire date. We are confident that you as PI of the project and your esteemed organization will monitor the ethical implication of the project as it is stipulated in the latest approved document.

With Regards,

Awol Hussein Mohammed
Research Ethics Directorate
Acting Director

CC

- HE the Minister
- HE the State Minister(Technology and Research Sector)
- STI, Capacity Building, Policy and Research Affairs Director General
- Chairperson, NRERC
- Dareskedar Tsehay (PI)
- Abraham Assefa (PI)
- Kidist Bobosha (PI)
- Diana NJ Lockwood (PI)

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ARMAUER HANSEN RESEARCH INSTITUTE (AHRI)
ALERT CAMPUS, ETHIOPIAN MINISTRY OF HEALTH
Jimma Road
P.O. Box 1005, Addis Ababa
PHONE [251] 11 – 321 15 64
e-mail: ahri.ethiopia@yahoo.com

Ref.N°: ጊ/ሃ/196/ooc3/17
Date: 16/10/2017

To:-East Gojjam zone Health bureau
Debre Markos

Re: Request for collaboration

The Armauer Hansen Research Institute is conducting a study on leprosy entitled "The role of neutrophil Fc gamma receptors in the pathogenesis of Erythema Nodosum Leprosum". Dareskedar Tsehay is MSc. Student attached to our Institute and responsible for this study.

As the study requires enrollment of treated Lepromatous Leprosy patients who never developed reaction, it is found important to assess some sites in your zone including Bichena, Debre work and Mertule Maryam Woredas.

Therefore, this is to kindly request your permission to collect samples in the mentioned sites and write us support letters addressed to the woreda health offices.

Best regards

Sponsored By the Government of Ethiopia Norway & Sweden

Tilahun Tolera Balcha (Dr.)
Director General
Armauer Hansen Research Institute

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East Gojjam Zonal Health Department

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ለጤናችን በጋራ እንስራ !!

የምሥራቅ ጎጃም ዞን ጤና ጥበቃ መምሪያ
East Gojjam Zonal Health Department

ቁጥር 202/ጠ/ጠተ/1

ቀን 09/02/2010

ለ እንማይ መረዳ ጤና ጥበቃ ጽ/ቤት
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ለጤናችን በጋራ እንሰራ !!

Annex II: information sheet and informed consent (English &Amharic)

Part 1: Participant Information sheet

Study title:THE ASSOCIATION OF COPY NUMBER VARIATION OF FCγRIIIB GENE WITH THE RISK OF ENLIN LEPROMATOUS LEPROSY PATIENTS FROM SELECTED SITES IN ETHIOPIA

Invitation paragraph

You are being invited to take part in a research study. Before you decide 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 and to talk to others about the study, if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

The purpose of the study

The underlying cause of ENL is poorly understood. Understanding the cause of this condition and identifying risk factors which contribute to its development is useful for developing strategies to reduce the morbidity and mortality caused by ENL. This knowledge is crucial for the development of new and better treatments as well as proper management of ENL patients through improving diagnosis and treatment. In the project we are investigating the immunological changes that occur when lepromatous leprosy (LL) patients develop ENL reactions. We want also to quantify the Fcg receptor copy numbers and correlate with the clinical observation. The information obtained from this study will also be used to develop better patient treatment.

Why you have been chosen?

You are invited to participate in this study as a leprosy patient with ENL or without ENL. We want to know the role of neutrophil Fc_{γ}receptors in the pathogenesis of ENL. In this study 94 patients with ENL and 94 LL patients without ENL will participate.

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.

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. These are: history of treatment related to your current case, symptoms and time of the onset of the disease. In addition you will be asked your age, current address and educational status. You will be asked these questions as a normal treatment procedure even if you will not take part in this study.

Then you will be asked to give 2.5 ml blood (equivalent to about half tea spoon) which 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 (LL or ENL) irrespective of this study by your doctor.

What I have to do?

You will be expected to attend the donation of 2.5 mL blood and subsequent clinical investigation which included demographic data collection.

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

By participating in this research project, you may feel that it is inconvenient that you have to come once but you are normally expected to be followed for 1 year treatment for your sickness (leprosy) and come to the hospital even if you do not take part in this study.

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.

What are the possible benefits of taking part?

If you participate in this research, you may not get any direct benefit but anything regarding your leprosy treatment case will be followed with greater care based on your laboratory results. In addition, your participation is likely to help us in understanding the disease process of ENL which may benefit future patients by developing treatments for ENL reactions.

Compensation

You will not be provided any incentive to take part in this research. However, you will be given 50 ETB for lunch andyour travel expenseswill be covered if you come to participate in the study. This means that if we call you are called to participate in this study; we will cover all your expenses. If you come to the Hospital by yourself to seek treatment and agreed to participate in the study, you will be compensated only for lunch. If you are called by us to take part in the study, full accommodation will provided or paid if you come from far area and unable to return on the same day. We will cover your transport expense between 30-300 ETB. You are not expected to provide receipt for transportation. The above amount does not include accommodation service.

What happens when the research study stops?

On completing the study no further participation is required.

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this issue is given in part 2 in this booklet.

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.

Contact details

Principal investigator

Dareskedar Tsehay

Mobile no: 0913 46 39 39

AHRI, Ethiopia

Study clinician

Dr.Saba Lambert

Mobile no: 0911824438

ALERT Hospital, Ethiopia

Local Study Manager

Dr. Abraham Aseffa

Tel. +251 113211334

AHRI, Ethiopia

AHRI/ALERT Ethical Committee:

Tel No: 0113 481285 AHRI, Ethiopia

This completes part 1 of the information sheet. If the information in part 1 has interested you and you are considering participation, please continue to read the additional information in part 2 before making any decision.

Part 2

What if relevant information becomes available?

If the study is stopped for any reason, you will be told why and your care will not be affected by the discontinuation of the study.

What will happen if I don't want to carry on with the study?

If you withdraw from the study, we will remove and destroy all your identifiable samples but we will need to use the data collected up to your withdrawal.

What if something goes wrong?

The London School of Hygiene and Tropical Medicine hold insurance policies which apply to this study. If you are harmed due to someone's negligence, then you may have grounds for legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the local project manager (Edessa negera), project coordinator (Dareskedar Tsehay) or the study physician (Saba lambert).

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 majority of the samples are immediately used but some will be stored after Semi-processed for as a backup until the final document is produced (not for more than 6 months). Any unused samples will be destroyed within 6months. The data collected will be written and published in peer reviewed scientific journals. A list of publications can be accessed on the LSHTM website. Blood samples will be transferred to collaborating laboratories abroad (LSHTM) for genotyping since this method needs both expertise and advanced facilities which are not available in the country.

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 and international meetings. No information containing your name will be disclosed.

Who is organizing and funding the project

The MALTALEP funded the project and London School of Hygiene and Tropical Medicine will organize the project

Who has reviewed the study?

This study was given a favourable ethical opinion by the AHRI/ALERT Research Ethics committee and by the London School of Hygiene and Tropical Medicine Research Ethics committee. You will be given a copy of the information sheet and a signed consent form to keep.

INFORMED CONSENT FORM

Full Title of Project: THE ASSOCIATION OF COPY NUMBER VARIATION OF FCγRIIIB GENE WITH THE RISK OF ENLIN LEPROMATOUS LEPROSY PATIENTS FROM SELECTED SITES IN ETHIOPIA

Name of the principal investigator: Dareskedar Tsehay

1. I Confirm that I have read and understand the participant information sheet dated .....….. (Version .....) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered fully.	
2. I understand that my participation is voluntary and I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	
3. I understand that sections of my medical notes and data collected during the study may be looked at by responsible individuals from the London School of Hygiene & Tropical Medicine, AHRI, regulatory authorities or from ALERT hospital, where it is relevant to my taking part in this research. I give permission for these individuals to access my records. I agreed that my samples can be used for the intended research only.	
4. I agree for the blood/DNA samples to be transported and used at London School of Hygiene & Tropical Medicine for the intended research project mentioned above.	
5. I agree to take part in the above study.	

Name of Participant *(printed)*	Signature/Thumbprint	Date
Name of Person taking consent	Signature	Date
Principal Investigator	Signature	Date

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

Name of Impartial Witness *(if required)*	Signature	Date

copy for participant; 1 copy for Principal Investigator; 1 copy to be kept with hospital notes

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Annex III: Clinical data collection form

Clinical Data Collection sheet **PatientID------------------**

PATIENT DETAILS					PHYSICIAN		
Patient ID		New ENL case□	Recurrent ENL case□	Chronic ENLcase□			
Date	/ /						
Gender	Male□	Female□	Postmenopause Yes□ No□	Date of birth	/ /	Age	
LEPROSY DETAILS							
Date of diagnosis of leprosy	/ /	Family history	Duration	Ridley Jopling	BB □	BL □	LL□

Bacterial index at diagnosis			Mean BI	____	Highest BI at anysingle site	____		
Current BI			Mean BI	____	Highest BI at anysingle site	____		
MB treatment	DDS	/ /			MB DDS	/ /_		
	MDT	/ /			MB MDT	/ /_		
Relapse		□		DDS resistance		□	Defaulter	□
Presented with reaction at leprosy diagnosis		NO□	YES□	On treatment ----------	After completion of treatment ----------	NEURI TIS□		
Date of onset of most recent T1R or neuritis		/ /			No previous T1R or neuritis□			
Other illnesses			TB □	Diabetes □	HIV □		Other (specify)	

Current ENL treatment (drug and dose)		Date started	/ /	<u>Previous ENLtreatments:</u> Prednisolone Clofazimine Thalidomide Pentoxifylline Aspirin(or NSAID) Other (please state)		
Duration of treatment with prednisolone			Reason for prednisolone uptake			

Current symptoms	Fever □	Skin lesions □	Other localised swelling (not skin) □ _____	Peripheral oedema □	Insomnia□	Anorexi□	
	Weight loss□	Nasal stuffiness□	Depression□	Malaise□	Epistaxis□	Joint swelling □	
Pain symptoms	Skin □	Bone □	Digits □	Testes □	Eyes □		
	Muscles□	Lymph nodes □	Nerves □	Joints □			
Where is pain worse?							
Nerve symptoms	Pain □	Weakness □	Reduced sensation □	Paraesthesia □		Hyperaesthesia □	
	New NFI □	Old NFI □	G2D				
EXAMINATION			Urinalysis	Protein □	Blood □	EHF Score	___
WBC count	___	Neutrophil	_____		BP	/	
Type ENL skin lesions	Papules □	Nodules □	Vesicles □	Bullae □	Pustules □		
	Plaques □	Ulcerated □	Necrotic □	Sub-cutaneous nodules □	EM like lesions □	Scar due to ENL	
Size of largest lesion (mm)			Describe unusual skin lesions such as EM-like				

Number of ENL skin	5 or less □	6-10 □	11-20 □	21-50 □	>50 □		
Number of ulcerated/necroti							
Location of ENL skin	Head/neck□	Trunk □	Upper limbs □	Lower limbs			

Oedema	Face □	Hands □	Lower limbs □	Localised □	Specify:		
Dactylitis	Yes □	No □					
Arthritis	Yes □	No □	Large joints □	Small joints			
Orchitis	Left □	Right □					
Eyes							
Lagophthalmos	Left □	Right □					
Madurosis	Left □	Right □					
Conjunctivitis	Left □	Right □					
Anterior uveitis	Left □	Right □					
Scleritis	Left □	Right □					
Episcleritis	Left □	Right □					
Glacoma	Left □	Right □					
Trichiasis	Left □	Right □					
Visual acuity	Left	/	Right	/			
Lymphadenitis	Yes □	No □	Site/s:				
Liver tenderness	Yes □	No □					
Rhinitis	Yes □	No □					
Other signs associated with ENL	Yes □	No □	Specify:				

Annex IV: protocol for DNA extraction

Protocol for DNA Extraction

Protocol: Purification of Genomic DNA from Human Whole Blood Collected into PAXgene Blood DNA Tubes The PAXgene Blood DNA Kit is used for purification of genomic DNA from 2.5 ml of human whole blood, collected in PAXgene Blood DNA Tubes PAXgene Blood DNA Tubes and the PAXgene Blood DNA Kit are an integrated system for collection of whole blood and isolation of genomic DNA.

Important note before starting

• All centrifugation steps should be carried out at room temperature (15–25°C) in a swing-out rotor.

Things to do before starting

• Thaw frozen PAXgene Blood DNA Tubes in a wire rack at ambient temperature (18–25°C) for approximately 2 hours or at 37°C in a water bath for approximately 15 minutes. Carefully invert the thawed PAXgene Blood DNA Tubes 10 times before beginning the procedure.

• Heat a heating block or water bath to 65°C for use in steps 8 and 17

• Add 1.4 ml Buffer BG4 (resuspension buffer) to lyophilized PreAnalytiX Protease. Dissolved PreAnalytiX Protease should be stored at 2–8°C or in aliquots at –20°C

• For every sample, mix 1.5 ml Buffer BG3 (digestion buffer) and 15 µl reconstituted PreAnalytiX Protease.

Procedure

1. Pour all the blood from one PAXgene Blood DNA Tube into a Processing Tube containing 7.5 ml Buffer BG1. Close the tube. To avoid cracking the blue lids of the Processing Tubes, do not overtighten them. Tighten the lid only until the first sign of resistance is felt. Mix by inverting the tube 5 times. If the blood in the PAXgene Blood DNA Tube has separated into plasma and red blood cells, invert the tubes carefully 10 times to homogenize the sample.
2. Centrifuge for 5 min at 2500 x g in a swing-out rotor.

3. Carefully discard the supernatant and place the tube in a rack. In rare cases the pellet may be loose, so pour slowly.

3. Add 1.5 ml Buffer BG2, close the tube, and wash the pellet by vortexing vigorously for 5 s.
4. Centrifuge for 3 min at 2500 x g in a swing-out rotor.
5. Carefully discard the supernatant and place the tube back in the rack. In rare cases the pellet may be loose, so pour slowly.
6. Add 1.5ml Buffer BG3/PreAnalytiX Protease (see “Things to do before starting”), close the tube, and vortex for 20 s at high speed. Vortexing for 20 s is essential to dissolve the pellet completely. Shorter vortexing times may lead to incomplete resuspension of the pellet and reduced DNA yield or purity. After this step, samples can be stored for at least 7 days at 2–8°C. After storage, resume the procedure at step 8.

7. Place the tube in a heating block or water bath and incubate at 65°C for 10 min. The sample changes color from light red to light green, indicating that protein digestion has occurred.
8. Vortex again for 5 s at high speed.
9. Add 1.5 ml isopropanol (100%) and mix by inverting the tube at least 20 times until the white DNA strands clump visibly together. Complete mixing with isopropanol is essential to precipitate the DNA and should be checked by inspection. Only tightly clumped DNA strands can be efficiently pelleted by centrifugation. Do not vortex as this might reduce DNA yield.
10. Centrifuge for 3 min at 2500 x g.

11. Discard the supernatant and leave the tube inverted on a clean piece of absorbent paper for 1 min. In rare cases the pellet may be loose, so pour slowly. Inverting the tube onto absorbent paper minimizes backflow of isopropanol from the rim and sides of the tube onto the pellet.

12 Add 1.5 ml 70% (v/v) ethanol and vortex for 1 s at high speed. 14. Centrifuge for 3 min at 2500 x g.

13 Discard the supernatant and leave the tube inverted on a clean piece of absorbent paper for at least 5 min. In rare cases the pellet may be loose, so pour slowly. Inverting the tube onto absorbent paper minimizes backflow of ethanol from the rim and sides of the tube onto the pellet.

14 14. Carefully dab the tube onto absorbent paper to remove ethanol from the rim, and leave it inverted for a further 5 min to allow the DNA pellet to dry. Avoid over drying the pellet, since over dried DNA is very difficult to dissolve.

15 Add 0.3 ml Buffer BG4 and dissolve the DNA by incubating for 1 h at 65°C in a heating block or water bath, followed by incubation overnight at room temperature. Highly concentrated, high-molecular-weight genomic DNA samples may not redissolve completely after an incubation of 1 h at 65°C, therefore an additional overnight incubation at room temperature is recommended.

16 Put the DNA in eppindorf tubes and store at -20°C.

Annex V: Standard operating procedure for Measurement of CNV

SOP for CNV measurement using qPCR

Purpose of this SOP

The purpose of this Standard Operating Procedure (SOP) is to describe about measurement of Copy Number Variation of a gene using qPCR technique.

Principle of CNV

A CNV has been defined as a DNA segment that is 1 kb or larger and present at variable copy number in comparison with a reference genome with the usual copy number of N = 2. CNV may arise from Deletions, insertions, duplications and complex multi-site variants leading to quantitative variation in expression. CNV will be measured using Rotor-Gene3000 real-time

thermal cycler. Specific primers and probes will be used. For this specific purpose, a reference gene which doesn't show variation in copy number is used.

Materials and equipment required

- Rotor- Gene 3000, Real Time-PCR machine
- Robot
- 1.5 ml Centrifuge tubes (RNase-free)
- PCR tubes (200 µl)
- Pipets
- DNAse free Filter tips
- Nitrile gloves
- Safety cabinet

Reagents required

- Primers for FCGRIIIB and FOXP2
- Fluorescent labeledprobesfor FCGRIIIB and FOXP2
- Master mix (PerfeCTaqPCRToughMix)
- PCR grade water
- Template genomic DNA

No.	Gene	Sequence
1.	FCGRIIIB FP	AGTTTGAGATGCCTTGGGTTC
	FCGRIIIB RP	CCATCTTGGCTTGTCCTGGTA
	FCGR3B probe	5'-HEX-CCACAGCTATAGATGTGGTGAGGGG-BHQ1-3'
2.	FOXP2 FP	TCACTACTAACAATTCCTCCTCGACTAC
	FOXP2 RP	GATGAGTTATTGGTGGTGATGCTT
	FOXP2 probe	5'-FAM-TCCTCCAACACTTCC-BHQ3-3'

Procedures

1. Dilute DNA to the concentration of 10 ng/µl for qPCR reaction.

2. Dilute PCR primers and use at a final concentration of 0.4 µMin the reaction mixture.

3. Dilute PCR probes to final volume of 0.2 µMin the reaction mixture

3. Final volume of PCR reaction will be 10 µl.

Reagent name	Amount in microliters
Master mix	5
FCGR3B forward primer	0.4
FCGR3B reverse primer	0.4
FCGR3B probe	0.2
FOXP2 forward primer	0.4
FOXP2 reverse primer	0.4
FOXP2 probe	0.2
Molecular grade Water	2
Template DNA/ water	1

4. Perform PCR in duplicate.

The temperature profile was

Initial 95 °C for 15 minutes followed by 40 cycles of denaturation at 95 °C for 10 ,annealing at 50°C for 45 s and extension at 72 °C for 30 s.

5. Standard curve will be generated for each run from Ct values of serially diluted template gDNA to check reaction efficiency of the real time PCR reaction.

Result and interpretation

The signal will be detected in real time. The machine will detect the fluorescence emitted when the fluorescent dye is detached from the probe during extension.

$\Delta Ct = Ct \text{ of FCGRIIIB} - Ct \text{ of FOXP2}$

$\Delta\Delta CT = \Delta Ct \text{ of cases} - \Delta Ct \text{ of controls}$

$2^{-\Delta\Delta CT}$ will be calculated

References

1. Traherne JA, Martin M, Ward R, Ohashi M, Pellett F, Gladman D, et al. Mechanisms of copy number variation and hybrid gene formation in the KIR immune gene complex. Hum Mol Genet. 2010;19(5):737-51

2. Redon R, Ishikawa S, Fitch KR, Feuk L, Perry GH, Andrews TD, et al. Global variation in copy number in the human genome. Nature. 2006;444(7118):444-54.

3. Ma L, Chung WK. Quantitative analysis of copy number variants based on real-time LightCycler PCR. CurrProtoc Hum Genet. 2014;80:Unit 7 21

Declaration

I, the undersigned, declare that this M.Sc. research thesis is my original work, it has not been presented for a degree in this or any other university and that all sources of materials used for the research proposal have been duly acknowledged.

M.Sc. candidate: **Dareskedar Tsehay (B.Sc.)**

Signature: _____

Date of submission: _____

Place Addis Ababa, Ethiopia.

This researchthesis has been submitted with our approval as advisors.

Name of advisor: Aster Tsegaye (MSc, PhD,)

Signature _____

Place: Department of Medical Laboratory Sciences, Addis Ababa University

Date of submission_____/_____/_____

Name of advisor: Kassu Desta (MSc, PhD candidate)

Signature _____

Place: Department of Medical Laboratory Sciences, Addis Ababa University

Date of submission_____/_____/_____

Name of advisor: Kidist Bobosha (MSc, PhD)

Signature _____

Place: Armauer Hansen Research Institute

Date of submission_____/_____/_____