THE LEVEL OF LL37 PROTEIN AND RNA GENE EXPRESSION AMONG LEPROSY PATIENTS AND THEIR CONTACTS ATTENDING ALL AFRICA LEPROSY,TUBERCULOSIS, REHABILITATION AND TRAINING(ALERT) HOSPITAL,ETHIOPIA

A Thesis submitted to graduate studies, Addis Ababa University, College of Health Sciences, School of Medicine, Department of Microbiology, Immunology and Parasitology, in partial fulfillment of the requirements for the degree of Masters of Sciences in Medical Microbiology.

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This is to certify that the thesis prepared by Mahlet Osman entitled: The level of LL37 protein and RNA gene expression among leprosy patients and their contacts attending All Africa Leprosy, Tuberculosis, Rehabilitation and Training (ALERT) Hospital, Ethiopia is submitted to Addis Ababa University in partial fulfillment of the requirements for Master of Science degree in Medical Microbiology and complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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List of acronyms and abbreviations

AAERC	AHRI/ALERT Ethics Review Committee
AFB	Acid-fast bacilli
AHRI	Armauer Hansen Research Institute
AIDS	Acquired Immune Deficiency Syndrome
ALERT	All Africa Leprosy Rehabilitation and Training Centre
BB	Borderline Borderline leprosy
BT	Borderline Tuberculoid
CMI	Cell Mediated Immunity
cDNA	Complementary Deoxyribonucleic acid
DNA	Deoxyribonucleic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ENL	Erythema Nodosum Leprosum
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
HHC	House Hold Contact
HuPo	Human acidic ribosomal Protein
HIV	Human Immunodeficiency Virus
IFN-γ	Interferon gamma
IL	Interleukin
LL	Lepromatous Leprosy
MB	Multibacillary

MDT	Multi Drug Therapy
MLWCS	*M. leprae* Whole Cell Sonicate
MRNA	Messenger RNA
M. leprae	*Mycobacterium leprae*
PB	Paucibacillary
PBMCs	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
RNA	Ribonucleicacid
RR	Reversal Reaction
RT	Room Temperature
RTPCR	Real time Polymerase Chain Reaction
SSS	Slit-Skin Smear
T1R	Type 1 Reaction
T2R	Type 2 Reaction
Th1	T helper 1 cell
Th2	T helper 2 cell
TLR	Toll like Receptor
TNF-α	Tumour Necrosis Factor alpha
Tregs	Regulatory T-cells
TT	Tuberculoid leprosy
WHO	World Health Organization

VCT Voluntary Counseling and Testing

Abstract

Background: The antimicrobial peptide cathelicidin, also calledLL37, has broad-spectrum antimicrobial activity against bacteria, fungi and viruses.LL37 is the only human cathelicidin antimicrobial peptide encoded by the gene CAMP. It is referred to as LL37, since it has 37 amino acid sequences starting with two leucines. There are few reports that showed its Level, pro and anti-inflammatory immunomodulatory activity of LL37 in mycobacterial infections; tuberculosis and leprosy that indicates the need for further investigation of the role of LL37.

Objective: To investigate the level of LL37 in different forms of leprosy.

Methods: Across sectional study was conducted from January2017toSeptember2018 at ALERT hospital. A total of 74 HIV negative individuals consisting of 26 leprosy patients (11tuberculoid leprosy/borderline tuberculoid (TT/BT), 15 borderline lepromatous/lepromatous leprosy (BL/LL)), 23 Household contacts and 25 healthy controls were included in this study, by convenience sampling. LL37 expression was measured at protein and mRNA level in newly diagnosed leprosy patients attending ALERT hospital, their household contacts and healthy controls.

Result: Plasma level LL37 was significantly higher in household contacts compared to lepromatous patients (p=0.017) and healthy controls (p=0.007). However, there was no statistically significant differences in mRNA expression level of LL37between the three groups; patients and contacts (p=0.3244), patient and controls (p=0.5560), contact and controls (p=0.0758) and within the two polar forms of leprosy; tuberculoid and lepromatous (p=0.3842).

Conclusion: - Our finding demonstrates that the low plasma level of LL37 in lepromatous patients may give a clue why there is a delay in clearing the bacilli from their body. However, measuring the level of LL37 at the infection site would be more informative.

Key words: Leprosy, *M. leprae*, Cathelicidin, LL37

1. Background

1.1.Introduction

Leprosy is a curable infectious disease caused by *Mycobacterium leprae (M. leprae)*which affects hundreds of thousands of people every year and it attacks mainly the skin and peripheral nerves where macrophages and Schwann cells are the target cell(1). There are five different clinical forms of leprosy; the tuberculoid leprosy (TT), Borderline tuberculoid Leprosy (BT), borderline borderline leprosy (BB), Borderline lepromatous Leprosy (BL) and Lepromatous leprosy (LL)(2). The world Health organization (WHO) has simplified the classification by grouping leprosy patients into paucibacillary (PB) and multibacillary (MB)based on the number of skin lesions, nerves involved and BI (Bacterial index) for treatment purpose(3).

Treatment for leprosy is administered as multi-drug therapy (MDT) which is a combination of rifampicin, clofazmine and dapsone; which is efficient and has contributed a lot in the declining of the prevalence of leprosy globally except the prolonged treatment period: 6 months for PB and 12 months or above for MB(4).Other than the difference in clinical forms, the occurrence of leprosy reactions (type 1 and type 2) is the major challenge in the management of leprosy which can occur before, during or after MDT in 30–50 % of the patients, and may lead to severe nerve function impairment and disability(5, 6). Corticosteroids such as prednisolone are the preferred drugs so far to manage reactions(6).

In the absence of strain differences in *M. leprae,* the host immunological responses determine the clinical forms of leprosy(7). In TT/BT patients, the IL-15 dependent killing of the bacilli via the vitamin D dependent antimicrobial peptides activities is dominant, whereas in BL/LL patients, IL-10 dependent phagocytosis with the absence of phagosome-lysosome fusion, leaves the macrophages filled with host and pathogen derived lipids leading to bacilli survival(8, 9).

LL37 is the only human cathelicidin antimicrobial peptide encoded by the gene CAMP and referred to as LL37, since it has 37 amino acid sequences starting with two leucines; this antimicrobial peptide has a broad-spectrum activity against bacteria, fungi and viruses (10).Besides, its antimicrobial properties, it plays a central role in innate immune response and inflammation (10, 11). The presence of dominant IL-10 dependent pathway in lepromatous

patients and the potential of LL37 to inhibit IL-10(8) show the possibility of using LL37 as a therapeutic agent to enhance pro inflammatory type responses in these patients which is important in the killing process of the bacilli.

Hence, with the current challenges in leprosy: lengthy treatment period, delayed bacterial clearance in lepromatous patients and complications of reactions, detail investigations of the role of LL37 and analyzing the potential of such agents in the disease management along with the MDT is essential.

In this study, we measured the mRNA expression and protein level of LL37in periphery of Leprosy patients with the different forms of leprosy and their household contacts.

1.2 Statement of the problem

Leprosy remains a significant public health problem in a number of developing countries and continues to be the leading infectious cause of disability worldwide(3).In Ethiopia, where the disability rate is 14%, more than 700 Ethiopians are disabled every year, and around 4000 new cases of leprosy per year on average are reported; this number could be far higher than this figure if active case detection and proper diagnosis are incorporated in the national tuberculosis and leprosy control program(12).

The disability together with self and society driven psychological distresses leave leprosy affected persons with a life time of misery and pain(3, 13).Importantly, a key factor for leprosy-induced disability is a delay in diagnosis and treatment (3).Lack of simple diagnostic tools to detect leprosy early and the absence of primary prevention like specific vaccine against leprosy are some of the many reasons for delayed diagnosis(14).

The treatment duration especially for multibacillary cases is 12 months which is too long(14, 15).Such patients can't easily clear the bacteria because immune response of a host, phagocytosis with the absence of phagosome-lysosome fusion that leads macrophages to be filled with lipids favors survival of the bacteria; there is very limited antimicrobial activity with this Leprosy form(8, 16).

Occurrence of reactions during the natural course of the disease worsen the condition which happens in all forms of the spectrum but predominately occur in multi bacillary Leprosy patients and these immunological episodes are responsible for irreversible nerve damage, deformities and increase the disease burden and associated stigma(5, 17). These reactions may occur before, during or after MDT and no clinical or laboratory tests can accurately predict who is most likely to develop a reaction or when it might occur(17, 18).

The first gap is lack of literatures which give information about host factors which could possibly play a role in immune modulation in leprosy where one of them is LL37.

1.3 Study rationale

As early detection of leprosy and prompt treatment with MDT help to reduce transmission, and thus have a major impact on preventing nerve damage, disabilities and deformities, investigating potential host factors may lead to identification of potential diagnostic or prognostic tools.

In this study, we aimed to explore the level of LL37 in the different forms of leprosy including leprosy patients and their household contacts. The level of LL37 in the household contact group may generate useful information to further investigate the possibility of using LL37as a prognostic marker.

Moreover, this study fills the existing knowledge gap concerning the association between the antimicrobial peptide LL37and Leprosy.

2. Literature review

Leprosy, which is also known as Hansen's disease, is a chronic infection caused by *Mycobacterium leprae* that leads to clinical manifestations ranging from cutaneous manifestations to disfigurement, deformity and disability and stigma(16, 19). The burden of disease associated with *M. leprae* infection in humans stems from the ability of this bacterial pathogen to induce severe injury of peripheral nerves (Schwann cells) and skin(20).The clinical spectrum of leprosy is further classified based on the host immune responses against the bacilli ranging from tuberculoid, borderline, and lepromatous forms(21).

Once the infection is established, the occurrence of leprosy reactions, which have inflammatory impact on the peripheral nerves, constitutes an important contributor to sensory loss and dysfunction (22).

2.1. Epidemiology of leprosy

According to WHO report for the year 2017, about 210,671 new leprosy cases have been registered globally in recent years. South-East-Asia region has the largest number of new cases, followed by the Americas and the African countries(23). Among the newly reported cases, MB cases dominated in most of the regions (24).

Table1. Number of new cases during 2017, by WHO Region(23)

WHO Region	Number of new cases detected (new case detection rate/100 000 population) during 2017
African	20, 416
Americas	29, 101
Eastern Mediterranean	3, 550
South-East Asia	153, 487
Western Pacific	4, 084
Europe	33
Global total	210, 671

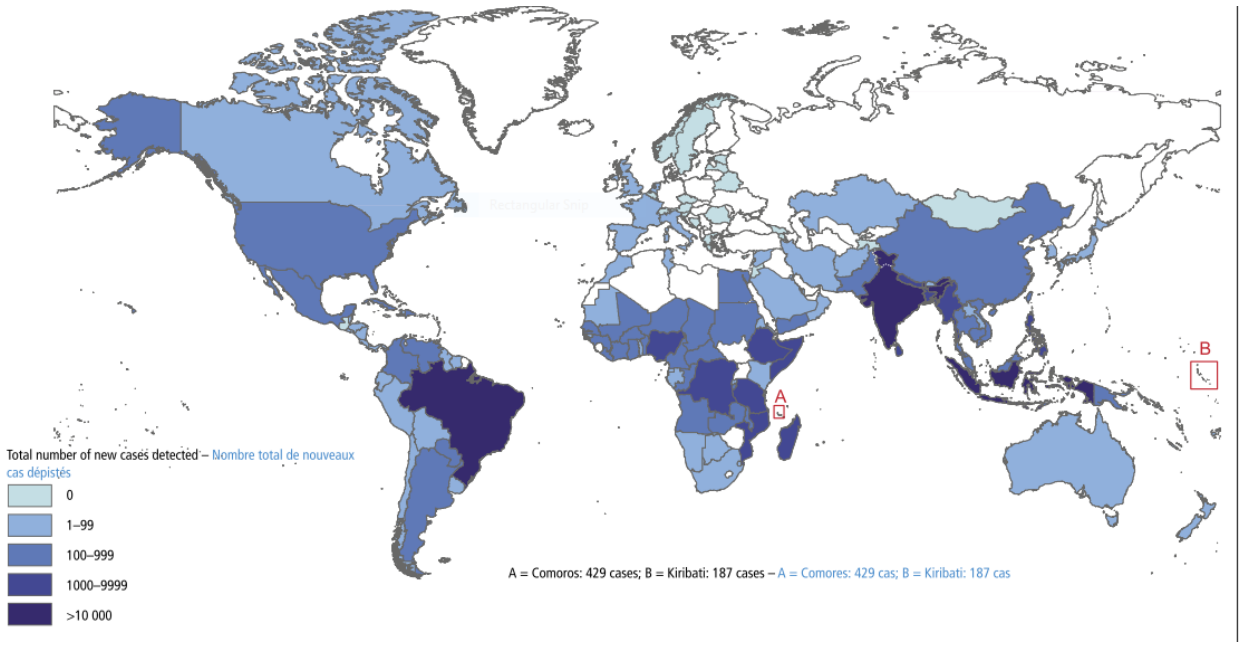


Figure 1. Geographical distribution of new leprosy cases,2017(23)

2.2. Characteristics of *M. leprae*

M. leprae is an obligate intracellular, acid-fast, capsulated, rod shaped bacillus which belongs to the order Actinomycetales, family Mycobacteriaceae and genus *Mycobacterium*(25). Unlike any other species in this genus, *M. leprae* replicates every 12-14 days and it has never been grown in artificial media/*in vitro*(16*).*

2.3. Forms of Leprosy

The spectrum of clinical manifestations of Leprosy is correlated mainly with the level of cell mediated and humoral immunity and the bacterial load; depending on clinical, hisopathological and immunological criteria, there are five forms (18).

Tuberculoid leprosy (TT) is at one pole of the spectrum, characterized by restricted growth of the pathogen and high CMI(19).At the opposite pole is lepromatous leprosy (LL), in which the CMI is strikingly absent, affecting minimally resistant hosts with a predominantly humoral immune response in a T helper (Th) 2 pattern and a widespread dissemination of the bacilli(22, 26). Between these two poles, there are immunologically unstable borderline forms: borderline tuberculoid, borderline-borderline and borderline lepromatous(18, 26).

2.4Leprosy reactions

Leprosy reactions are sudden acute immune-inflammation episodes against *M leprae* superimposed on the chronic course of leprosy and may occur before, during or after MDT(27).Understanding the pathology and identifying risk factors for these episodes are important for developing strategies to reduce nerve damage (21).There are two types of reactions: Type 1 or Reversal reaction (RR) and Type 2or Erythema Nodosum Leprosum (ENL)(28).

2.4.1. Type 1 Leprosy reaction (T1R) (RR)

It is a type IV hypersensitivity reaction that occurs in borderline leprosy patients(26) with cellular immune responses to *M. leprae* antigenic determinants, and is characterized by acute inflammation of pre-existing skin lesions or by the appearance of new lesions and/or neuritis(27),(29).

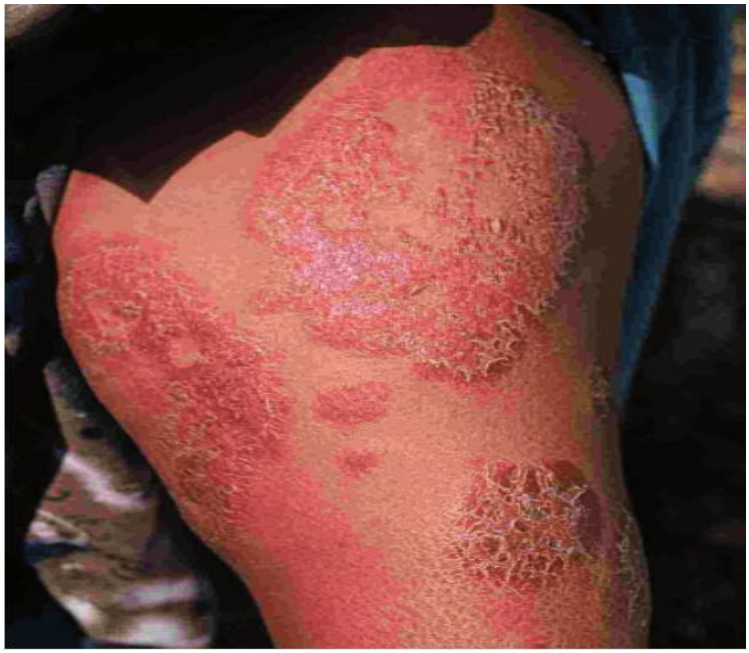


Figure 2. Type one reaction(22)

2.4.2 Type 2 Leprosy reaction (T2R) or Erythema nodosum leprosum (ENL)

It is an immune complex mediated complication of lepromatous leprosy (LL)(28). T2R presents with skin lesions (red, painful, and tender subcutaneous lesions), fever, and systemic inflammation that may affect the nerves, eyes, joints, testes, and lymph nodes(30).

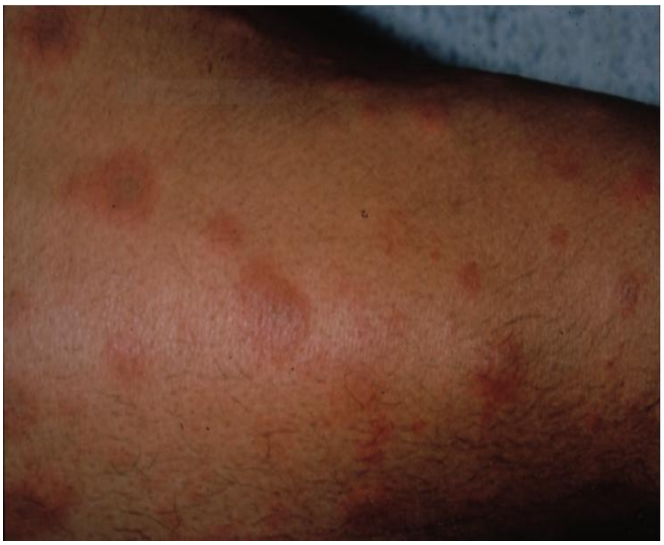


Figure 3. Type 2 reaction(22)

2.5 Host immune response

It is clear that immunological responses play a critical role in controlling each stages of an infection. Resistance to intracellular pathogens such as mycobacteria is associated with the ability to mount Th1 responses (21). The first line of interaction of *M. leprae* with humans is mediated by host receptors that recognize Pathogen Associated Molecular Patterns (PAMPs) of mycobacteria; thus, TLR on phagocytic cells, especially TLR-2, is activated by *M. leprae* lipoproteins and the ability to start protective responses is directly associated to interleukin (IL)-12/23 secretion and differentiation of macrophages and dendritic cells, consequently preventing the development of severe forms of the disease(31).

The above process can lead to expansion and differentiation ofTh1 interferon (IFN)g-producing cells that, in turn, induce *M. leprae* killing and control of disease spread(32). Th1 cells which are dominant in lesions of TT patients produce proinflammatory cytokines mainly IFN-Ɣ, IL-2, TNF and other Th1 associated factors that play an important role in activating macrophages to initiate their microbicidal activity(22).

On the other hand, in LL patients suppressor type CD8+ T cells are present which are important in down regulating macrophage activation and suppressing CMI(33). There is high production of antibody and theTh2 type response is mainly characterized by the production of IL-4, IL-5,IL-10 and IL-13 and lacks IL-2 and IFN-γ(17).

Furthermore, the dichotomy of Th1 versus Th2 as an explanation for clinical features observed among tuberculoid and lepromatous leprosy patients, respectively and also reflects the ability to mount an effective cellular immune response against the mycobacteria(22). Leprosy response patterns can be modified along the course of the disease(34).

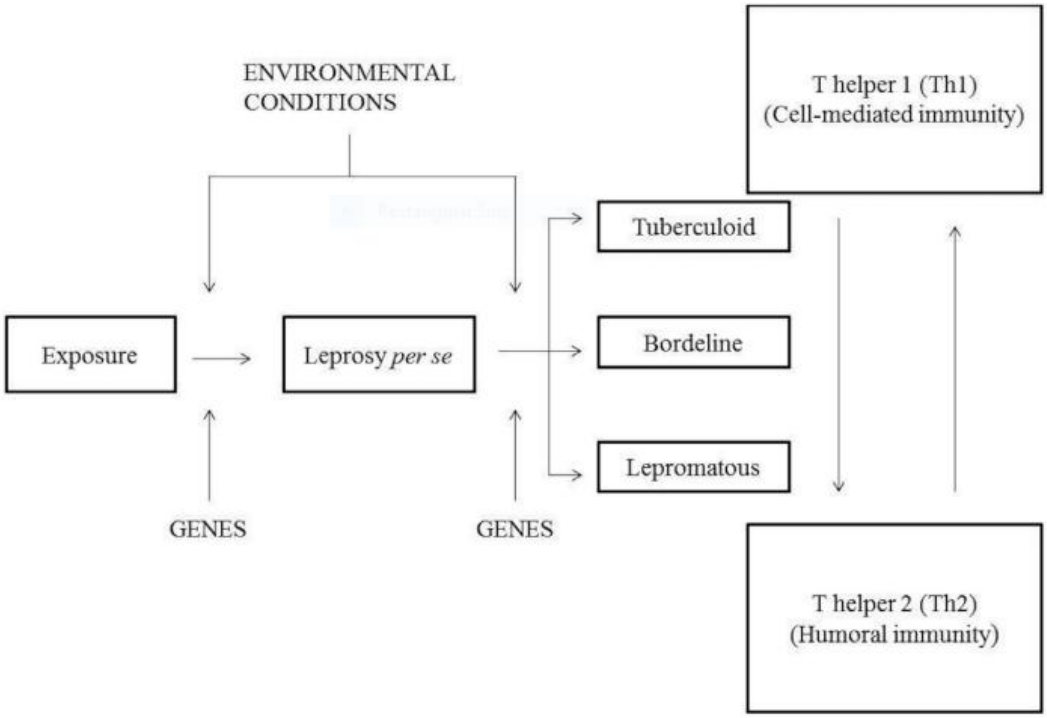


Figure 4. Immune response in different Leprosy spectrums(35)

2.6Transmission

Understanding the mode of *M. leprae* transmission has been complicated due to the long incubation time of leprosy as well as the lack of tests that can detect asymptomatic *M. leprae* infection(36).Person-to-person spread via nasal droplets is believed to be the main route and the source of infections are mainly untreated MB patients(3).Close contacts of patients are at the highest risk of infection(37).

Genetic susceptibility to leprosy has been addressed in few literatures where PARK2, PACRG and NRAMP1 genes were reported to have association with susceptibility(35).

2.7Antimicrobial peptides (AMPS)

Antimicrobial peptides (AMPs) are abundant in nature and have a broad spectrum activity against many pathogens to the extent of killing cancerous cells(38).These small amphipathic peptides are part of the innate immune system and act against bacteria, fungi and viruses(39).They can be grouped according to their size, conformational structure or predominant amino acid structure; Nevertheless, the diversity of these molecules is so enormous that it is difficult to categorize them in a generally accepted classification(40).

2.7.1 Mechanism action of antimicrobial peptides

The exact mechanism by which AMPs exert their antimicrobial properties is yet unknown, but it is generally accepted that cationic AMPs interact by electrostatic forces with the negatively charged phospholipids'head groups on the bacterial membrane and cause disruption of the membrane(41).Furthermore, it neutralizes bacterial toxins(42).

2.7.2.The human cathelicidin LL37

In mammals, there are two distinct groups of AMPs; Defensins are more representatives and cathelicidin form the second group(43).hCAP18/LL37 is the only known human cathelicidin(44). It was first described in 1995 in bone marrow cells and it is 18 kDa peptide, cationic (+6), amphipathic α-helical peptide, encoded by the same genes with that of CRAMP; the sole cathelicidin in mice; this encoding similarity has contributed a lot to generate data on LL37 using mouse model(44, 45).

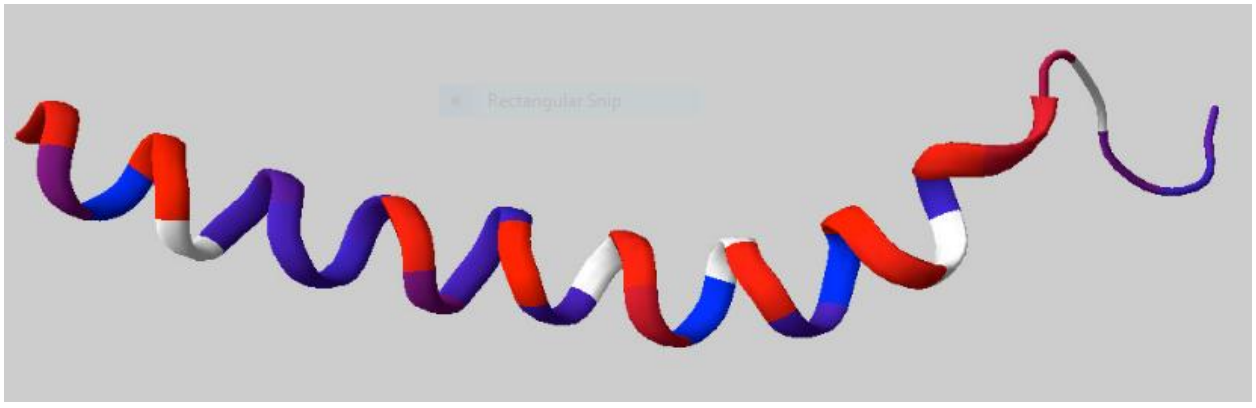


Figure 5.α- helical structure of LL37(44).

LL37 is produced by circulating immune cells, bone marrow progenitor cells, and epithelial surfaces of immunologic barrier sites, such as the respiratory, gastrointestinal, and genitourinary epithelium, in response to infectious processes (10, 41, 46).

Beside antimicrobial properties, LL37 plays a central role in innate immune responses and inflammation(46). It has been identified as a potent chemo attractant for mast cells, monocytes, T lymphocytes and neutrophils using formyl-peptide receptor–like 1 (FPRL1)(44, 47). It also promotes wound healing, angiogenesis and arteriogenesis(46).

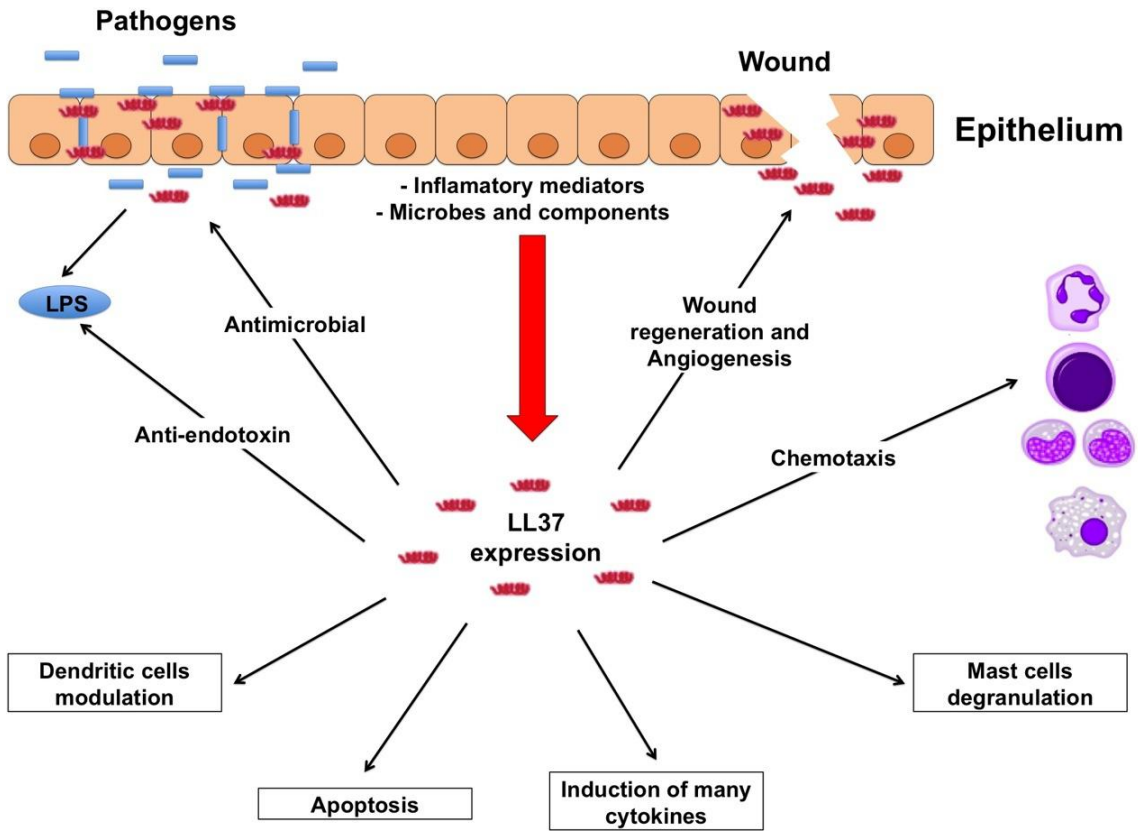


Figure 6. Biological activities of LL37(44)

2.8. LL37and Mycobacterial infections

Few literatures reported previously thatLL37 can control the expression of proinflammatory and anti-inflammatory cytokines to modulate the response of macrophage during mycobacterial infections(48).

In addition to its capacity to inhibit mycobacterial growth, the cathelicidin hCAP18/LL37 exhibits its bactericidal activity against M. tuberculosis(49).In this infection, type I IFN cytokine is required for the induction of vitamin D antimicrobial pathway and the subsequent induction of cathelicidin LL37 so that making macrophages phagocytic in IL-10 dependant pathway (50).

The role of different types of antimicrobial peptides; defensins, hepcidin and the cathelicidin LL37 in leprosy was reported in few studies; the association of defensin genes variation with severity of leprosy and the up regulation of hBD3 in type 1 reaction are some evidences to show their relevance in leprosy(51).As to LL37, decreased serum level was reported(11) in untreated leprosy patients probably associated with up regulation of micro RNA hsa-mir-21expression in infected monocytes(51).

3. Objectives

3.1 General Objective

- To investigate the association of LL37 in different forms of leprosy.

3.2 Specific Objectives

- To determine the mRNA expression level of LL37 inperipheral blood ofdifferent forms of leprosy patients, household contacts and healthy controls.
- To determine the plasma level of LL37 in different forms of leprosy patients, household contacts and healthy controls.
- To determine the level of LL37 from stimulated whole blood of Leprosy patients.

4. Hypotheses

LL37 expression level is lower in multibacillary (BB/BL/LL) than pauciliary (TT/BT) clinical forms of leprosy.

5. Materials and Methods

5.1 Study area

This study was conducted at ALERT Hospital Addis Ababa, Ethiopia; the only leprosy referral centre in Ethiopia. Patients were recruited at Red Medical Clinic (RMC) of ALERT Hospital. Leprosy patients are seen at Red Medical Clinic (RMC). RMC is a special clinic within the hospital dedicated for leprosy, diagnosis, treatment and rehabilitation. Clinical sample processing such as peripheral blood mononuclear cells (PBMCs) isolation, storage, H&E staining, ELISA and qPCR have been done at the Armauer Hanssen Research Institute (AHRI), Addis Ababa, Ethiopia; which is a biomedical research institute located in ALERT campus.

5.2 Study Design and period

A cross-sectional study was conducted from January 2017 to September 2018 to measure the expression level of LL37 from patients sicking medical treatment at ALERT Hospital Addis Ababa, Ethiopia.

5.3 Source and Study Population

5.3.1 Source population

All patients coming with Dermatological complaints to dermatology ward of ALERT Hospital, Addis Ababa with their HHCs and apparently healthy individuals coming to VCT center of ALERT were source population.

5.3.2 Study population

Study population: all newly diagnosed (untreated) leprosy patients attending ALERT Hospital, Addis Ababa, household contacts of leprosy patients and apparently healthy individuals coming to VCT center of ALERT who fulfilled the inclusion criteria.

Patients were diagnosed based on the cardinal signs of leprosy (clinical examination by dermatologist and AFB staining) 1.Skin lesions with definite sensory loss. The lesion could be raised or flat, light or pigmented 2. Thickened or enlarged peripheral nerve(s) with loss of sensation and /or weakness of the muscles by that nerve 3. Presence of acid-fast bacilli (AFB) in a slit skin smear or tissue biopsy. Any one of these signs has been regarded as sufficient for the

diagnosis of leprosy. The standard diagnostic procedure is H&E staining. It is used as a confirmatory test and mostly used for research purpose. Patients visiting the ALERT leprosy clinic were asked by the nurses for their willingness to take part in the study. Those who fulfilled the study criteria were enrolled. Clinical examination, consenting, demographic and clinical data collection were done at the leprosy clinic. Biopsy and blood samples were taken from those who consented at AHRI by experienced research nurses.

Newly diagnosed (untreated) leprosy patients (TT/BT n=11; BB/BL/LL n=15); Household contacts (n=23) and healthy controls (n=25), a total 74 HIV negative individuals who fulfilled the inclusion criteria were considered for the study.

5.4 Eligibility

5.4.1 Inclusion criteria

- Leprosy patients: newly diagnosed and clinically confirmed
- Household contacts: who have been living at least 6 months and above with an index case and no previous treatment history of leprosy and TB
- Healthy controls: apparently healthy, no contact with an index case and no previous treatment history of leprosy and TB
- HIV negative individuals
- 18-60 years of age
- Willingness to participate in the study by signing a consent form

5.4.2 Exclusion Criteria

Critically ill patients, HIV positive individuals, pregnant women, Lactating mothers, anemic patients and patients with parasitic infections were excluded from the study.

5.5 Study Variables

Socio-demographic and clinical variables such as age, sex and Leprosy status were considered as independent variables, whereas the level of LL37 at protein and mRNA were considered as dependent variables.

5.6 Measurement and Data collection

5.6.1. Sample size determination and sampling method

We decided to take TT/BT n=11; BL/LL n=15; Household contact n=23 and healthy controls n=25, a total of 74 HIV negative individuals who fulfill the inclusion criteria based on convenient sampling technique where new patients coming consecutively to the leprosy clinic of ALERT are recruited because of time and budget limitation.

5.6.2 Data collection tools

All study participants were properly informed about the study and also asked for written informed consent. Structured clinical form was used for clinical data recording for each participant. This form included core points such as demographic information, clinical feature such as: skin lesion, number of nerves involved, type of reaction and diagnostic information set.

5.6.3 Sample collection, processing and storage

Peripheral blood (17.5ml) in heparinized vacutainer tubeand 2.5 ml blood in Paxgene tubes from all participants was collected. Five mm of skin punch biopsy from skin lesions of leprosy patients was taken by experienced health personnel(annex16).The 3ml blood from heparinized vacutainer tube was used for whole blood Assay(WBA)(annex16)from which the supernatant was used for ELISA. The remaining heparinized blood was used for Plasma (for ELISA) and PBMC isolation (annex16) for further flowcytometry work. The PAX gene Blood RNA tubes were kept at room temperature for 2 hours and then transferred to -20^{o}C for storage until used for RNA isolation. The biopsy specimen for H&E staining (annex16) were fixed in 10% formalin and kept at ambient temperature till used for histology.

RNA was extracted from blood sample in Paxgene tube using Qiagen RNA extraction kit.(Annex 16).These isolated RNA samples were measured using NanoDrop 2000 Spectrophotometer (Thermo Scientific, Epsom, UK) to determine the quality and quantity of RNA yield. Complementary DNA (cDNA) was synthesized from RNA using High Capacity cDNA Reverse Transcriptase Kit (AB Applied Biosystems, UK) on the same dayto avoid the risk of RNA degradation during storage. Reactions consisted of 10x RT buffer (1.0ml), 25x dNTP mix(100nm), 10x RT random primer(1.0ml), RNase inhibitor(100µl) (all from AB Applied Biosystems, UK), reverse transcriptase 50 U/µL, 10µl template RNA and nuclease-free water to

a total volume of 20µl. Reactions were incubated in an ABI9700 Programmable Thermal Cycler (Applied Biosystems, Foster City, California) for 10 minutes at 25ºC followed by 120 minutes at 37^{o}C and 5 minutes at 85^{o}C then cooling to 4ºC according to the insert kit provided (Annex16)and the expression of LL37assessed using Real time PCR.

5.6.4 ELISA Determination of cathelicidin LL-37 levels

We used LL37 ELISA kit (HK321; Human LL-37 ELISA Kit, Hycult Biotechnology, Uden, the Netherlands) for detection and quantification of LL-37 from plasma (from leprosy patient, HHC and healthy controls) and supernatants obtained by stimulating whole blood from Leprosy patients with *M. leprae* WCS (NR-19329 b/e/i resources).WCS is an Irradiated armadillo-derived *M. leprae* whole cell which is ready to use. The assay was performed according to the manufacturer's instructions (Ref Annex 16).LL-37 was detected from a range of 0.1 ng/ml to100 ng/ml. The working volume of 100 µl/well was the standard for the dilution. Plasma samples were diluted 1:20, duplicate were assayed for each diluted plasma sample. The human LL37 ELISA, a solid-phase enzyme linked Immunosorbent assay based on the sandwich principle was performed. Micro titer wells were coated with antibody to recognize the human LL37, 100 µL of the sample was added to each well and the plates were incubated for 60 min at 25°C, the samples were washed four times, there after a Biotinylated tracer antibody (100 µl) was added to bind and capture the peptide and incubated for 60 min at 25°C andthe samples were washed four times. Streptavidin-peroxidase conjugate (100 µl) was added to bind to the tracer; the samples were again washed four times. A substrate (Tetra Methylbenzidine (TM solution) (100µl) was added to react with the conjugate and incubated for 30 min at 25°C. Finally, the enzyme reaction was stopped by adding100µl of oxalic acid stop solution and read within 30minutes.Optical density was read using Elisa reader at 450nm wave length and converted to concentration (pg/ml) by micro plate manager 6.The data was analyzed using Graph pad prism version 6(Annex6).

5.6.5 Quantitative Real-Time PCR (qRT-PCR)

Quantitative real time PCR (qRT-PCR) was performed on the Rotor-Gene™ 3000 programmable thermal cycler (Corbett Life Science (Qiagen), Crawley, UK)using Roter-gene® SYBR® Green PCR Kit (Qiagen, Crawley, UK) to analyze the relative mRNA expression of LL37 in whole blood. PCR reactions consisted of 1x Rotor-gene SYBR Green PCR Master Mix, 1µM forward

primer, 1µM reverse primer, 2.5µl cDNA and nuclease free water to a total volume of 12.5 µl. The master mix contained Hot StarTaq plus DNA polymerase, Rotor-gene SYBR Green PCR buffer, dNTP mix and SYBR Green I fluorescent dye. All the PCR reaction mixtures were prepared using Corbett Robotics (Corbett Research, Australia) and run in duplicates including non-template controls. Before running the samples, PCR optimizations were done. The qRT-PCR cycling was carried out under the following conditions: initial Taq enzyme heat activation step at 95^{o}C for 15 minutes, followed by 40 repeats of three step cycling, denaturation step 95°C for 5 seconds, primer annealing step 60°C for 10 seconds and final extension/ polymerization step at 72°C for 20 seconds.

Primers

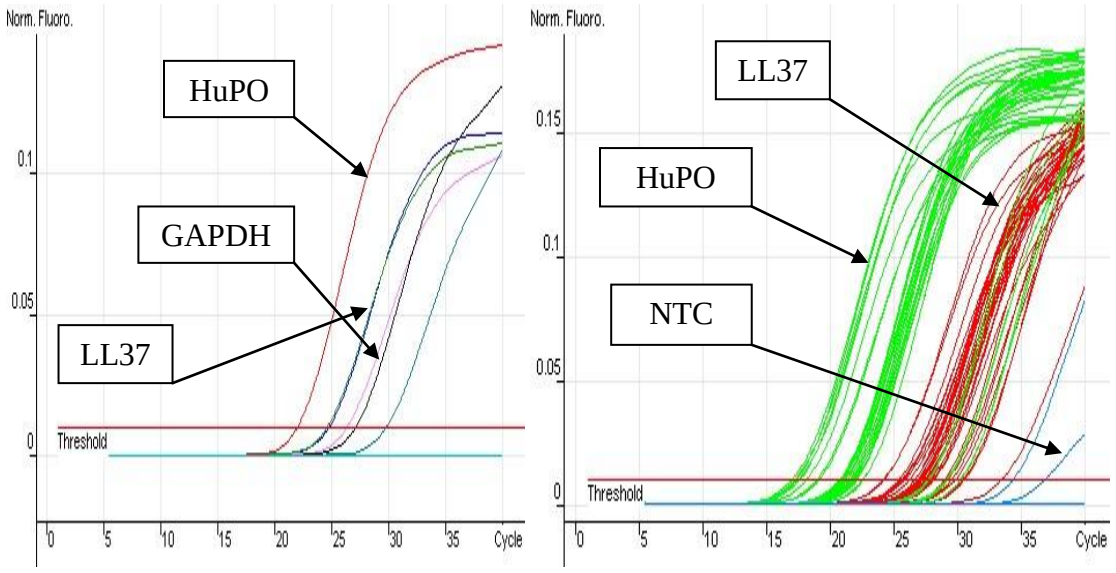
The Primer stock solution was prepared according to the manufacturer's recommendation.
Working stocks of 10µM were prepared and kept at -20°C until used.

Table 1. Primer sequence used

Primer(forward and reverse)	Length	Sequence(5'-3')
LL37(FW)	18	GGACCCAGACACGCCAAA
LL37(RV)	23	GCACACTGTCTCCTTCACTGTGA
HuPO(FW)	18	GCTTCCTGGAGGGTGTCC
HuPO(RV)	20	GGACTCGTTTGTACCCGTTG

In all the experiments, data were acquired at extension phase (on FAM/Sybr channel) and analyzed using Rotor-Gene Real-Time analysis Software 6.0 (Corbett Research, Australia).
We used human acidic ribosomal protein (HuPO) as a housekeeping gene because of its less variability relative to other housekeeping gene like GAPDH based on our optimization results. An assay control was included from RNA extraction to the amplification steps. For RNA extraction, one assay control per batch was used. The assay control included all buffers except the sample and was processed under identical conditions with the samples. The same assay control was used during cDNA synthesis and real-time quantitative PCR.
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 end point of qRT-PCR. These values were obtained for the target gene and reference gene (HuPO) for each patient and healthy sample at each time point.



A B

C

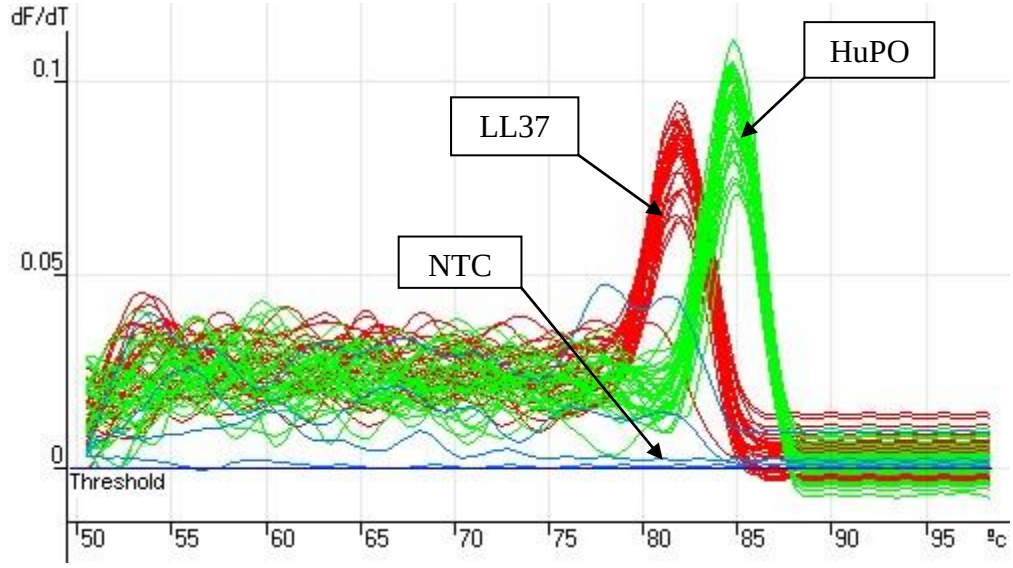


Figure 7. Real time PCR results A:shows the cycle threshold of housekeeping gene (HuPO), (GAPDH) and target gene (LL37) on optimization step in log scale. B: shows the cycle threshold of housekeeping gene (HuPO), target gene (LL37) and non-template control (NTC) in log scale.

C: shows the melt curve analysis; the peak curve indicates the primer amplified the same region of all samples and primer dimer is not detected as there is only one such peak.

The relative gene expression was analyzed by using the $2^{-\Delta\Delta}$ Ct(52)method. The Ct value is the threshold number for the amplification of the target gene. Briefly, there were healthy control groups; leprosy patient group and house hold contact groups: all Ct values were initially normalized to the Ct values of HuPO. Then; the difference in Ct value was obtained by subtracting the CT of the reference gene (Hupo) from the Ct of the target gene (LL37) and designated as ΔCt. To compare the target gene expression in leprosy patients (cases) and house hold contacts, ΔΔCt was obtained by subtracting the ΔCt of healthy control from the ΔCt of the leprosy patient (cases) and ΔCt of household contacts respectively. Then, the fold change was obtained by using the formula $2^{-\Delta\Delta}$ Ct. The computed number indicates the fold change in the expression of a target gene(LL37) in the different groups relative to endemic control group as shown below:

Table2.Relative quantification calculations; fold change (Fc) $2^{-(\Delta\Delta Ct)}$

Groups	Serial No	LL37	Hupo	ΔC_t (LL37-Hupo)	ΔC_t Average ($\Delta C_{t\ 1+}\ \Delta C_t 2/\ 2$)	$\Delta\Delta C_t$ ($\Delta C_{t\ -}\ \Delta C_t$ Average)	$2^{-(\Delta\Delta Ct)}$
Healthy control	1	22.52	16.625	5.895	5.6375	0.2575	0.837
	2	23.82	18.44	5.38		-0.2575	1.195
Leprosy patient	1	25.275	21.74	3.535	-	-2.1025	0.232
	2	27.015	22.82	4.195	-	-1.4425	2.718
House hold contact	1	25.875	17.35	8.525	-	2.8875	0.135
	2	25.585	17.91	7.675	-	2.0375	0.244

5.7. Data Quality Control

Data quality was addressed by following Standard operational procedures (SOPs) for each laboratory activity to minimize the possible errors. All tests were performed by skilled professionals and also the qualities of laboratory works were checked against experimental positive and negative controls. Cross-checking and data cleaning was done. In addition, all laboratory experiments and procedures were recorded in hard copies (laboratory log book) while conducting laboratory work and made backups using softcopies (computer, CD, flash disks).

5.8. Data Analyses and Interpretation

Statistical analysis and graphs were performed using GraphPad Prism version 7.01 (GraphPad Software, La Jolla California USA, www.graphpad.com) by exporting Data (Fc($2^{(-\Delta\Delta Ct)}$) from qRT-PCR, from micro plate manager 6and SPSS version 20 for further statistical analyses.

Categorical variables such as clinical and socio-demographic data were presented in numbers and percentages. Mann-Whitney (U) test was used to compare the fold change in the relative expression of LL37.A P-value < 0.05 was considered statistically significant.

5.9 Ethical Considerations

This study was carried out after receiving ethical approval from Addis Ababa University, College of Health Sciences (CHS), Department of Microbiology, Immunology and Parasitology Ethical Review Committee and AHRI/ALERT Ethics Review Committee (AAERC) (Registration numbers005/09 andP005/16).

5.10 Dissemination of results

The result of this study will be disseminated to National TB and Leprosy control program; it will also be present in different conferences. The scientific community will be reached via a publication in a national & international journal.

5.11. Operational definitions

Newly diagnosed leprosy patient: clinically confirmed leprosy patients who have never been on MDT.

Household contacts of leprosy patients: individuals living at least 6 months and above with an index case and no sign and symptoms of leprosy at enrollment.

Critically ill: Patient that has lost reactivity, reactions to stimulations, reflexes = Comatous

Healthy control: apparently healthy individuals.

6. Results

6.1 Socio- demographic and Clinical characteristics of the study population

Socio-demographic and Clinical data were collected from 74 participants (26 Leprosy patients, 23 household contacts and 25 healthy controls) (Table 4).Sixty six percent of the study participants were males. Only 4(15.38%) patients were with reaction and the rest all were free of reactions during initial diagnosis. As a whole none of these parameters had significant association with LL37 result.

Table3. Characteristics of socio-demographic and clinical data of study population, Addis Ababa, Ethiopia, 2018

Gender	n(percent)
Male	59(66.22%)
Female	25(33.78%)
Age group	
<21	4(5.41%)
21-30	35(47.30%)
31-40	18(24.32%)
41-50	8(10.81%)
51-60	9(12.16%)
Duration of symptom	
1-3 months	4(15.38%)
3-6 months	3(11.54%)
6-12months	8(30.77%)
Above 12 months	11(42.31%)

BCG	n(percent)
Yes	17(33.33%)
No	21(41.18%)
Not sure	13(25.49%)

Number of lesion	
Five or less skin lesion	9(34.62%)
Six or more skin lesion	17(65.38%)

Disability	
Yes	15(57.69%)
No	11(42.31%)

6.2. LL37 mRNA Expression in the Peripheral Blood of Leprosy Patients, house hold contacts and Healthy controls.

In order to assess whether the level of LL37 gene expression correlates with leprosy disease, we compared the level of LL37 gene expression between leprosy patients, household contacts and Healthy controls. First the LL37 gene expression was normalized against the reference gene, HuPO and then the relative gene expression was analyzed and presented as fold change. Our data showed no significant difference in LL37 gene expression between leprosy patients, house hold contacts and Healthy controls. We also observed heterogeneity in the LL37 gene expression among household contacts than in leprosy patients and endemic controls as shown in figure 8.

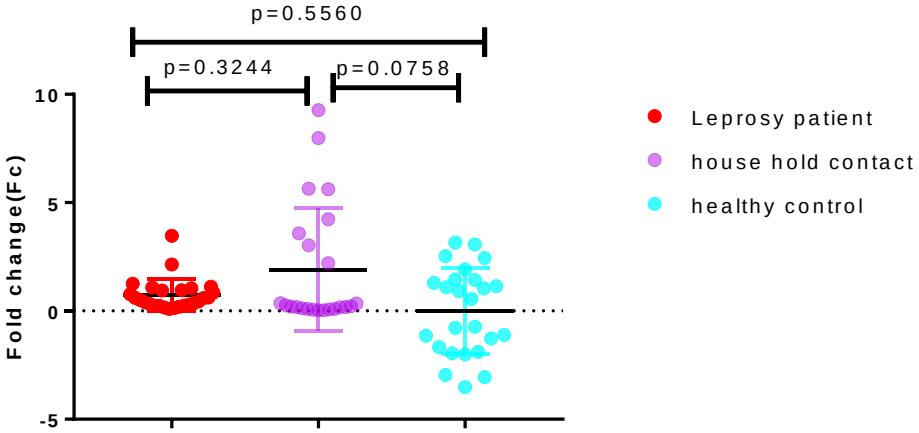


Figure 8 .LL37 mRNA expression level in peripheral blood of Leprosy patients, HHCs and Healthy controls. Horizontal line indicates median of the groups (filled circles). Data were analyzed using nonparametric Mann-Whitney test with p-values (p=0.3244) indicating non-significant difference between leprosy patients and house hold contacts, (p=0.0758) between house hold contact and Healthy controls, (p=0.5560) between leprosy patient and Healthy control.

6.3. LL37 mRNA Expression in the Peripheral Blood of PB and MB

In order to assess whether the level of LL37 gene expression correlates with the clinical form of leprosy disease, we compared the level of LL37 gene expression between PB and MB leprosy patients first the LL37 gene expression was normalized against the reference gene, HuPO and then the relative gene expression was analyzed and presented as fold change. Our data showed no significant difference in LL37 gene expression between PB and MB (P=0.3842) as shown in figure 9.

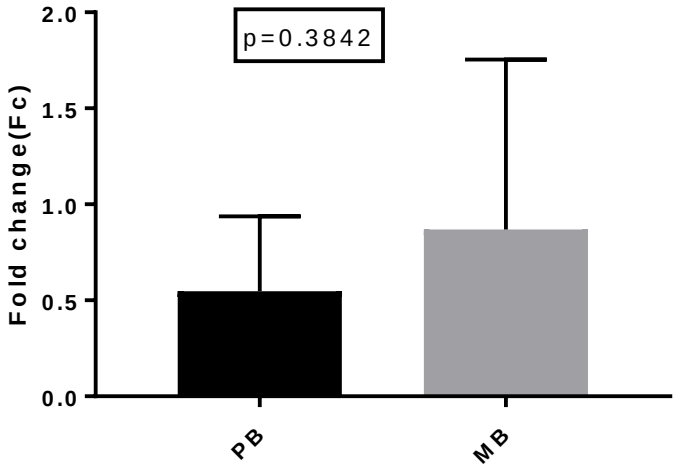


Figure9.LL37 mRNA expression level in peripheral blood of leprosy patients PB (TT/BT) vs MB (BL/LL).Data were analyzed using nonparametric Mann-Whitney t test (p=0.3842) indicating non-significant difference.

6.4. Plasma Level of LL37 in different forms of leprosy, HHC and Healthy controls.

The protein level of LL37 in the plasma of newly diagnosed leprosy patients (TT, BT, BL and LL), HHC of untreated MB patients and apparently healthy controls was measured using ELISA and there was no significant difference between patient groups (TT/BT Vs BL/LL p=0.08). However, a significant difference was found in the level of LL37 between BL/LL patient group and HHC (p=0.017). In addition, the level of LL37 in HHC is significantly higher compared to Healthy controls (p=0.007) as shown in figure 10.

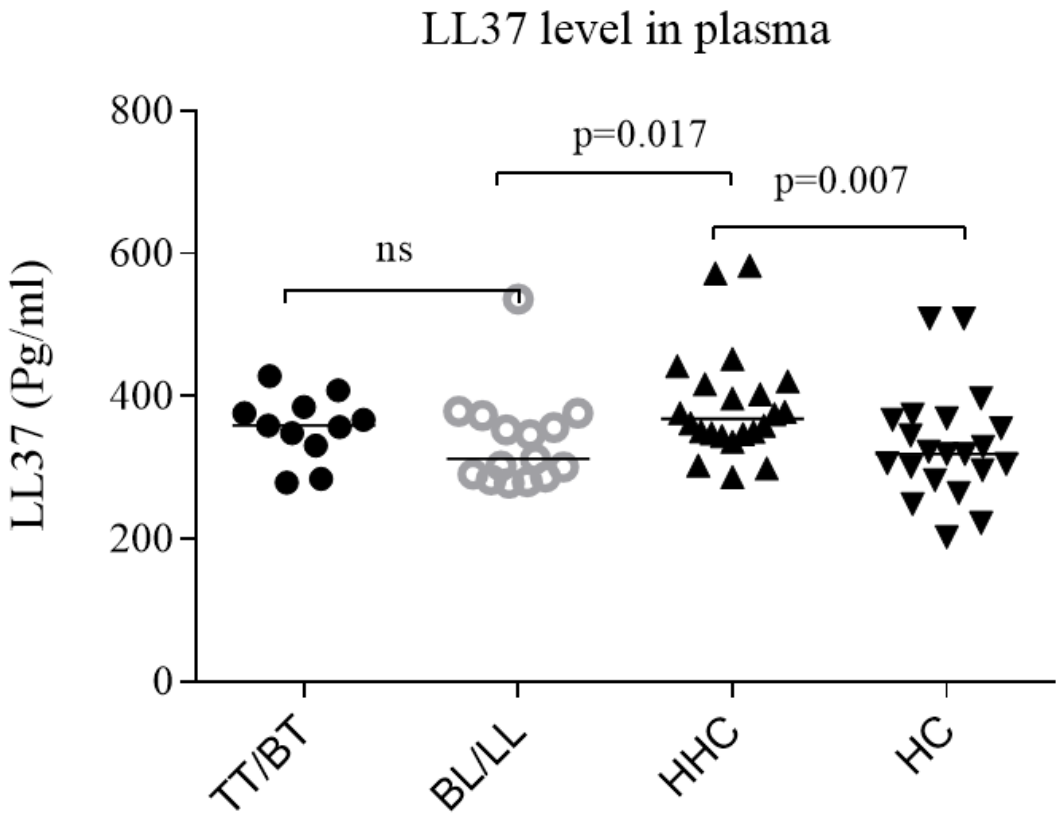


Figure 10.LL37 levels (pg/ml) in plasma of PB (TT/BT), MB (BL/LL) leprosy patients, HHCs and healthy controls. Horizontal line indicates median of the groups.

6.5. LL37 level in *M. leprae* WCS stimulated whole blood of leprosy patients

Whole blood samples collected from TT/BT and BL/LL patients were stimulated with *M. leprae* WCS (10ug/ml final concentration) for 24 hrs in 37^{o}C with 5% CO_2 to measure *M. leprae* specific level of LL37 in plasma. The level of LL37 between TT/BT and BL/LL patients didn't show significant difference (p=0.7360) although an increased LL37 level was measured in stimulated samples compared to unstimulated plasma as shown in figure 11.

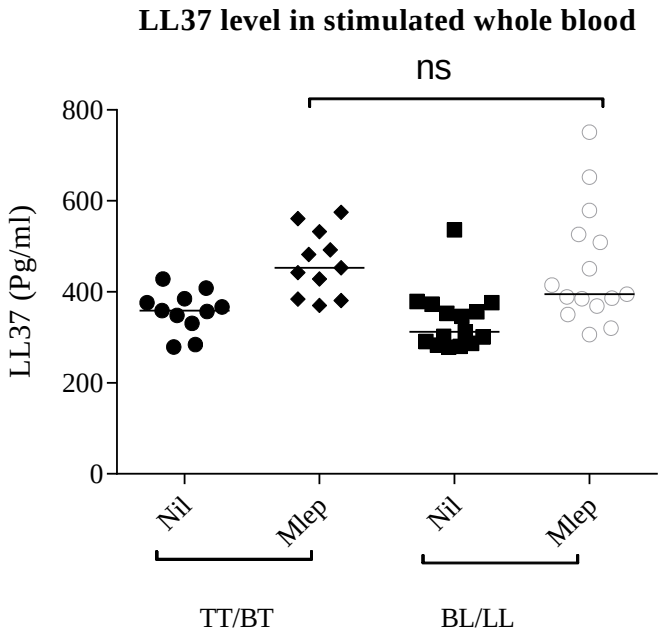


Figure11. LL37 levels (pg/ml) in M.leprae WCS stimulated whole blood of PB (TT/BT) and MB (BL/LL) leprosy patients. Horizontal line indicates median of the groups.

7. Discussion

To the best of our knowledge this is the first study to report on the plasma level and mRNA expression level of LL37 in leprosy in Ethiopia and in general, only few studies are available globally on Cathelicidin in relation to leprosy(11). There are few studies that have shown a profound anti-microbial activity of LL37 against *M. tuberculosis*(11, 53).

In our study, we investigated leprosy patients (n = 26) presenting the full spectrum of clinical forms of leprosy using ELISA technique and a significantly lower levels of LL37 was measured in untreated lepromatous patients compared to household contacts (n=23) (p=0.017) but no significance difference with Healthy controls. A similar study in Yemen ,which involved 29 treated and untreated Yemenian leprosy patients, low levels of serum LL37 in untreated Leprosy patients relative to control groups were reported using the same technique ELISA(11). The lower serum or plasma concentration of LL37 correlates with high bacillary load as, lepromatous leprosy is the most severe clinical manifestation of leprosy with high bacterial load in the body. The absence of a critical concentration of this cathelicidin LL-37 abstain it from accumulation on the bacterial surface ,alteration of membrane structure which leads to formation of ion channels or aqueous pores, leading to the bacteria death through hypo osmotic lysis(49).There is a need for in depth investigation if the complete absence of leprosy specific cellular mediated response is as a result of lower level of LL37 in *in vitro* experiments.

In other study, it has been also reported that individuals with inflamed dental pulp tissue have significantly lower LL37 level than the healthy ones (p=0.03) when measured by the same technique ELISA(54).

There are also some studies that showed the association of LL37 with susceptibility to diseases such as the diminished expression seen during atopic dermatitis patients compared with healthy individuals(55) and an increased susceptibility to skin infections in mice with disrupted Cnlp, the gene coding for cathelin-related antimicrobial peptide (CRAMP)(46).

Whereas, when we compare between the two clinical forms, tuberculoid and lepromatous leprosy forms, which constitute opposite poles of leprosy spectrum, being clinically, immunologically and histologically distinct from each other (8), no significant differences were seen in the

level/amount of LL37 in their plasma (p=0.18).This may require further investigation in larger sample sizes as the insignificant difference between these distinct groups might have been overlooked through using smaller sample size or LL37 might play a different immunomodulatory role in different leprosy forms like in TB by interacting with receptors and different cytokines.

Similarly, the level of LL37 in *M. leprae* WCS stimulated whole blood samples from TT/BT and BL/LL patients didn't differ significantly. Considering, the well known poor Th1 responses of LL patients specifically to *M. leprae* proteins(56), which commonly known as T cell anergy(20), the comparable LL37 level of BL/LL patients with TT/BT patients in this study could be due to various reasons. One reason could be the small sample size which didn't allow us to group patients clearly in to the five forms where the polar forms are combined with borderline patients. This might have over shadowed the differences especially between polar forms. Level of LL37 alone might not give the clear picture about the scenario unless and otherwise some functionality assays are done.

On the other hand, LL37 plasma concentrations in healthy controls showed significantly lower (p=0.007) level compared with household contacts of untreated multibacillary leprosy patients. These results might suggest that LL37 level increment is associated with the degree of exposure to *M. leprae* and immunological profile of the individual.

We have also demonstrated that LL37 expression at mRNA level irrespective of the clinical forms; there were no difference on the expression level between all groups. The level in leprosy patients was lower but not statistically significant as compared to household contacts and healthy controls. Even the two clinical forms, tuberculoid and lepromatous leprosy forms related insignificantly (p=0.3842).Our suggestion for this is the same like that of the protein level; that is, LL37 might have different role in the different clinical forms even if the concentration does not matter. In contrary, in a study done in TB patients; LL37 mRNA expression was elevated in the peripheral blood of TB Patients compared to non-TB Controls(53).And in the skin of psoriasis patients which is characterized by skin inflammation(57).the strong up regulation of hCAP18/LL37 upon injury or infection, indicates that LL37 assists the immune system.

The level of LL37 at protein and mRNA level show us there is variability across the group. Leprosy patients' and healthy controls' LL37 protein level in plasma was lower as compared to household contacts. This might be due to the post-translational modifications that may or may not be occurring at that moment(58) or the degradation of the protein is higher in this groups relative to the others.

In general, in this study, all assays were done using peripheral blood which represents the situation in the circulation only. Therefore, mRNA expression level of LL37 should also be assessed on samples collected from infection sites (on biopsy samples from active skin lesions of leprosy patients) and also using immunoflourecence assay like immunohistochemistry (IHC).On the other hand, as LL37 has an immunomodulatory role, its level in periphery or at the active sites may not be a good indicator about its role. The level of LL37 in different forms of leprosy could be comparable but the role played in different forms may vary. Hence, in depth investigation of the role of LL37 in different forms of leprosy is important.

8. Strengths and Limitations of the Study

8.1Strength

This is the first study which measured LL37 level in leprosy patients in Ethiopia and one of the few studies globally that studied LL37 in leprosy.

8.2 Limitations

The limitation factor for this study was getting untreated leprosy patients in the specified time table and small sample size in the different clinical forms.

9. Conclusion and Recommendations

9.1Conclusion

The study has shown that expression level of LL37 at mRNA level in periphery blood does not differ significantly between leprosy patients, household contacts of untreated multibacillary leprosy patients and healthy controls. Whereas LL37 at protein level showed a significant difference between HHCs of untreated multibacillary leprosy patients and leprosy patients. And also with healthy controls which might be an indication for level of *M. leprae* exposure.

9.2 Recommendation

The role and association of LL37 with leprosy should be further investigated at infection sites. This may provide better evidence to understand its role in leprosy. Similarly, investigating the effect of MDT on the level of LL37 through enrolling untreated and treated leprosy patients is essential to know if there is any association between LL37 and MDT and to know if there is a possibility to use as therapeutic agent. Increasing the sample size may also be required to gain more information about LL37 in relation with Leprosy. In general further research is needed to fully understand the big effects of this little peptide on immune system function so that potential therapeutic uses can be explored.

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11. Annexes

Annex 1: English version of participant's information sheet for Leprosy patients

The correlation of LL37 expression with different forms of Leprosy

Participant Information

Background

Leprosy is a chronic infectious disease caused by Mycobacterium leprae. It affects more than 200,000 people every year. Understanding the interaction and role of some compounds like LL37 in our body is important to improve leprosy diagnostics and therapeutics.

Aim of the study

-To investigate the association of LL37 in different forms of leprosy.

Duration of the study

The duration of the study will be from January one 2017 to September 2018 and participants will have two visits: before treatment and after completion of treatment

Procedure

Experienced nurse will take 20 ml (2 table spoon) blood samples and 5mm (like a size of pea) biopsy samples from confirmed lesions of leprosy patients before and after treatment completion.

HIV testing

The study requires HIV screening. Therefore, participants will be asked for voluntarily HIV testing. If they don't wish to pass through the HIV testing, they can refuse to take part in the study. Appropriate health personnel will give Pre and post counseling. The HIV result will be kept confidential unless the participants want to know their status. HIV negative individuals will be involved in the study. Those individuals who turn out to be HIV positive will be offered the available care in their nearby Hospital or health center.

Risks and complications

Minimal risks Such as bruise, pain, bleeding, scar formation may happen in taking the samples. If these things happen the participants have the right to get the necessary medical care and the cost will be covered.

Benefits

Participants in this study will receive no direct benefit from the study and as they are voluntarily participating in this study; there will be no inducement. However, the outcome of the study will contribute to understand more about the compound which is important for future use in diagnostics and therapeutics of Leprosy. There will be 50 birr for every participant for Lunch and also maximum of 300 birr for transportation pay according to their Traveling distance which is confirmed by the ticket they come with.

Participant Right

The participants have the right to withdraw from the study at any time they need but they will not deprived of their right to get the proper service they need. Participants also have the right to ask and know information about the study.

Confidentiality

All personal records will be kept confidential. No personal information will appear in any report from this study. All test results will be treated confidentially with use of coded labels on specimens. The treating physician may only make links to persons and AHRI Data Manager, where identity documents will be kept locked in cabinets.

If you have any question associated with the study, you can contact the PI and Secretary of the Armauer Hansen research institute with the following address

Mahlet Osman (Prinicipal investigator)

Armauer Hansen Research Institute

Mobile No. 0910539625

Mr. Hailemichael Getachew

Secretary, AAERCTel. 0118-962183

Annex 2 Participant information sheet in Amharic (for Leprosy Patients)

The correlation of LL37 expression with different forms of Leprosy

ለተሳታፊዎች ስለጥናቱ የሚገልጽ መረጃ

የጥናቱ መንደርደርያ

የስጋ ደዌ ማይኮባክቴሪየም ሌፕሬ በሚባል ተህዋስ የሚመጣ ተላላፊ በሽታ ነው ። በሽታው በየአመቱ 200000 ሰዎችን ያጠቃል ። በሰውነታችን ውስጥ ያለ እንደ ‹ LL37 › አንዳንድ ንጥረ ነገሮችን ከስጋ ደዌ ጋር ያላቸውን ተዛምዶና ሚና ማጥናት የስጋ ደዌን በሽታ የበለጠ ለመረዳት፣ የምርመራ ዘዴዎችንና መድሀኒቶችን ለማሻሻል ይረዳል ።

የጥናቱ አላማ

‹LL37› **የተባለውን ንጥረነገር ከስጋ ደዌ በሽታጋር ያለውን ተዛምዶና ሚና ማጥናት**

የጥናቱ ጊዜ

ጥናቱ የሚካሄደው ከጥር 2009-መስከረም2011 ይሆናል ።በዚ ጊዜ ዉስጥ ተሳታፊዎች ሁለት ጊዜ ይመጣሉ፡- ከመዳህኒት በፊትናበኃላ።

ናሙና አወሳሰድ

ከስጋደዌ ህመማን 20 ሚሊ (ሁለት የሻይማንኪያ) ደም እና በተጨማሪ የበሽታው ምልክት ካለበት ቦታየአተር ፍሬ የሚያክል የቆዳ ናሙና (ባዮፕሲ) ከህክምና(ከመድሃኒት)በፊትም በኃላም ልምድ ባለው /ባላት ነርስ የሚወሰድ ይሆናል።

የኤችኤይቪ ምርመራ

ይህ ጥናት የኤች አይቪ ምርመራ ውጤት ያስፈልገዋል ። ስለዚህም የጥናቱ ተሳታፊዎች በፍቃደኝነት ላይ የተመሰረተ የኤችኤይቪ ምርመራ እንዲያደርጉ ይጠየቃሉ ። ተሳታፊዎቹ የኤችኤይቪ ምርመራ ማድረግ ካልፈለጉ በጥናቱ ውስጥ አለመሳተፍ ይችላሉ ። ተሳታፊዎቹ የቅድመና የድህረ ምርመራ ምክር ይሰጣቸዋል ውጤቱም በሚስጥር ይያዛል ፤ ውጤታቸውን ማወቅ ከፈለጉ ብቻ ይነገራቸዋል ። የኤችኤይቪ ቫይረስ በደማቸው ውስጥ የሌለባቸው በጥናቱ ውስጥ ይካተታሉ ። የኤችኤይቪ ቫይረስ በደማቸው ውስጥ እንዳለባቸው የተረጋገጠ ከሆነ

አስፈላጊው ምክርና በአቅራቢያው ካለው ሆስፒታል ወይም ጤና ጣቢያ ለኤችኤይቪ ህሙማን የሚሰጠውን አገልግሎት እንዲያገኙ ይደረጋል ።

ናሙና በመውሰድ የሚከተል ስጋት ወይም ጉዳት

ናሙናዎቹ በሚወሰዱበት ጊዜ መጠነኛ ጉዳት ሊኖር ይችላል። ምናልባት የከፋ የህመም ስሜትና ቁስለት የቆዳ ናሙና የተወሰደበት ቦታ ላይ ከቆየ በአቅራቢያው ወዳለ ጤና ጣቢያ ወይም ወደ አለርት ሆስፒታል በመምጣት ህኪም ማየት ያስፈልጋል ። ለዚህምየሚወጣው ወጪ ይሸፈናል።

ተሳታፊው ከጥናቱ የሚያገኘው ጥቅም

ተሳታፊዎች ከጥናቱ በቀጥታ የሚያገኙት ጥቅም የለም በጥናቱ የሚሳተፉት በፈቃደኝነት ስለሆነ ምንም ዓይነት ማግባብያ አይሰጣቸውም ።ነገርግን የዚህ ጥናት ውጤት የስጋ ደዌ በሽታን ጉዳት ከማስከተሉ በፊት ቀድሞ ለማወቅ የሚረዱ ዘዴዎችን ለማግኘት የሚደረገውን ጥረት በማገዝ የአገርአቀፍ የቁጥጥር ፕሮግራሙን ና ህብረተሰቡን ይጠቅማል። ሆኖም ለእያንዳንዱ ተሳታፊ 50ብር ለምሳ እና እስከ 300ብር በደረሰኝ የሚወራረድ የትራንስፖርት ወጪ ይሸፈናል።

የተሳታፊዉ መብት

ተሳታፊዎች በማንኛዉም ሰአት በጥናቱ ውስጥ አለመሳተፍ ይችላሉ ።በዚህም ምክንያት ማግኘት ከሚገባቸዉ ተገቢ ህክምና አይከለከሉም። የጥናቱንም ውጤትመጠየቅና ማወቅ ይቸላሉ።□

የጥናቱ ሚስጥራዊነት

ሁሉምየተሳታፊ መረጃዎች በሚስጥር ይያዛሉ ። ከተሳታፊው መረጃዎች ውስጥ ምንም አይነት የግል መረጃዎች የትኛውም ሪፖርት ላይ አይወጡም ። ሁሉም የምርመራ ውጤቶች ምስጥራዊ መለያ ቁጥር ስለሚሰጣቸው መረጃዎች ምስጢርነታቸው የተጠበቀ ነው ። የተሳታፊው የግል መረጃዎች በሚቆለፉ ሳጥኖች ስለሚቀመጡ መረጃዎቹን በቀጥታ የሚያገኙት ሃኪሙና የአህሪ ዳታ ማኔጀሩ ብቻ ናቸው ።

ከጥናቱ ጋር ተያይዞ ማወቅ የሚፈልጉት ጉዳይ ካለ ወይም ጥያቄ ካልዎት ዋና አጥኚዋን ወይም የአርማዎር ሃንሰን ሪሰርች ኢንስቲትዩትን ጸሀፊ በሚከተለው አድራሻ ማነጋገር ይችላሉ ።

ማህሌት ኡስማን (ዋና አጥኚ)
አርማዎር ሃንሰን ሪሰርች ኢንስቲትዩት
ስልክ ቁጥር 0910539625
አቶ ሀይለሚካኤል ጌታቸው
አርማዎር ሃንሰን ሪሰርችኢንስቲትዩት /አለርት ኤቲክስ ኮሚቴ
ስልክ ቁጥር 0118-962183

Annex 3 Participant information sheet (For HHC)

The correlation of LL37 expression with different forms of Leprosy

Participant Information

Background

Leprosy is a chronic infectious disease caused by Mycobacterium leprae. It affects more than 200,000 people every year. Understanding the interaction and role of some compounds like LL37 in our body is important to improve leprosy diagnostics and therapeutics.

Aim of the study

To investigate the association of LL37 in different forms of leprosy.

Duration of the study

The duration of the study will be: from January 2017 to September 2018.

Procedure

Experienced nurse will take 20 ml (2 table spoon) blood samples. There is no need of revisiting.

Who are Household contacts?

In this study, household contacts are individuals who have been living together with leprosy patients for at least 6 months before the patients start treatment. The household contacts should be 18 years old and above and willing to participate.

HIV testing

The study requires HIV screening. Therefore, participants will be asked for voluntarily HIV testing. If they don't wish to pass through the HIV testing, they can refuse to take part in the study. Appropriate health personnel will give Pre and post-test counseling. The HIV result will be kept confidential unless the participant wants to know his/her status. If they turn out to be HIV positive, they will be excluded from the study but they will be offered the available care in their nearby Hospital or health center.

Risks and complications

Minimal risks Such as bruise, pain, bleeding may happen in taking the samples.If these things happen the participant has the right to get the necessary medical care and the cost will be covered.

Benefits

Participants in this study will receive no direct benefit from the study and as they are voluntarily participating in this study; there will be no inducement. However, the outcome of the study will contribute to devise early diagnosis technique for leprosy which is important for national leprosy control program and the society. There will be 50 birr for every participant for Lunch and also maximum of300 birr for transportation pay according to their Traveling distance which is confirmed by the ticket they come with.

Participant Right

The participants have the right to withdraw from the study at any time they need but they will not deprived of their right to get the proper service they need. Participants also have the right to ask and know information about the study.

Confidentiality

All personal records will be kept confidential. No personal information will appear in any report from this study. All test results will be treated confidentially with use of coded labels on specimens. The treating physician may only make links to persons and AHRI Data Manager, where identity documents will be kept locked in cabinets. Regarding the HIV results, only the participant and the physician may know participant's HIV result. The HIV results will be reported to the counseling physician who will discuss the finding with the respective participant.

If you have any question associated with the study, you can contact the PI and Secretary of the Armauer Hansen research institute with the following address

Mahlet Osman (Principal investigator)

Armauer Hansen Research Institute

Mobile No. 0910539625

Mr. Hailemichael Getachew

Secretary, AAERCTel. 0118-962183

Annex 4 Participant information sheet Amharic (HHC)

The correlation of LL37 expression with different forms of Leprosy

ለተሳታፊዎች ስለጥናቱ የሚገልጽ መረጃ

የጥናቱ መንደርደርያ

የስጋ ደዌ ማይኮባክቴሪየም ሌፕራ በሚባል ተህዋስ የሚመጣ ተላላፊ በሽታ ነው ። በሽታው በየአመቱ 200000 ሰዎችን ያጠቃል ። በሰውነታችን ውስጥ ያለ እንደ ‹ LL37 › አንዳንድ ንጥረ ነገሮችን ከስጋ ደዌ ጋር ያላቸውን ተዛምዶና ሚና ማጥናት የስጋ ደዌን በሽታ የበለጠ ለመረዳት፣ የምርመራ ዘዴዎችንና መድሀኒቶችን ለማሻሻል ይረዳል ።

<u>የጥናቱ ዓላማዎች</u>

ላላ37 የተባለውንንጥረነገርከሥጋደዌበሽታጋርያለውንተዛምዶናሚናማጥናት

<u>የጥናቱ ጊዜ</u>

ጥናቱ የምካሄደው ከጥር 2009 አስከ መስከረም2011**ይሆናል።**

ናሙና አወሳሰድ

20 ሚሊ (ሁለት የሻይማንኪያ) ደም ልምድ ባለው /ባላት ነርስ የሚወሰድ ይሆናል።ተመልሰዉ መምጣት አ□□በቅባቸዉም።

ከሥጋደዌ ህመምተኞች ጋር አብረው የሚኖሩ ማለት ምን ማለት ነው?

በዚህ ጥናት ከሥጋደዌ ህመምተኞች ጋር አብረው የሚኖሩ ማለት የሥጋደዌ ህመምተኞች መድሃኒት ከመውሰዳቸው በፊት አብረዋቸው ቢያንስ ለ6 ወራት የኖሩ ናቸው። በጥናቱም ለመሳተፍ ዕድሜአቸው 18 ዓመትና ከዚያ በላይ ሊሆናቸው ይገባል።

<u>የኤች አይቪ ምርመራ</u>

ይህ ጥናት የኤች አይቪ ምርመራ ውጤት ያስፈልገዋል። ስለዚህም የጥናቱ ተሳታፊዎች በፈቃደኝነት ላይ የተመሰረተ የኤች አይቪ ምርመራ □ንዲያደርጉ ይጠየቃሉ። ተሳታፊዎቹ የኤች አይቪ ምርመራ ማድረግ ካልፈለጉ በጥናቱ ውስጥ ያለመሳተፍ ይችላሉ። ተሳታፊዎቹ የቅድመና ድህረ ምርመራ ምክር ይሰጣቸዋል ውጤቱም በሚስጥር ይያዛል፣ ውጤ□ቸውን

ማወቅ ከፈለጉ ብቻ ይነገራቸዋል። የኤችአይቪ ቫይረስ በደማቸው ውስጥ የሌለባቸው ተሳታፊዎች በጥናት ውስጥ ይካተታሉ። የኤችአይቪ ቫይረስ በደማቸው ውስጥ □ንዳለባቸው የተረጋገጠ ከሆነ ከጥናቱ ይወጣሉ ነገርግን አስፈላጊው ምክር □ና በአቅራቢያው ካለው ሆስ□ታል ወይም ጤና ጣቢያ ለኤችአይቪ ህሙማን የሚሰጠውን አገልግሎት □ንዲያገኙ □□ረ□ል።

<u>ናሙና በመውሰድ የሚከተል ስጋት ወይም ጉዳት</u>

ናሙና በሚወሰዱበት ጊዜ መጠነኛ ጉዳት ሊኖር ይችላል።ምናልባት የከፋ የህመም ስሜትና ቁስለት ካለ በአቅራቢያው ወዳለ ጤና ጣቢያ ወይም ወደ አለርት ሆስፒታል በመምጣት ህኪም ማየት ያስፈልጋል ። ለዚህ የሚወጣውም ወጪ ይሸፈናል።

<u>ተሳታፊው ከጥናቱ የሚያገኘ□ □ቅም</u>

ተሳታፊዎች ከጥናቱ በቀጥታ የሚያገኙት ጥቅም የለም በጥናቱ የሚሳተፉት በፈቃደኝነት ስለሆነ ምንም ዓይነት ማግባብያ አይሰጣቸውም። ነገርግን የዚህ ጥናት ውጤት የስጋ ደዌ በሽታን ጉዳት ከማስከተሉ በፊት ቀድሞ ለማወቅ የሚረዱ ዘዴዎችን ለማግኘት የሚደረገውን ጥረት በማገዝ የአገርአቀፍ የቁጥጥር ፕሮግራሙን ና ህብረተሰቡን ይጠቅማል።ሆኖም ለእያንዳንዱ ተሳታፊ 50ብር ለምሳ እና እስከ 300ብር በደረሰኝ የሚወራረድ የትራንሰፖርት ወጪ ይሸፈናል።

የተሳታፊዉ መብት

ተሳታፊዎች በማንኛዉም ሰአት በጥናቱ ውስጥ አለመሳተፍ ይችላሉ ።በዚህም ምክንያት ማግኘት ከሚገባቸዉ ተገቢ ህክምና አይከለከሉም። የጥናቱንም ውጤት መጠየቅና ማወቅ ይቸላሉ።□

<u>የጥናቱ ምስጢራዊነት</u>

ሁሉም □ተሳታፊው መረጃዎች በሚስጥር ይያዛሉ። ከተሳታፊው መረጃዎች ውስጥ ምንም አይነት የግል መረጃዎች የትኛውም ሪፖርት ላይ አይወጡም። ሁሉም የምርመራ ውጤቶች ምስ□ጢራዊ መለ□ ቁጥር ስለሚሰጣቸው መረጃዎቹ ምስጢርነታቸው የተጠበቀ ነው። □ተሳታ□□ የግል መረጃዎች በሚቆለፉ ሳጥኖች ስለሚቀመጡ መረጃዎቹን በቀጥታ□ □ሚ□□ኝ

ት ሃኪሙና የአሀሪ ዳታማኔጀሩ ብቻ ናቸዉ። የኤችአይቪ ውጤትን አስመልክቶ ተሳታፊውና ሃኪሙ ብቻ የተሳታፊውን የኤችአይቪ ውጤት የሚያውቁ ይሆናሉ። የኤችአይቪ ውጤት ለአማካሪው ሃኪም ይላካል ሃኪሙም ከተሳታ□□ ጋራ ይመካከራል።

ከጥናቱ ጋር ተያይዞ ማወቅ የሚፈልጉት ጉዳይ ካለ ወይም ጥያቄ ካለዎት ዋና አጥኚዋን ወይም የአርማዎር ሃንሰን ሪሰርች ኢንስቲትዩትን ጸሀፊ በሚከተለው አድራሻ ማነጋገር ይችላሉ።

ማህሌትአስማን(ዋና አጥኚ)

አርማዎር ሃንሰን ሪሰርች ኢንስቲትዩት

ስል□ ቁ□ር 0910539625

አቶ ሀይለሚካኤል □ታቸዉ

አርማዎር ሃንሰን ሪሰርች ኢንስቲትዩት

ስል□ ቁ□ር 0118-962183

Revision Date 25/7/17

Annex 5 Participant information sheet (for Healthy control)

The correlation of LL37 expression with different forms of Leprosy

Participant Information

Background

Leprosy is a chronic infectious disease caused by Mycobacterium leprae. It affects more than 200,000 people every year. Understanding the interaction and role of some compounds like LL37 in our body is important to improve leprosy diagnostics and therapeutics.

Aim of the study

- To investigate association of LL37 in different forms of leprosy.

Duration of the study

The duration of the study will be: from January 2017 to September 2018.

Procedure

Experienced nurse will take 20 ml (2 table spoon) blood samples. There is no need of revisiting.

HIV testing

The study requires HIV screening. Therefore, participants will be asked for voluntarily HIV testing. If they don't wish to pass through the HIV testing, they can refuse to take part in the study. Appropriate health personnel will give Pre and post counseling. The HIV result will be kept confidential unless the participant wants to know his/her status. HIV negative individuals will be involved in the study. Those individuals who turn out to be HIV positive will be offered the available care in their nearby Hospital or health center.

Risks and complications

Minimal risks Such as bruise, pain, bleeding may happen in taking the samples. If these things happen the participant has the right to get the necessary medical care and the cost will be covered.

Benefits

Participants in this study will receive no direct benefit from the study and as they are voluntarily participating in this study; there will be no inducement. However, the outcome of the study will contribute to understand more about the compound which is important for future use in diagnostics and therapeutics. There will be 50 birr for every participant for Lunch and also maximum of 300 birr for transportation pay according to their Traveling distance which is confirmed by the ticket they come with.

Participant right

The participants have the right to withdraw from the study at any time they need but they will not deprived of their right to get the proper service they need. Participants also have the right to ask and know information about the study.

Confidentiality

All personal records will be kept confidential. No personal information will appear in any report from this study. All test results will be treated confidentially with use of coded labels on specimens. The treating physician may only make links to persons and AHRI Data Manager, where identity documents will be kept locked in cabinets.

If you have any question associated with the study, you can contact the PI and Secretary of the Armauer Hansen research institute with the following address

Mahlet Osman (Prinicipal investigator)

Armauer Hansen Research Institute

Mobile No. 0910539625

Mr. Hailemichael Getachew

Secretary, AAERC

Tel. 0118-962183

Annex 6 Participant information sheet in Amharic (for Healthy controls)

The correlation of LL37 expression with different forms of Leprosy

ለተሳታፊዎች ስለጥናቱ የሚገልጽ መረጃ

የጥናቱ መንደርደርያ

የስጋ ደዌ ማይኮባክቴሪየም ሌፕራ በሚባል ተህዋስ የሚመጣ ተላላፊ በሽታ ነው ። በሽታው በየአመቱ 200000 ሰዎችን ያጠቃል ። በሰውነታችን ውስጥ ያለ እንደ ‹ LL37 › አንዳንድ ንጥረ ነገሮችን ከስጋ ደዌ ጋር ያላቸውን ተዛምዶና ሚና ማጥናት የስጋ ደዌን በሽታ የበለጠ ለመረዳት፣ የምርመራ ዘዴዎችንና መድሀኒቶችን ለማሻሻል ይረዳል ።

የጥናቱ አላማ

‹LL37› **የተባለውን ንጥረነገር ከስጋ ደዌ በሽታጋር ያለውን ተዛምዶና ሚና ማጥናት**

የጥናቱ ጊዜ

ጥናቱ የሚካሄደው ከጥር 2009-መስከረም2011ይሆናል ።

ናሙና አወሳሰድ

20 **ሚ.ሊ. (ሁለት የሻይማንኪያ) ደም ልምድ ባለው /ባላት ነርስ የሚወሰድ ይሆናል።ተመልሰዉ መምጣት አ□□በቅባቸዉም።**

የኤችኤይቪ ምርመራ

ይህ ጥናት የኤች አይቪ ምርመራ ውጤት ያስፈልገዋል ። ስለዚህም የጥናቱ ተሳታፊዎች በፍቃደኝነት ላይ የተመሰረተ የኤችኤይቪ ምርመራ እንዲያደርጉ ይጠየቃሉ ። ተሳታፊዎቹ የኤችኤይቪ ምርመራ ማድረግ ካልፈለጉ በጥናቱ ውስጥ አለመሳተፍ ይችላሉ ። ተሳታፊዎቹ የቅድመና የድህረ ምርመራ ምክር ይሰጣቸዋል ውጤቱም በሚስጥር ይያዛል ፤ ውጤታቸውን ማወቅ ከፈለጉ ብቻ ይነገራቸዋል ። የኤችኤይቪ ቫይረስ በደማቸው ውስጥ የሌለባቸው በጥናቱ ውስጥ ይካተታሉ ። የኤችኤይቪ ቫይረስ በደማቸው ውስጥ እንዳለባቸው የተረጋገጠ ከሆነ አስፈላጊው ምክርና በአቅራቢያው ካለው ሆስፒታል ወይም ጤና ጣቢያ ለኤችኤይቪ ህመ·ማን የሚሰጠውን አገልግሎት እንዲያገኙ ይደረጋል ።

ናሙና በመውሰድ የሚከተል ስጋት ወይም ጉዳት

ናሙና በሚወሰዱበት ጊዜ መጠነኛ ጉዳት ሊኖር ይችላል፡፡ምናልባትየከፋ የህመም ስሜትና ቁስለት ካለ በአቅራቢያው ወዳለ ጤና ጣቢያ ወይም ወደ አለርት ሆስፒታል በመምጣት ሀኪም ማየት ያስፈልጋል ፡፡ ለዚህ የሚወጣውም ወጪ ይሸፈናል፡፡

ተሳታፊው ከጥናቱ የሚያገኘው ጥቅም

ተሳታፊዎች ከጥናቱ በቀጥታ የሚያገኙት ጥቅም የለም በጥናቱ የሚሳተፉት በፈቃደኝነት ስለሆነ ምንም ዓይነት ማግባብያ አይሰጣቸውም፡፡ ነገርግን የዚህ ጥናት ውጤት የስጋ ደዌ በሽታን ጉዳት ከማስከተሉ በፊት ቀድሞ ለማወቅ የሚረዱ ዘዴዎችን ለማግኘት የሚደረገውን ጥረት በማገዝ የአገርአቀፍ የቁጥጥር ፕሮግራሙን ና ህብረተሰቡን ይጠቅማል፡፡ ሆኖምለእያንዳንዱ ተሳታፊ 50ብር ለምሳ እና እስከ 300ብር በደረሰኝ የሚወራረድ የትራንሰፖርት ወጪ ይሸፈናል፡፡

የተሳታፊዉ መብት

ተሳታፊዎች በማንኛዉም ሰአት በጥናቱ ውስጥ አለመሳተፍ ይችላሉ ፡፡በዚህም ምክንያት ማግኘት ከሚገባቸዉ ተገቢ ህክምና አይከለከሉም፡፡ የጥናቱንም ውጤትመጠየቅና ማወቅ ይቸላሉ፡፡□

የጥናቱ ሚስጥራዊነት

ሁሉም የተሳታፊው መረጃዎች በሚስጥር ይያዛሉ ፡፡ ከተሳታፊው መረጃዎች ውስጥ ምንም አይነት የግል መረጃዎች የትኛውም ሪፖርት ላይ አይወጡም ፡፡ ሁሉም የምርመራ ውጤቶች ምስጥራዊ መለያ ቁጥር ስለሚሰጣቸው መረጃዎች ምስጢርነታቸው የተጠበቀ ነው ፡፡ የተሳታፊው የግል መረጃዎች በሚቆለፉ ሳጥኖች ስለሚቀመጡ መረጃዎቹን በቀጥታ የሚያገኙት ሃኪሙና የአህሪ ዳታ ማኔጀሩ ብቻ ናቸው ፡፡

ከጥናቱ ጋር ተያይዞ ማወቅ የሚፈልጉት ጉዳይ ካለ ወይም ጥያቄ ካልዎት ዋና አጥኚዋን ወይም የአርማዎር ሃንሰን ሪሰርች ኢንስቲትዩትን ጸሀፊ በሚከተለው አድራሻ ማነጋገር ይችላሉ ፡፡

ማህሌትአስማን (ዋና አጥኚ)

አርማዎር ሃንሰን ሪሰርች ኢንስቲትዩት

ስልክ ቁጥር *0910539625*

አቶ *ሀይለ*ሚካኤል ጌታቸዉ

አርማሞር ሃንሰን ሪሰርች ኢንስቲትዩት/አለርት ኤቲክስ ኮሚቴ

ስልክ ቁጥር 0118-962183

Annex 7 Consent form English (For Leprosy Patients)

ALERT Hospital No._____

AHRI No._____

AHRI has planned to investigate LL37 peptide's role in leprosy to investigate its potential in leprosy diagnostics and therapeutics. As a leprosy patient, I am asked to participate in this study and I have read the information given about the study / the information given is read to me. I am informed that the study requires 20ml (2 table spoon) blood samples and5mm (like a size of pea) biopsy from skin lesions. up on this procedure I am also informed that there might be a slight pain. In addition, I have agreed to inform my close household contacts about my status and ask their willingness to take part in the study. I am also informed that there is a follow up visit (once) after completing my medication where20ml (2 table spoons) blood samples and5mm (like a size of pea) biopsy from skin lesion will be collected.

I am also informed that I have the right to withdraw from the study at any time and this will have no influence in the medical care I am supposed to get. I am also informed that all data obtained from me will be kept confidential. I have the right to get any information regarding the progress or results from study if I need. I have received clarification of matters that were not clear to me and taking into consideration all points mentioned above, I agree to participate in this study.

Participant's Signature_____

Physician's Name _____ Signature_____

Witnesses' Name_____ Signature_____

Date_____

Annex 8.Consent form Amharic (For Leprosy Patients)

የስምምነት ቅጽ_____

አለርት ሆስፒታል ቁጥር _____

የእህሪ ቁጥር _____

አርማወር ሃንሰን የምርምር ተቋም ‹LL37› የተባለውን ንጥረ ነገር ከስጋ ደዌ ጋረ ያለውን ተዛምዶ ና ሚና ማጥናት ታቅዷል ። ይህም ንጥረ ነገሩ ለስጋ ደዌ በሽታ ምርመራ ወይም ህክምና ሊኖረው የሚችል ጥቅም ካለው የሚጠና ይሆናል ። የስጋ ደዌ ህመምተኛ በመሆኔ በዚህ ጥናት ውስጥ እንድሳተፍ ተጠይቅያለው ። ስለጥናቱ የሚገልፀውን መረጃ አንብቢያለሁ ወይም ተነቦልኝ ተረድቻለው ። ለጥናቱ 20 ሚሊ (ሁለት የሻይማንኪያ) ደምና የበሽታው ምልክት ካለበት ቦታየአተር ፍሬየሚያክል የቆዳ ናሙና የሚወሰድ መሆኑ ተነግሮኛል።ናሙና በሚወሰዱበትም ጊዜ መጠነኛ ጉዳት ሊኖር እንደሚችል ተነግሮኛል። በተጨማሪም የስጋደዌ ህመምተኛ መሆኔን አብሮኝ ለሚኖር ሰው ማሳወቅና መጥቶ በጥናቱ እንዲሳተፍ ለመጠየቅ ተስማምቻለው ። ለጥናቱ ህክምናዬን ከጨረስኩ በኃላም 20 ሚሊ (ሁለት የሻይማንኪያ) ደምና የበሽታው ምልክት ካለበት ቦታየአተር ፍሬየሚያክል የቆዳ ናሙና እንደምሰጥ ተነግሮኛል ።

በዚህ ጥናት የመሳተፍ ወይም ያለመሳተፍ መብት እንዳለኝም አውቂያለው ። በጥናት ውስጥ መሳተፍ ጀምሬ ማቀረጥ ብፈልግም ላገኝ የሚገባኝ የህክምና አገልግሎት እንደማይጓደልብኝ አውቅያለው ። እኔን የተመለከቱ መረጃዎች በሚስጥር እንደሚያዙ አውቅያለው ። ስለ ጥናቱ ውጤት መረጃ ማግኘት ብፈልግ ማግኘት እንደምችል ተነግሮኛል ።

ግልፅ ላልሆነልኝ ጉዳይ በሙሉ ማብራሪያ ተሰጥቶኛል ። ከላይ በተጠቀሰውም መሰረት በጥናት ውስጥ ለመሳተፍ ተስማምቻለው ።

የተሳታፊው ፊርማ _____

የሃኪሙ ስም_____
ፊርማ_____
የአማኙ ስም _____
ፊርማ_____

ቀን _____

Annex 9 Consent form English (household contacts)

ALERT Hospital No._____

AHRI No._____

AHRI has planned to investigate LL37 peptide's role in leprosy to understand the peptide more and identify its potential in diagnostics and therapeutics. As a close household contact of untreated leprosy patient, I am asked to participate in this study and I have read the information given about the study / the information given is read to me. I am informed that the study requires 20ml (2 table spoon) blood samples.

I am also informed that I have the right to withdraw from the study at any time and this will have no influence in the medical care I am supposed to get. I am also informed that all data obtained from me will be kept confidential. I have the right to get any information regarding the progress or results from study if I need. I have received clarification of matters that were not clear to me and taking into consideration all points mentioned above, I agree to participate in this study.

Participant's Name _____ Signature_____

Physician's Name _____ Signature_____

Witnesses' Name___ _____ Signature_____

Date ----------

Annex 10 Consent form Amharic (Household contact)

የስምምነት ቅጽ _____

አለርት ሆስፒታል ቁጥር _____

የእህሪ ቁጥር_____

አርማወር ሃንሰን የምርምር ተቋም ‹LL37› የተባለውን ንጥረ ነገር ከስጋ ደዌ ጋረ ያለውን ተዛምዶ ና ሚና ማጥናት ታቅዷል ። ይህም ንጥረ ነገሩ ለስጋ ደዌ በሽታ ምርመራ ወይም ህክምና ሊኖረው የሚችል ጥቅም ካለው የሚጠና ይሆናል።ከስጋ ደዌ ህመምተኛ ጋር አብሬ የምኖር በመሆኔ በዚህ ጥናት ውስጥ እንድሳተፍ ተጠይቅያለው ። ስለ ጥናቱ የሚገልፀውን መረጃ አንብቢያለው ወይም ተነቦልኝ ተረድቻለው ። ለጥናቱ 20 ሚሊ (ሁለት የሻይማንኪያ) ደም የሚወሰድ መሆኑ ተነግሮኛል ።

በዚህ ጥናት የመሳተፍ ወይም ያለመሳተፍ መብት እንዳለኝም አውቂያለው ። በጥናት ውስጥ መሳተፍ ጀምሬ ማቀረጥ ብፈልግም ላገኝ የሚገባኝ የህክምና አገልግሎት እንደማይጓደልብኝ አውቅያለው ። እኔን የተመለከቱ መረጃዎች በሚስጥር እንደሚያዙ አውቅያለው። ስለ ጥናቱ ውጤት መረጃ ማግኘት ብፈልግ ማግኘት እንደምችል ተነግሮኛል ።

ግልፅ ላልሆነልኝ ጉዳይ በሙሉ ማብራሪያ ተሰጥቶኛል ። ከላይ በተጠቀሰውም መሰረት በጥናት ውስጥ ለመሳተፍ ተስማምቻለው ።

የተሳታፊው ፊርማ _____

የሃኪሙ ስም_____

ፊርማ_____

የአማኙ ስም_____

ፊርማ_____

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Annex 11. Consent form English (Healthy control)

ALERT Hospital No._____

AHRI No._____

AHRI has planned to investigate LL37 peptide's role in leprosy to understand the peptide more and identify its potential in diagnostics and therapeutics. As apparently healthy individual, I am asked to participate in this study and I have read the information given about the study / the information given is read to me. I am informed that the study requires 20ml (2 table spoon) blood samples.

I am also informed that I have the right to withdraw from the study at any time and this will have no influence in the medical care I am supposed to get. I am also informed that all data obtained from me will be kept confidential. I have the right to get any information regarding the progress or results from study if I need. I have received clarification of matters that were not clear to me and taking into consideration all points mentioned above, I agree to participate in this study.

Participant's Signature_____

Physician's Name _____ Signature_____

Witnesses' Name_____ Signature_____

Date ----------

Annex 12 Consent form Amharic (Healthy control)

የስምምነት ቅጽ

አለርት ሆስፒታል ቁጥር _____

የእህሪ ቁጥር _____

አርማወር ሃንሰን የምርምር ተቋም ‹LL37› የተባለውን ንጥረ ነገር ከስጋ ደዌ ጋረ ያለውን ተዛምዶ ና ሚና ማጥናት ታቅዷል ። ይህም ንጥረ ነገሩ ለስጋ ደዌ በሽታ ምርመራ ወይም ህክምና ሊኖረው የሚችል ጥቅም ካለው የሚጠና ይሆናል ። ጤነኛ በመሆኔ በዚህ ጥናት ውስጥ እንድሳተፍ ተጠይቅያለው ። ስለጥናቱ የሚገልፀውን መረጃ አንብቢያለሁ ወይም ተነቦልኝ ተረድቻለሁ። ለጥናቱ 20 ሚሊ (ሁለት የሻይማንኪያ) ደም የሚወሰድ መሆኑ ተነግሮኛል ።

በዚህ ጥናት የመሳተፍ ወይም ያለመሳተፍ መብት እንዳለኝም አውቂያለው ። በጥናት ውስጥ መሳተፍ ጀምሬ ማቀረጥ ብፈልግም ላገኝ የሚገባኝ የህክምና አገልግሎት እንደማይጓደልብኝ አውቅያለው ። እኔን የተመለከቱ መረጃዎች በሚስጥር እንደሚያዙ አውቅያለው ። ስለ ጥናቱ ውጤት መረጃ ማግኘት ብፈልግ ማግኘት እንደምችል ተነግሮኛል ።

ግልፅ ላልሆነልኝ ጉዳይ በሙሉ ማብራሪያ ተሰጥቶኛል ። ከላይ በተጠቀሰውም መሰረት በጥናት ውስጥ ለመሳተፍ ተስማምቻለው ።

የተሳታፊው ፊርማ _____

የሃኪሙ ስም _____

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የአማኙ ስም _____

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Annex 13 Data acquisition form for leprosy patients

Leprosy Patients Identification & Clinical Examination Form

Individual Study Code

Individual's Hospital No.

Date Seen _____

Sex 1.M 2.F Age (Y

Address:Region_____Woreda_____Kebele_____ House No._____

Leprosy Classification

Number of lesions 1. Five or less skin lesions 2. Six or more skin lesions

Number of nerves involved

Disability 1. Yes 2. No

If Yes, (Disability grade) 1. Eye Right Left

2. Hand Right Left

3. Foot Right Left

Bacterial Index M logical Index

1. TT 2. BT 3.BB 4.BL 5. LL

Duration of the Symptom

1. 1-3 months 2.3-6 months 3 months over 12 months

Do they have Reaction? 1. Yes 2. No

If Yes which type? Type I Type II

Does the individual have a BCG vaccination scar? 1. Yes 2. No . Not sure

Examining Physician: _____ Signature_____

AHRI No.

Annex 14.House Hold Contact Identification & Clinical Examination Form

Name _____

Individual Study Code

Individual's Hospital No.

Date Seen _____ (First Time visit)

Sex 1.M 2.F Age (Yrs.)

Address: Woreda_____ Kebele_____ House No._____

Relationship with the MB patient

For how long have the contact lived with the MB patient (Index)

1. for 6 months 2. 6- 12 months 3.more than 12 months

Does the individual have a BCG vaccination scar? 1. Yes 2. No 3. Not sure

History of leprosy? 1. Yes No

History of TB? 1. Yes 2. No

Examining Physician:_____ Signature_____

AHRI No.

Annex. 15. Healthy controls Identification & Clinical Examination Form

Name _____

Individual Study Code ☐☐☐☐

Individual's Hospital No. ☐☐☐☐☐☐

Date Seen _____

Sex 1.M ☐ 2.F ☐ 3.Age (Yrs.) ☐☐

Address: Woreda_____ Kebele_____ House No._____

Does the individual have a BCG vaccination scar? 1. Yes ☐ 2. No ☐ 3. Not sure ☐

History of leprosy? 1. Yes ☐ 2.No ☐

History of TB? 1. Yes ☐ 2. No ☐

Examining Physician:_____ Signature_____

AHRI No. ☐☐☐☐☐☐

Annex 16.Standard operating procedures for laboratory tests

Skin biopsy collection

1. The lesion was punched out using a disposable or sterilizable punch of varying sizes.

2. The area was anaesthetized and the skin is stretch in a direction perpendicular to the resting skin lines.

3. The punch was push into the skin by rotatory movements until a “give away” feel is perceived. After this

4. We fix into the proper fixative according to the intended work needed.

Whole blood assay procedure

1. Prepare a 96 well plate and label it (if not available and sample size is low,using cryotubes is possible)

2. Mix the whole blood in a 50ml falcon tube

3. Add 450ml of the whole blood for each well (for better accuracy you can done in duplicate wells

4. Add 50µl of RPMI (as negative control) PHA (positive control) and WCS (Antigen) for each well respectively

5. Incubate at 37 o C 5% CO2 for 24 hours

6. Collect the supernatant carefully and kept at -20 Oc if not processed on the same day.

Hematoxyline & Eosin staining

This procedure was performed with the slides in glass staining racks and the solutions in square glass staining jars; we seal the jars with parafilm while not in use; we use fresh solutions which prepared in time.

We dip the glass racks with slides (from cryosection or -80 0C) serially in the following jars:

1.95% EtOH, for 2 minutes

2. 70% EtOH, for 2 minutes

3. Running water, for 5 minutes

4. Gill's Haematoxylin, for 3 minutes

5. Running water, for 5 minutes

6. Scott's solution, for 3 min

7. Running water, for 5 minutes

8. Eosin 1x, for 1.5 minute

9. 70% EtOH, for 30 seconds

10 .95% EtOH, for 30 seconds

11.100% EtOH, for 30 seconds

12. Xylene, for 5 min

13. Lastly we mount with Permount (Fisher) and coverslip to see.

RNA isolation from PAXgen Blood RNA Tubes

PAXgene Blood RNA Kit was used to isolate RNA from blood stored in PAXgene Blood RNA Tubes. PAXgene Blood RNA tubes was brought to safety cabinet and allowed to reach room temperature (18-22oC) for approximately two hours or overnight. After thawing, the tubes were carefully inverted 10 times. Then the PAXgene Blood RNA Tubes was centrifuge for 10 minutes at 5000g using a swing-out rotor. The supernatant removed by decanting and 4ml RNase-free water (Qiagen) was added to the pellet and the tube closed using a fresh secondary Hemogard™ (Qiagen) closure followed by vortex until the pellet completely dissolve and centrifuged for 10 minutes at 5000g. The supernatant discarded and 350µl Buffer BR1 (Qiagen) was added. Then the sample was pipette into a 1.5 ml RNase-free microcentrifuge tube. Followed by addition of 300µl BufferBR2 and 40µl proteinase K (Qiagen) and mixed by vortex for 5 seconds and then we incubate at 55oC for 10 minutes on a shaker set at 400rpm. The lysate was directly pipette into a PAXgene Shredder spin column (Qiagen) which was placed in a 2ml processing tube. The

lysate centrifuged for 3 minutes at maximum speed (20,000g). The entire supernatant of the flow-through was carefully transferred to a fresh 1.5ml microcentrifuge tube without disturbing the pellet in the processing tube. Then 350µl ethanol was added and centrifuge briefly (1-2 seconds at 1000g) to remove drops from the inside of the tube lid. Then, 700µl of sample (resuspended in 350µl ethanol) was pipette into the PAXgene RNA spin column (red) placed in a 2ml processing tube, and centrifuge for 1 minute at 20,000g. The old processing tube containing flow-through was discarded and the spin column place in a new 2mL processing tube. Three hundred and fifty microliters of Buffer BR3 (Qiagen) was pipette into the PAXgene RNA spin column, centrifuged for 1 minute at 20,000g and the old processing tube containing flow-through discarded and the spin column was placed in a new 2mL processing tube. Then 10µl DNase I stock solution was added to 70µl Buffer RDD in a 1.5ml microcentrifuge tube and mixed gently by flicking the tube and centrifuged briefly to collect the residual liquid from the sides of the tube. DNase I incubation mix (80µl) was pipetted directly onto the PAXgene RNA spin column membrane (containing the sample) and placed on the bench-top at 20-300C for 15 minutes. Then 350µl Buffer BR3 pipetted into the PAXgene RNA spin column and centrifuged for 1 minute at 20,000g. The old processing tube containing flow-through was discarded and the spin column placed in a new 2mL processing tube. Five hundred microliter Buffer BR4 added to the PAXgene RNA spin column and centrifuged for 1 minute at 20,000g followed by replacing the old processing tube as described and the procedure was repeated once but this time for 3 minutes. After replacing the old processing tube, without adding any buffer, the spin column was centrifuged for 1 minute at 20,000g. The tube containing the flow-through discarded and followed by addition of 40µl Buffer BR5 directly onto the PAXgene RNA spin column membrane, centrifuged for 1 minute at 20,000g to elute the RNA and this step was repeated once. Then, the elute RNA was incubated for 5 minutes at 650C and followed by immediate chilling. Finally, the RNA yield was determined using a NanoDrop 2000, spectrophotometer.

Procedure for ELISA

Sandwich ELISA was run for detection and quantification of LL37 in the supernatants obtained by stimulating whole blood from patients and controls with M. leprae WCS.

Bring all reagents to room temperature (20 - 25°C) before use.

1. Determine the number of test wells required, put the necessary microwell strips into the supplied frame, and fill out the data collection sheet. Return the unused strips to the storage bag with desiccant, seal and store at 2 - 8°C.

2. Transfer 100 µl in duplicate of standard, samples, or controls into appropriate wells. Do not touch the side or bottom of the wells.

3. Cover the tray and tap the tray to eliminate any air bubbles. Be careful not to splash liquid onto the cover.

4. Incubate the strips or plate for 1 hour at room temperature. 5. Wash the plates 4 times with wash/dilution buffer using a plate washer or as follows*: a. Carefully removes the cover avoid splashing. b. Empty the plate by inverting plate and shaking contents out over the sink, keep inverted and tap dry on a thick layer of tissues.

c. Add 200 µl of wash/dilution buffer to each well, wait 20 seconds, empty the plate as described in 5b.

d. Repeat the washing procedure 5b/5c three times.

e. Empty the plate and gently tap on thick layer of tissues.

6. Add 100 µl of diluted tracer to each well using the same pipetting order as applied in step 2. Do not touch the side or bottom of the wells.

7. Cover the tray and incubate the tray for 1 hour at room temperature.

8. Repeat the wash procedure described in step 5.

9. Add 100 µl of diluted streptavidin-peroxidase to each well, using the same pipetting order as applied in step 2. Do not touch the side or bottom of the wells.

10. Cover the tray and incubate the tray for 1 hour at room temperature.

11. Repeat the wash procedure described in step 5.

12. Add 100 µl of TMB substrate to each well, using the same pipetting order as applied in step 2. Do not touch the side or bottom of the wells.

13. Cover the tray and incubate the tray for 30 minutes at room temperature. It is advised to control the reaction on the plate regularly. In case of strong development the TMB reaction can be stopped sooner. Avoid exposing the micro well strips to direct sunlight. Covering the plate with aluminium foil is recommended.

14. Stop the reaction by adding 100 µl of stop solution with the same sequence and timing as used in step 12. Mix solutions in the wells thoroughly by gently swirling the plate. Gently tap the tray to eliminate any air bubbles trapped in the wells.

15. Read the plate within 30 minutes after addition of stop solution at 450 nm using a plate reader, following the instructions provided by the instrument's manufacturer.

High-Capacity cDNA Reverse Transcription Kits

Catalog Numbers 4368813, 4368814, 4374966, and 4374967

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Product description

The Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit uses the random primer scheme for initiating cDNA synthesis. The kit has been tested extensively and validated against various RNA templates, including G/C-rich and A/U-rich RNA species. An essential requirement for the relative quantitation of cDNA is that the reverse transcriptase reaction generates products in a manner directly dependent on the amount of input RNA template.

Contents Cat. Nos. 4368813 and 4374967 Cat. Nos. 4368814 and 4374966 Storage

10X RT Buffer, 1.0 mL 2 tubes 1 tube–25°C to –15°C

10X RT Random Primers, 1.0 mL 2 tubes 1 tube

25X dNTP Mix (100 mM) 1 tube, 1.0 mL 1 tube, 0.2 mL

MultiScribe™ Reverse Transcriptase, 50 U/µL 1 tube, 1.0 mL 2 tubes, 0.1 mL

RNase Inhibitor, 100 µL [1] 10 tubes 2 tubes

[1] Included in Cat. Nos. 4374966 and 4374967only.

Workflow

Prepare 2X reverse transcription master mix

↓

Add RNA to reverse transcription reactions

↓

Perform reverse transcription in a thermal cycler:

Use the reverse transcription reactions

(cDNA) directly for quantitative or other PCR applications

Store the reverse transcription reactions

(cDNA) at:

• 2°C to 6°C for short-term storage

• −25°C to −15°C for long-term storage

Reverse transcription reaction guidelines

The kit contains reagents that, when combined, form a 2X reverse transcription (RT) master mix. An equal volume of RNA sample should be added. To avoid RNase contamination, RNase-free reagents and consumables must be used.

Prepare the 2X RT master mix

1. Allow the kit components to thaw on ice.

2. Calculate the volume of components needed to prepare the

required number of reactions.

Note: Prepare the RT master mix on ice.

IMPORTANT! Include additional reactions in the calculations to provide excess volume for the loss that occurs during reagent transfers.

3. Place the 2X RT master mix on ice and mix gently.

Settings (Step 1 Step 2 Step 3 Step 4 at Temperature of 25°C 37°C 85°C 4°C and

Time 10 minutes 120 minutes 5 minutes ∞ respectively.)

Important Licensing Information: Corporate entity: Life Technologies Corporation | Carlsbad, CA 92008 USA | Toll Free in USA 1 800 955 6288

Declaration

I, the undersigned, declare that this M.Sc. research thesis is my original work, 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 Mahlet Osman

Signature ----------

Date of submission: ----------

Place Addis Ababa, Ethiopia.

This research thesis has been submitted with our approval as advisors.

Name of advisor: Adane Mihret (DVM,M.Sc,PhD, Researcher)

Signature ----------

Place: Addis Ababa University, School of Medicine Department of Microbiology, Parasitology, and Immunology

Date of submission: ----------

Name of advisor: Kidist Bobosha (M.Sc,PhD, Researcher)

Signature ----------

Place: Armauer Hansen research institute

Date of submission: ----------

Name of advisor: Markos Abebe (M.Sc,PhD, Researcher)

Signature ----------

Place: Armauer Hansen research institute

Date of submission: ----------