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Immuno-hematological profile of individuals with podoconiosis in Yilmana  
Densa Woreda, West Gojjam, Ethiopia

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**This is to certify that the thesis prepared by Aytnew Atnaf, entitled:**

*Immuno-hematological profile of individuals with podoconiosis in Yilmana Densa Woreda, West Gojjam ,Ethiopia* and submitted in partial fulfillment of the requirements for Master of Science degree in Clinical Laboratory Sciences (Hematology and Immunohematology) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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## Abbreviations

AAU	Addis Ababa University
Baso	Basophil
CBC	Complete Blood Count
CD	Cluster Of Differentiation
Ck	Cytokine
CSA	Central Statistics Agency
DMLS	Department of Medical Laboratory Science
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
Eos	Eosinophil
FACS	Fluorescent activated cell sorting/sorter
Gran	Granulocytes
HCT	Hematocrit
HGB	Hemoglobin
IFN	Interferon
IL	Interleukin
IOCC	International Orthodox Christian Charity
Lymp	Lymphocyte
MCH	Mean Cell Hemoglobin
MCHC	Mean Cell Hemoglobin Concentration
MCV	Mean Cell Volume
MF	Microfilaria
MID	Mixed population of cells
Mono	Monocyte

MPV	Mean Platelet Volume
NEUT	Neutrophil
OD	Optical Density
PBS	Phosphate Buffer Solution
PF	Plasmodium falciparum
PLT	Platelet
Podo	Podoconiosis
PV	Plasmodium vivax
QC	Quality Control
RBC	Red blood cells
RDT	Rapid Diagnostic Test
RDW	Red Cell Distribution Width
SOP	Standard Operating Procedure
SPSS	Statistical Package for the Social Sciences
SST	Serum Separator Tube
STH	Soil Transmitted Helminthes
Th	T-helper
TMB	Tetramethyl Benzidene
TNF	Tumor Necrosis Factor
WBC	White Blood Cell
WHO	World Health Organization

## **Abstract**

**Background:** Podoconiosis is non-filarial elephantiasis, is suggested as an inflammatory disease caused by prolonged contact with irritant soil. It is prevalent in tropical Africa, including Ethiopia.. However, little is known about the immuno-hematological changes in this disease.

**Objective:** To assess the immuno-hematological profiles of individuals with podoconiosis (Podo) in Yilmana Densa woreda, West Gojjam.

**Method:** A case-control study was conducted from August 29, 2017-June 15, 2018 on 120 adults (53 Podo cases and 67 controls). Socio-demographic variables and associated factors for podoconiosis were collected. Stool and blood samples were analyzed for parasite identification, hematological parameters and cytokines levels. Data were entered and analyzed using SPSS 20.

**Results:** Of the cases, 64.2% (n=53) and of the controls 67.2% (n=67) were males. Age, length of years of residence in the kebele and educational status had significant association with podoconiosis ( $P<0.05$ ). Most of the cases had blood type A and B whereas controls had A and O ( $p>0.05$ ). Significantly less number of cases than controls had washed their legs daily (60.4% vs 71.6%,  $p=0.014$ ) and had worn shoes (88.7% versus 98.5%,  $p=0.023$ ). However, less than half (45.3%) of the cases and 82.1% of the controls were wearing shoes during interview ( $p<0.01$ ). Age at first leg swelling (22.6years) was less than at first shoe wearing (27.1 years). Cases had significantly lower mean WBC, GRAN, RBC, HGB, MCH, MCHC and higher mean LYMP and mixed cell population (MID) counts than controls ( $p<0.01$ ). The mean CD4 count and percent, level of cytokines (IL-4, IL-6, IL-10,IL-17, IFN $\gamma$ ) of cases were not significantly different from controls ( $p>0.05$ ). 37.74% of the cases and 32.84 % of the controls ( $p=0.576$ ) were positive for intestinal parasites. Hookworm was the most prevalent intestinal parasite in both groups. The mean HGB level of Hook worm positive podo cases and Hook worm negative podo cases was 11.69g/dl and 12.21g/dl respectively,( $F=0.061,p=0.805$ ).

**Conclusion:** Remarkable increase in MID and lymphocyte counts (%) but decrease in granulocyte counts (%), HGB, MCH and MCHC were seen in Podo cases. Appropriate interventions are needed to prevent multiple burdens in Podo patients and intestinal parasites in controls.

**Key words:** *Podoconiosis, CBC, Cytokines, CD4, STH*

# 1. Introduction

## 1.1. Background:

The term podoconiosis was coined by Ernest Price, derived from two Greek words *podos* and *konos*, which mean foot and dust, respectively. Podoconiosis (endemic non-filarial elephantiasis) is a geochemical, non-infectious and neglected tropical disease. It results in bilateral swelling of the lower legs in individuals with bare foot, as a result of long-term exposure to irritant alkaline red clay soil derived from volcanic rocks (1).

Historically, podoconiosis has been a known disease for more than 1000 years and it was described by Romans as “swollen legs”. In the eighteenth and nineteenth centuries, the pathogenesis of elephantiasis was considered due to only filarial parasites. Towards the end of the 19th century, the distribution of elephantiasis and filaria in continents varied so that researchers were initiated to revise the theory. This variation changed their assumptions on the causative agent of the disease from filarial to bacterial, i.e. streptococci infection but the tests were negative for both agents. This discrepancy alters their thinking on elephantiasis and observed that elephantiasis is associated with walking barefoot in endemic areas. Due to difficulties of knowing the cause of the disease, Cohen in 1960 stated it as “idiopathic lymphedema” in place of the local terms “verruous lymphatica” in Kenya and “mossy foot” in Ethiopia. Finally, Earnest Price described the etiology, pathology and natural history of non-filarial elephantiasis in the 1970's and discovered a term podoconiosis in the 1988 (2).

The pathogenesis of podoconiosis is not yet completely known. Most researchers suggest colloid sized mineral particles in the soil (phyllosilicate minerals, particularly clay (smectite and kaolinite) and mica groups, quartz (crystalline silica), iron oxide, and zirconium) and genetic susceptibility had important role for the generation of the disease (2, 3). These particles have been demonstrated in the lower limb lymph node macrophages of both patients and non-patients living barefoot on the clays. Mineral particles present in red clay soils are absorbed through the skin of the foot, engulfed by macrophages in the lower limb lymphatic and then induce an inflammatory response in the lymphatic vessels, leading to fibrosis and obstruction of the lymphatic vessel lumen leads to lymphedema and finally elephantiasis(2).

The early symptoms of podoconiosis include recurrent episodes of burning and itching of the skin of the forefoot and brushing of the toes (lymph edema of the foot or lower leg), especially after periods of intense physical activity. The early signs include; swelling of the foot, skin changes over the foot like lichenification, increased skin markings, damp skin and mossy foot. The later symptoms include soft or water bag lymphoedema and hard or leathery leg ‘elephantiasis. The effect is a debilitating lymphoedema of the lower leg, with or without skin changes (hyperkeratosis and ‘mossy’ papillomata) and fibrotic nodule formation (4).

Geographically, podoconiosis is found in tropical Africa (affecting about 10 countries), Central and south America and northwest India. High prevalence of podoconiosis was detected in highlands with high altitudes of >1500m above sea level, annual rain fall of > 1500mm and mean annual temperature of 19-20 °c (5). The prevalence is high in Uganda(6), Tanzania (7), Kenya (8), Rwanda, Burundi, Sudan, and Ethiopia (9). Podoconiosis has been reported in Equatorial Guinea (10), Cameroon, Chad, Niger and Nigeria, on the islands of Bioko, Sao Tome as well as Principe and Cape Verde (11). It has also been reported in the Central American highlands in Mexico and Guatemala and South America, Ecuador, and Brazil (12).

In Ethiopia, podoconiosis is endemic in one-fifth of the country's land surface area. The overall prevalence of podoconiosis in Ethiopia was estimated to be 3.4% in 2013. Its prevalence varied by region: 4.8% in SNNPR, 4.4% in Harari, 3.0% in Amhara, 2.5% in Oromia, 1.6% in Tigray, 0.6% in Gambella, 0.4% in Benishangul Gumuz and 0.4% in Dire Dawa (5).

### **Prevention and control**

In 2012, the World Health Organization (WHO) published targets for the elimination of neglected tropical diseases or reductions in their impact to levels at which they are no longer considered public-health problems (13, 14). Podoconiosis is unique in being an entirely preventable non-communicable disease. Specific strategies for podoconiosis control may be divided into primary, secondary and tertiary prevention (5, 15). Primary prevention i.e. the prevention of contact between feet and irritant soil that trigger the inflammatory process includes the use of shoes, regular foot hygiene and floor coverings. Secondary and tertiary prevention are based on the management of the lymphoedema-related morbidity and include foot hygiene, foot care, wound care, compression, exercises, elevation of the legs and treatment of acute attacks (16).

## 1.2. Statement of the Problem

WHO has started the progress in the elimination of Non-filarial elephantiasis in 2016. But, the global burden of the disease is not clearly described. However, there were available estimates that suggest 4 million people are affected by podoconiosis worldwide where barefoot is a common practice in 5 to 10% of the population in endemic areas. Environmental factors and other climatic factors that affect the generation of red clay soil can help to predict the occurrence of podoconiosis and vulnerability of individuals to the disease is also aggravated by poverty and insufficient access to water for foot hygiene (17, 18).

Studies have shown that the prevalence of podoconiosis is 7.8% in Africa, particularly in Ethiopia where 34.9 million people (43.8%) lived in areas of environmentally suitable for podoconiosis occurrence and 19.2 million people are expected to be at risk of this disease (19, 20). Studies also suggested that 500,000 to 1 million people are affected by podoconiosis. For instance, in Wolayita zone, 18% of the population are at risk through exposure to the irritant soil and majority of affected individuals with podoconiosis were found in productive age group (16-45 years), the economic sector of the population (21).

The economic burden of podoconiosis was estimated in endemic areas in Ethiopia and it indicated that the total productivity loss for a patient per year amounted to 45% of total working days which corresponds to US\$ 63, and the direct cost per patient per year was US\$ 143, giving a total loss of 206 US\$ per patient per year. In a zone of 81,000 podoconiosis affected patients, the total overall annual cost of the disease was estimated to be exceeded US\$ 16 million per year (22).

The heritability of podoconiosis was estimated to be more than half percent (63%) as a result siblings are expected to have the disease five times more likely than other individuals. The antigens responsible for the risk of heritability of the disease are Human Leukocyte antigens (HLA-DRB-1, HLA-DQA1 & HLA-DQB1) and the gene was identified as an autosomal co-dominant major gene (23).

Social stigma against people with podoconiosis is most common activity, patients being excluded from school, discriminated from participation in local meetings, churches and mosques, and barred from marriage with unaffected individuals (24). Podoconiosis patients can also be affected by mental distress, females are the most affected (25). Histological studies revealed that Podoconiosis had distinctive changes on the pathogenesis of chronic lymphedema with extensive

sclerosis, loss of elastic fibers, verrucous acanthosis (not human papilloma virus induced) and reactive changes of eccrine structures, mast cells, macrophages and altered blood vessels. The domination of lymphocytes, particularly T cells (70%), increased mast cells and activated factor XIII(factor-XIIIa) fibrocytes were demonstrated by immunohistochemistry of irreversible nodular podoconiosis (26).

Unavailability of point of care devices for diagnosis of podoconiosis and the inaccurate diagnosis and staging of the disease by community health workers, especially in early stages were the major problems in its diagnosis (1, 27).

However, there are no global and local published data found on the immunologic and hematology profiles on podoconiosis patients.

### **1.3. Significance of the study**

This study will provide basic information to Physicians on immunologic and hematological changes related to podoconiosis. The findings will contribute to the understanding of immunopathogenesis of podoconiosis. In addition, it may help both clinicians and patients to indicate the possible prevention and control mechanisms of comorbidities. It may also help researchers as a baseline data for further investigations.

## **2. Literature Review**

Research on podocniosis, one of the most neglected tropical diseases, is getting attention only recently though its description by Price dated back in 1970's (28, 29). Available researches mainly focus on disease epidemiology, associated social and psychological factors and clinical staging of the disease and its management (1, 2, 18). Few genetic studies were carried out but the immunological and hematological aspect is less investigated. The literature review here covers podocniosis epidemiology and associated factors, available information on disease pathogenesis, diagnosis, staging and related articles on chronic inflammatory conditions, as this is one of the suggested immunological mechanisms in the pathogenesis of podocniosis.

### **2.1. Podocniosis epidemiology and associated factors**

WHO identified 17 major NTDs on the third report of 2015 and set elimination targets regionally and internationally by the year 2030. These include: buruli ulcer, chagas disease, dengue, dracunculiasis (guinea-worm disease), echinococcosis, endemic treponematoses, foodborne trematodiasis, human african trypanosomiasis (sleeping sickness), leishmaniasis, leprosy, lymphatic filariasis, onchocerciasis (river blindness), rabies, schistosomiasis, soil-transmitted helminthiasis, taeniasis (neuro)cysticercosis and trachoma. However, podocniosis was again neglected in the report even if WHO incorporated it in the list of neglected tropical disease in 2011 (30, 31).

Podocniosis was found in 32 countries, 18 from the African Region, 3 from Asia and 11 from Latin America. However, the global prevalence of the disease is not exactly known but studies showed that it ranged from 0.10% to 8.08%. Its prevalence was highest in the African region, and more affects adults than children and adolescents. The highest reported prevalence had seen in Africa (in Cameroon (8.08%), Ethiopia (7.45%), Uganda (4.52%), Kenya (3.87%) and Tanzania (2.51%)). But recent studies showed decreased prevalence in Cameroon (0.5%), Ethiopia (4.04%), Uganda (0.10%) and Kenya (3.4%) (32, 33).

Podocniosis affects about 4 million people globally. It is prevalent in tropical Africa where irritant soils have been generated by environmental conditions of high altitude and are farm by very poor people who cannot afford shoes or clean water (34).

A survey conducted in Ethiopia in 2013 among 659 Woredas involved 129,959 study participants to assess the epidemiology and risk factors for podoconiosis. About 4% of the study participants had podconiosis and the male to female ratio was 0.7:1. People with podoconiosis had lived with leg swelling for an average of 19.8 (SD  $\pm$  14.2) years, men for 20.9 (SD  $\pm$  14.62) years, and women for 19.1 (SD  $\pm$  13.9) years ( $P < 0.001$ ) and about 23% of them mentioned at least one blood relative had the disease. Nearly half of the patients had stage two podoconiosis. The factors associated with podoconiosis included being female (OR= 1.3 (1.2–1.4)), age (OR = 1.02(1.02–1.03)) , being unmarried (OR = 1.4 (1.3–1.5)). The prevalence of podoconiosis increased steadily with age, and maximum prevalence was seen among individuals  $\geq 65$  years of age. However, factors associated with a decreased risk included attaining secondary or higher education, increased practice of foot hygiene, being employed and housing with covered floor (35).

A study conducted in 2012, Midakegn district, Central Ethiopia, among 1656 randomly selected individuals from 330 households. The prevalence of podoconiosis was 7.4% (123 / 1656) and almost identical among men and women. The mean age of cases and onset of the disease was 43.4 and 25.8 years (third decade) respectively. Majority of podoconiosis patients (94.3%) had bilateral swelling below the knees and almost one third of them (30.1%) had at least one first degree relative(s) with the disease. They had not consistently worn shoes since childhood, and 60.2% had never worn shoes in their life time (36).

Another study conducted in East and West Gojjam Zone, Northern Ethiopia, in 2012 among 17,553 households with 51,017 study participants aged  $\geq 15$  years indicated that the prevalence of podoconiosis was 3.3%. Almost all cases were in the age group 15-64 years, illiterate and farmers. The male to female ratio was 0.9:1. Over half of the study participants (54.5%) had aggravated pain of leg swelling during hot and dry seasons, whereas 20% was not seasonal. The most aggravating factors were long walk (72.2%), "mitch"(52.1%), laborious work (28.9%), and dust (13.2%). The median age of first use of shoes was 22 years. During interview, the type of shoes worn by study participants were covered hard plastic (33.3%) and open shoe (66.7%). However, gender and use of Protective shoes had statistically significant association. The average frequency of foot washing was everyday and had no significant difference between men and women ( $p=0.12$ ). Most of the cases had unilateral swelling with stage II (92.6%), followed

by bilateral and symmetric swelling with stage II (39.8%). The least clinical stage was bilateral and asymmetric swelling with one leg stage I, other leg stage III (0.2%) (37).

A case control study conducted in East Gojjam zone, Northern Ethiopia in 2013, among 460 podoconiosis individuals and 707 unaffected controls showed level of education was significantly associated with frequency of feet washing ( $t=2.7$ ,  $p=0.008$ ) and in multivariate analysis; sex, marital status, wearing non-protective shoes, not wearing shoes before age 30 were strongly associated with podoconiosis. Among the cases, 54.8% of men and 52.1% of women had a family member affected by podoconiosis (38).

A similar case control study conducted in East and West Gojjam (Debremarkos and Durbetie) in 2015 among 379 cases and 734 controls. The findings related to the determinants of podoconiosis were having family history (AOR, 2.81 [95% CI: 1.7-4.64]), bare foot (AOR, 3.26 [95% CI: 2.03-5.25]) and poor foot hygiene (AOR, 2.68 [95 CI: 1.72 - 4.19]) (39).

## 2.2. Clinical Presentation

The clinical manifestations and course of podoconiosis vary based on time of presentation (early and late) and type of the lymphedema (water bag versus sclerotic or both)(40).



Figure 1: Early and late signs of lymphoedema (40)

(A) Early edema of the foot with splaying of the big toe. (B) Lichenification on the dorsum of the anterior foot. (C) Mossy growth on the lateral part of the foot in slippery distribution

**2.2.1. Early symptoms:** The early symptoms include burning and itching the dorsum of foot and brushing of the toes. The intermittent nocturnal burning sensation of foot is seen following intense physical activity on barefoot in the farm or field, alcohol consumption or start of menses. This pain may progress to the lower leg and occasionally extend to the thigh, producing tender

and fever in femoral lymph node. Repetitive scratching may lead to a breach in barrier function of the skin, which may lead to recurrent cellulites or lymphangitis (4, 41).

**2.2.2. Early signs:** could occur following any causes of lymphedema and early recognition of these signs enables early intervention and prevention of progression of the disease.

The early signs could include foot swelling (Figure 1A), skin changes over foot: lichenification: (Figure 1B) and increased skin markings : mossy foot: (Figure 1C) (4, 40).

### 2.2.3. Later symptoms

Increased leg diameter and establishment of lymphoedema seen after a series of burning episodes related to foot swelling. Conventionally, three main forms of lymphedema are distinguished in podoconiosis: (1) Soft and pitting (“water bag” type) – subdermal edema that is soft to the touch and pits with pressure. It has usually a narrow neck around the knee and wider base on the foot. The skin will have a smooth and dumpy surface with loss of normal hair, occasional lymphorhea especially on the foot which attracts flies (Figure 2A). (2) Hard and sclerotic/fibrotic or leathery leg ‘elephantiasis’: sclerosis governs the changes in the skin and sub cutis, which become woody hard and grossly thickened due to increasing hyperkeratosis, takes on the so-called ‘mossy’ appearance (Figure 2B). (3) Above knee podoconiosis: the vast majority of nonfilarial lymphoedema cases remain below the knee(4, 40)



**Figure 2: Forms of lymphedema in podoconiosis patients (40)**

**(A)** Water bag pitting. **(B)** Fibrotic swelling with nodularity. **(C)** Oozing and maceration on skin folds.

### **2.3. Pathogenesis of Podoconiosis**

The pathogenesis of podoconiosis is not yet clearly understood. Existing evidences point toward mineral particle induced inflammation to be the most accepted cause of podoconiosis in genetically susceptible individuals. The disease is linked to long-term exposure of bare-footed individuals to red clay soil. Interactions between genetic and environmental factors trigger an inflammatory response that leads to lymphoedema and fibrosis (42).

#### **2.3.1. Immunology of the pathogenesis of podoconiosis**

A research reviewed in United States on 'silica, silicosis and autoimmunity' stated that silica exposure activate alveolar macrophages to produce IL-1 $\alpha$  and results in nuclear factor (NF- $\kappa$ B) activation which leads to transcription and translation of pro-IL-1 $\beta$ . The pro-IL-1 $\beta$  and IFN- $\gamma$  are cytokines that cause pulmonary inflammation whereas IL-10 stimulates fibrotic response by limiting the inflammation induced by silicosis (43).

#### **Innate immunity**

The activation profiles of Macrophages can be categorized by two. These are classically activated (M1 macrophages); responsible for pro-inflammatory response and alternatively activated (M2 macrophages); responsible for pro-fibrotic responses. The M1 macrophages produce pro-inflammatory cytokines, such as interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-8, IL-6, IL-12 and TNF- $\alpha$ . In contrast, M2 macrophages are concentrated by the pro-fibrotic cytokines; IL-4 and IL-13. M2 macrophage induces the production of tissue-repairing IL-10. The development of podoconiosis is prolonged even if there is constant exposure to the irritant microparticles. This prolonged delay may be due to sequestration of microparticles by M2 macrophage scavenger receptors. When these abundant receptors are occupied by the microparticles, the free particles become engulfed by inflammatory M1 macrophages and stimulates the macrophages to release reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS), nuclear factor-kappa B (NF- $\kappa$ B) and activator protein-1 (AP-1)(44).

#### **Adaptive Immunity**

The Th1 and Th2 balance is important to modulate inflammatory response in fibrosis. The Th1 cytokines (IFN- $\gamma$  and IL-12) attenuated fibrosis, while the Th2 cytokines (IL-4, IL-5 and IL-13) linked with fibrogenesis (45).

#### **2.3.2. Podoconiosis as a chronic inflammation**

As podoconiosis is thought to be the result of abnormal chronic inflammatory response to one or more mineral particles in the red clay soil, and little is known of the mediators involved in the inflammatory process, studying the different groups of cytokines in podoconiosis patients could give some clue. In this regard, there is a suggestion that transforming growth factor- $\beta$  (TGF- $\beta$ ) may play a role, decreased in early and advanced stage of the disease (46). On the other hand, animal models done in Belgium suggested that inflammatory cells like neutrophils, macrophages and lymphocytes accumulated following pulmonary inflammation induced by silica particles. In addition, the expression of pro-inflammatory cytokine (TNF- $\alpha$ ) was pronounced in rats and anti-inflammatory (IL-10) increased in mice up on analyzed by standard ELISA (47).

Evidences are also available from studies in other diseases with inflammatory responses. For example, a review and meta-analysis done using 14 original articles on the association of chronic obstructive pulmonary disease (COPD) and systemic inflammation revealed that patients with COPD had higher C-reactive protein (CRP), circulating leukocytes and serum level of IL-6 with the standard mean difference was 0.53, 0.44 units and 13.10 ng/ml, respectively (48).

### **2.3.1. Podoconiosis and Hook worm co-infection**

A review done in Nigeria, in 2018 indicated that Hookworm co-infection may help to suppress inflammatory responses of podoconiosis and treatment of one exacerbates the other disease condition. Hook worm infection rates were higher in podoconiosis patients, especially in the early clinical stages. However, advanced stages of podoconiosis had lower Hook worm infection because the podoconiosis was advanced and complicated, preventing the patient working on the farm (reduced soil contact) (44).

A study in Ethiopia hypothesized that patients with podoconiosis might manifest an anemia of chronic disease, and that anemia might be more pronounced in the presence of anemia-causing intestinal helminthes. This study involved 677 podoconiosis patients and 236 controls in 2013, in rural Wolayita Zone to investigate the magnitude of soil transmitted helminthes infections (STH) and anemia. The study indicated that STH infections were higher in podoconiosis (47.6%) patients than controls (33.1%). Among the STH, Hook worm was the most prevalent (40.9% of patients and in 27.5% of controls) followed by *A.lumbricoids* (14.5% in patients and 9.3% in controls). The authors suggested that it may be due to 75% of the patients did not always wear shoes. Podoconiosis patients were more affected by Hookworm and *Trichuris trichiura*

infections than controls Adjusted odds ratio (AOR) = 1.74, AOR= 6.53, respectively. ‘Any STH’ and ‘hookworm’ infections were both associated with lower hemoglobin levels in patients. A significant reduction in hemoglobin level was observed among podoconiosis patients co-infected with hookworm and ‘non-hookworm STH (49).

#### **2.4. Diagnosis and staging**

The diagnosis of podoconiosis depends on signs and symptoms of the disease and exclusion of other causes of lymphedema of infectious and non-infectious agents. Lymphedema due to infectious agents can be seen in lymphatic filariasis, leprosy, mycetoma, Kaposi's Sarcoma, venereal infections and chronic recurrent erysipelas whereas non infectious causes of lymphedema can occur after surgery, radiation exposure, chronic inflammation etc.(50). For instance, to exclude lymphatic filariasis, the swelling usually started above the knee (descending) ,unilaterally and symmetrically but podoconiosis started below the knee (ascending) bilaterally and asymmetrically but groin involvement is rare. Its geographical location is usually in the low land areas where as podoconiosis is a disease of highlands. The detection of microfilaria antigen in blood confirms the lymphedema is due to filarial worm. In contrast to leprosy, lymphoedema, in podoconiosis had sensation in the toes and foot , tropic ulcers and hand involvement are absent (2,18). The nationwide mapping of lymphatic filariasis and podoconiosis done in 2013 in Ethiopia indicated that there are four weredas affected by lymphatic filariasis in Amhara region namely Metema, Quara, Tacharmachioo and West armachioo (51). As a result, there is no need of detecting lymphatic filariasis in our study area.

The staging of podoconiosis was done by Fasil Tekola through modification of Dreyer’s staging system of filarial lymphedema( seven stages to five stages). The staging system of podoconiosis is depending on features associated with duration of the disease, severity and its complications and regression of the disease with treatment. Stage1: swelling reversible overnight; Stage 2: below the knee swelling that is not completely reversible overnight and if present knobs, /bumps are below the ankle only; Stage 3: below the knee swelling that is not completely reversible overnight and if present knobs, /bumps are above the ankle; Stage 4: above knee swelling that is not completely reversible overnight and knobs, /bumps present at any location; Stage 5: Joint fixation; swelling at any place in the foot or leg. These stages indicate the severity of the disease, do not necessarily represent the disease process (52).

## **2.5. Treatment and Prognosis**

The conventional treatment for podoconiosis in poor setting consists of five components. (i) Foot hygiene: daily washing of legs with an antiseptic solution for 10 to 15 minutes and rinse it with clean water. (ii) Application of emollient and massaging the skin of the affected limb. (iii) Elevation of the foot above the hip whilst resting. (iv) Compression therapy with stretchable bandage. (v) Footwear: custom made shoes are advised where possible until the foot is of a size where more generic shoes can fit. If the above listed treatment options failed to regress the nodules, surgical removal ( Nodulectomy) is recommended. Surgical removal of nodules help patients to wear custom shoes and could limit disease progression (40).

The outcome of treatment and disease prognosis is monitored through indicators such as reduction of leg circumference (swelling) and clinical stage of the disease, improvement of skin texture, and rate of wound healing. The reduction in swelling of the foot and disappearance of bad odor were the most managed components of lymphoedema management success(53). A one year cohort study of podoconiosis patients in Southern Ethiopia had shown dramatic improvement in the quality of life of patients (54).

## **2.6. Prevention and Control**

The prevent strategies help to prevent the occurrence of podoconiosis. These can also be used to manage effects of the disease. The prevention mechanisms can be generally grouped into three. The primary prevention refers to prevention of irritant soil contact with barefoot. These can be achieved through regular foot hygiene, shoes and socks wearing and floor covering. Secondary and tertiary prevention are based on the management of the lymphoedema-related morbidity and include foot hygiene, foot care, wound care, compression, exercises, elevation of the legs and treatment of acute attacks (16, 41).

However, the immuno-hematological profile of the disease is still untouched and this investigation may fill the gap.

## 2.7. Conceptual Framework

The factors listed below may favor the progress of podoconiosis and this disease in turn may affect some of the factors such as socio-economic status and had a negative impact on the immune response of the patients, productivity and socializations. It may also predispose to physical disability and infections.

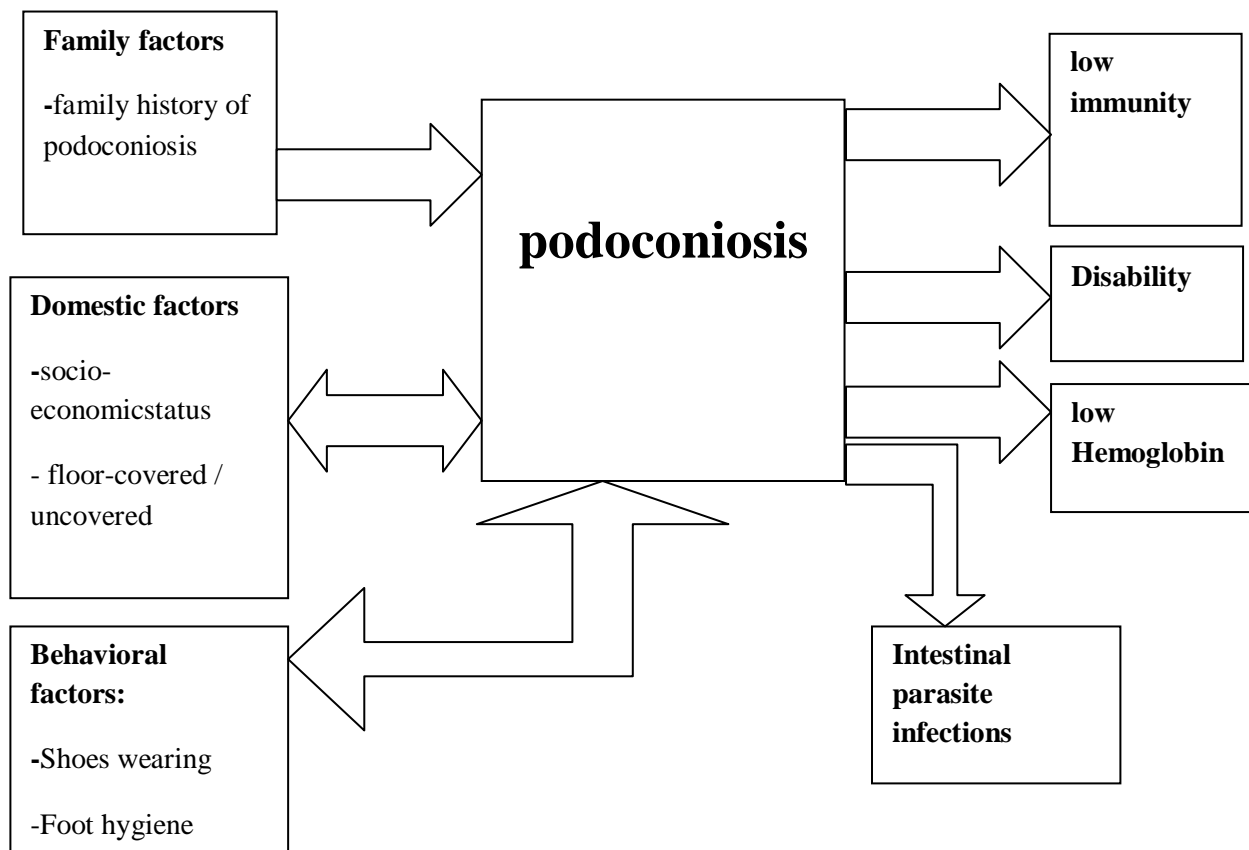


Figure 3: Conceptual framework for podoconiosis risk factors and its impact(summarized from the literatures reviewed )

### **3. Objectives**

#### **3.1. General Objective**

To assess the immuno-hematological profiles of individuals with podoconiosis in Yilmana Densa woreda, West Gojjam

#### **3.2. Specific Objectives**

- To assess complete blood count (CBC) and CD4 parameters between individuals with podoconiosis and apparently healthy subjects
- To compare selected inflammatory cytokine levels between individuals with podoconiosis and controls
- To determine magnitude of intestinal parasite infections among podoconiosis cases and controls

#### **4. Hypothesis**

H0: There is no significant difference in the level of CBC and CD4 between individuals with podoconiosis and controls

H0: There is no significant difference in selected Cytokine levels between individuals with podoconiosis and controls

H0: There is no significant difference in intestinal parasite infections between cases and controls

## **5. Materials and Methods**

### **5.1. Study Area:**

The study was conducted in Yilmana Densa woreda, West Gojjam zone. The capital city of this woreda is Adet which is located 43 km far from Bihar Dar, in the south direction. This town has a latitude and longitude of 11°16'North 37°29'East with an altitude of 2,216 meters above sea level. It is the largest settlement in Yilmana Densa woreda. This woreda had a total population of about 214,852, of whom 107,010 are men and 107,842 women; 19,169(8.92%) are urban inhabitants in the 2007 census and this population is projected to be 247,191 in 2014-2017(55, 56). The Woreda has one primary hospital (Adet Hospital) and 11 health centers. The reason why the study area was selected for this study is due to high prevalence of podoconiosis and cases are supported by International Orthodox Christian charity (IOCC) and its location is near to Bahirdar regional laboratory to perform hematological tests as soon as possible.

### **5.2. Study Design and period:**

A case control study was conducted from August 29,2017-June15,2018.

### **5.3. Population**

#### **5.3.1. Source population:**

All individuals who visited two Health centers and Adet Hospital in Yilmana Densa Woreda during the data collection period

#### **5.3.2. Study population:**

Voluntary individuals with and without podoconiosis, who fulfill the eligibility criteria and available during the data collection period.

### **5.4. Inclusion and Exclusion Criteria**

#### **5.4.1. Inclusion Criteria:**

Patients must:

1. Be 18 years or older;
2. Have been diagnosed with podoconiosis according to locally used criteria (57)
3. Not have received formal lymphoedema treatment prior to the study;

Individuals without podoconiosis must:

1. Be 18 years or older;
2. Not have been diagnosed with podoconiosis according to locally used criteria (57);
3. No family history of podoconiosis.

#### **5.4.2. Exclusion Criteria**

Individuals who lived in the area for less than 10 years were excluded from the study.

### **5.5. Study Variables**

#### **5.5.1. Dependent variables:**

- Levels of CBC and CD4
- levels of inflammatory cytokines

#### **5.5.2. Independent variables:**

- Age
- Sex
- Residence
- Religion
- Educational status
- Occupation
- Marital status
- Monthly income
- Floor covering status
- Frequency of legs washing
- Leg swelling history
- Habits of Shoe wearing
- Intestinal parasite infections
- Blood group
- Malaria status

### **5.6. Measurement and Data collection**

#### **5.6.1. Sample size and sampling method:**

The study consists of detailed immunological tests of different groups (pro-inflammatory and regulatory cytokines), and hematological tests; CBC & CD4. There were six different cytokines (IL-4,IL-6,IL-17,IFN $\gamma$ ,TNF $\alpha$  and IL-10) assayed. Thus, a total of 120 participants, 53 cases and 67 controls were conveniently enrolled. The samples were drawn from two health centers and Adet hospital.

Cases were recruited conveniently by the coordinating effort of the woreda health Office head through communicating people at church and kebele meetings. The process was announced

inviting those who are 18 years and above, had leg swelling ("keman anishe") for more than a year including those aided by IOCC. Potential participants as per the criteria announced were invited to avail themselves in either the two health centers namely Goshiye Health center in the morning (4 o'clock) and Debremewi Health center in the afternoon (8 o'clock) to get the service that will be provided. Potential participants were also informed that they could go to the nearest health center. Then the researcher reached to sites and explained the aim, benefits and risks of the study. The researcher got informed consent by the participants and continued to get information and samples from 53 cases. The information was filled on questionnaire, blood and stool samples were collected from them.

Sixty seven controls were selected from Adet Hospital which is located in between the above listed two health centers. Controls were apparently healthy people with 18 years and above, lived in the same areas of the cases for at least 10 years and had not been diagnosed with podoconiosis along with no family history of this disease. These study participants were voluntarily selected from the hospital coming to visit mothers in the delivery rooms and other purposes. The researcher got informed consent from them and filled questionnaires and collected samples from them for a month (August 29-September 28, 2017).

A convenient sampling method was used to select both the study participants and the health facilities.

### **5.6.2. Data collection procedure**

Data were collected from voluntary participants using predesigned structured questionnaire. The researcher explained the aim of the study using information sheet and obtained consents from the study participants. Socio-demographic data and associated risk factors for podoconiosis were collected by trained data collectors through interviewing study participants using translated Amharic questionnaire, filled on questionnaire and kept confidential. The consistency of filled questionnaires was checked every day. Four trained data collectors were involved in the data collection. Stool and blood samples were collected from voluntary study participants to detect parasites in both samples and to analyze hematologic parameters and immunologic markers in blood. The laboratory results were filled on data extraction format.

### **5.6.3. Laboratory analyses**

### **5.6.3.1. Specimen collection, processing and analysis**

#### **Stool collection, processing and examination:**

A stool cup was labeled with the study participant's name, code number and date of collection. Then labeled stool cup with screw cap was given for voluntary study participants to bring about a tea spoon full feces.

#### **a) Wet mount preparation:**

Wet mount preparation and examination was done in Adet hospital and surrounding health centers (Goshiye and Debremewi). A drop of normal saline was added on a microscopic slide and about 2mg of stool sample was added on it. The mixture was emulsified by applicator stick and covered with cover slip. The transparency of the preparation was checked and it should allow reading news paper under it. Then microscopic examination was done to assess presence of parasite eggs, larvae, trophozoite, cyst stage or adult worm, using 10X and 40X objectives (58).

#### **b) Formol-ether concentration technique**

The left over samples in the containers were preserved using 10% formalin, immediately after performing direct wet mount examinations. Formol-ether concentration technique was performed from formalin (10%) preserved stool sample to modify the density of parasites to be identified. The preserved stool samples were transported to Department of Medical Laboratory Science (DMLS), AAU for processing and examination(58).

**Principle:** Faeces are emulsified in formol water, the suspension is filtered to remove large faecal particles, ether or ethyl acetate is added, and the mixed suspension is centrifuged. Cysts, oocysts, eggs, and larvae are fixed and sediment and the faecal debris are separated in a layer between the ether and the formol water. Faecal fat is dissolved in the ether.

For procedure details refer to annex-V

Interpretation: the result were interpreted as follows;(58)

Ova /Larva/Cyst of .....seen or No O/p seen for negatives.

The species of parasite identified was recorded.

#### **Blood collection, processing and analysis**

About 10ml of venous blood was collected from voluntary study participants by vacutainer method, using ethylene diamine tetraacetic acid (EDTA) test tube and serum separator tube(SST)

tube. EDTA anti-coagulated blood was used to perform CBC, CD4 count, malaria screening (microscopy and RDTs), and blood group determination. Left over samples (EDTA anti-coagulated blood and blood in SST) were centrifuged (1000RPM for 10 minutes) to separate plasma and serum respectively, and stored at  $<-20^{\circ}$  c in Bahirdar Regional Laboratory up to the end of data collection date. Then it was transported to National Blood Bank Service, Addis Ababa for storage until tested for selected cytokine levels (IL-6, IL-10, IL-4, TNF- $\alpha$  ,IL-17 &IFN- $\gamma$ ).

From EDTA blood, two thin and one thick smear per individuals were prepared and labeled with code number. The one thin and one thick smear were used for malaria examinations, while the remaining was used for manual differential WBCs count. Thin and thick blood films preparation and staining was done in Adet hospital and surrounding health centers where as its examination was done in DMLS, AAU.

Onsite tests which include; blood smear preparation, fixation of thin smears for Giemsa staining, and RDT screening were done in Adet hospital and health centers.

EDTA blood samples were transported at room temperature to Bahirdar Regional Laboratory within 8 hours of collection. In this Laboratory, CBC and CD4 testing and blood group determination were done. In addition, dried fixed and unfixed smears were put in slide box and transported to Bahirdar Regional Laboratory to stain slides using Wright stain and Giemsa stain separately.

### **Malaria Screening**

**a) Giemsa staining of Blood films:** After drying of the smear, the thin smear will be fixed with methanol. Both the thin and thick smears were stained by Geimsa stain.

**Principle:** Giemsa solution is composed of eosin and methylene blue (azure). The eosin component stains the parasite nucleus red, while the methylene blue component stains the cytoplasm blue. The thin film is fixed with methanol. De-haemoglobinization of the thick film and staining take place at the same time.

Quality control: known malaria positive and negative smear slides were used to reassure the quality of Giemsa stain.

**For procedure please refer to annex-X**

Dried stained smears were stored in the slide box and transported to AAU, DMLS, Hematology laboratory for malaria detection and identification.

## **b) Rapid Diagnostic Kits**

### **Principle of the test**

RDT (Malaria P.f/Pan Antigen test, Humasis, Republic of Korea) for malaria is based on an immunochromatographic technique. As the test sample flows through the membrane assembly, after addition of the clearing buffer, the colored colloidal gold conjugates monoclonal anti-*P.falciparum* (HRP II specific) and monoclonal anti-pan (pLDH specific) complexes the HRP II/pLDH in the lysed sample. This complex moves further on the membrane to the region where it is immobilized by the monoclonal anti HRP II and monoclonal pLDH specific antibody coated on the membrane leading to formation of pink purple colored band, which confirms a positive result. Absence of colored band in the test region indicates a negative test result (59).

Quality control: The quality control procedure was done using known positive samples and negative samples.

**Test procedure:** refer to annex-XI

### **Interpretation of Results:**

The result is negative if a colored band visible only in the control region whereas it is positive if color bands are visible in the control region, and P.f region/ Pan region or in the three regions.

If there is no color in the control region, the result is invalid.

### **Manual differential count**

The rest well made thin smears were stained by Wright stain for manual differential white cell count and blood cell morphology (Refer to Annex-VIII).

### **Principle**

A stained smear is examined in order to determine the percentage of each type of leukocyte present and assess the erythrocyte and platelet morphology. Increases in any of the normal leukocyte types or the presence of immature leukocytes or erythrocytes in peripheral blood are important diagnostically in a wide variety of inflammatory disorders and leukemia. Erythrocyte abnormalities are clinically important in various anemias. Platelet size irregularities are suggestive of particular thrombocyte disorders. For procedure details refer to annex-VIII.

The stained and dried slides were stored in a labeled slide boxes and transported with malaria blood films to DMLS, AAU for examination.

### **Automated analysis of CBC**

The remaining anti-coagulated whole blood samples were transported at room temperature to Bahirdar Regional Laboratory, the current Amhara Public Health Institute (APHI) and analyzed for complete blood cell count within eight hours of collection using hematological analyzer (cell Dyne 1800 (Abbott diagnostics, USA)) after running the three levels of controls (Low, Normal, High). Cell dyne 1800 is CBC automation which is operated through electrical impedance principle (Coulter principle).

**Principle:** When a cell passes through an aperture, there is a change in resistance of the circuit and results in a change in voltage which is converted to an impulse. The number of impulses counted through the system is proportional to the number of cells passing through the aperture. The amplitude of the impulse is corresponding to the size of the cell. The impulse is analyzed through a computer system which can display the figure. Cell dyne 1800 has two counting chambers; one chamber for RBC and platelet counting (based on their size) and the other chamber for WBC counting and hemoglobin determination.

Daily maintenance procedures like; cleaning, priming and checking reagents for sufficiency were done according to the SOP (Annex-VII).

**Quality control:** prior to running the specimen, background count and controls (low, normal and high) were performed. When the background count and the controls were in the acceptable range, then the samples were run.

**Interpretation:** based on the reference range for age range and sex, the result of CBC was interpreted.

## **FACS count for absolute CD4 and % CD4**

BD FACS Count TM CD4 (Becton Dickinson company, USA) is used for enumeration of absolute counts and percentages of CD4 + T lymphocytes in unlysed whole blood.

Principle: When whole blood is added to the reagent tube, fluorochrome-labeled antibodies in the reagents bind specifically to white blood cell surface antigens, and a fluorescent nuclear dye binds to the nucleated blood cells. After a fixative solution is added, the sample is run on the instrument. During sample acquisition, the cells pass through the laser light, which causes the labeled cells to fluoresce. This fluorescent light provides the information necessary for the instrument to identify and count the lymphocytes and CD4+ T lymphocytes. In addition, the reagent tubes contain a known number of fluorescent reference beads to which a precise volume of whole blood is added. The software automatically identifies the lymphocyte populations of interest and calculates the CD4 counts (cells/ $\mu$ L) by comparing cellular events to bead events (60). Interpretation was done based on CD4 counts and CD4 percentages reference ranges (annex-IX)

## **Blood group Determination**

Blood group was determined using commercially prepared antisera (anti-A, anti-B and anti-D) and EDTA anti-coagulated blood. The slide method was applied to identify ABO and Rh blood group.

Principle: It is based on agglutination reaction; the blood group antigen present on the surface of red blood cells is agglutinated by the antiserum (supplied by the manufacturer) specific for the antigen.

A drop of blood was added on 3 areas of a slide (2 opposite ends and in the middle) and a drop of anti-A in the first drop of blood, anti-B in the second (middle) and anti-D in the last drop of blood was added. Then the contents were mixed with applicator stick. Agglutination reactions was observed and recorded within 2 minutes.

### **Quality control:**

The performance quality of the reagent was checked by using known cells.

### **Interpretation:**

The interpretation was based on the presence and absence of agglutination.

## **Cytokine measurement**

Cytokine levels were measured by quantitative sandwich ELISA assay and the absorbance of each sample was extrapolated to the corresponding concentration using online ELISA absorbance to concentration converter: <https://elisaanalysis.com/app>.

**Principle:** A monoclonal antibody specific for IL-10 has been pre-coated onto a microplate. Standards and samples are added into the wells and any IL-10 present is bound by the immobilized antibody. Following incubation unbound samples are removed during a wash step, and then a detection antibody specific for IL-10 is added to the wells and binds to the combination of capture antibody-IL-10 in sample. Following a wash to remove any unbound combination, and enzyme conjugate is added to the wells. Following incubation and wash steps, a substrate is added. A colored product TMB is formed in proportion to the amount of IL-10 present in the sample. The reaction is terminated by addition of acid and absorbance is measured. A standard curve is prepared from seven IL-10 standard dilutions and IL-10 sample concentration determined (similar for the rest cytokines) (61).

**Pre-analytical Quality assurance:**

The specimen containers were correctly labeled and double checked with the code number filled on the questionnaire. The blood was drawn using vacutainer method to minimize hemolysis. The transportation and storage temperature was maintained according to the SOPs. Daily maintenance and cleaning of ELISA washer was done.

**Quality control:**

A standard curve was incorporated into a sandwich ELISA assay by making serial dilutions of a standard cytokine protein solution of known concentration. Standard curves (also known as "calibration curves") were generally plotted as the standard cytokine protein concentration (typically nanogram or picogram of cytokine/ml) versus the corresponding OD value of samples.

**Post-analytical quality assurance:**

The result was printed out and translated to the corresponding concentration reading. Daily maintenance and shutdown procedures for equipments were performed.

### 5.6.4. The general work flow

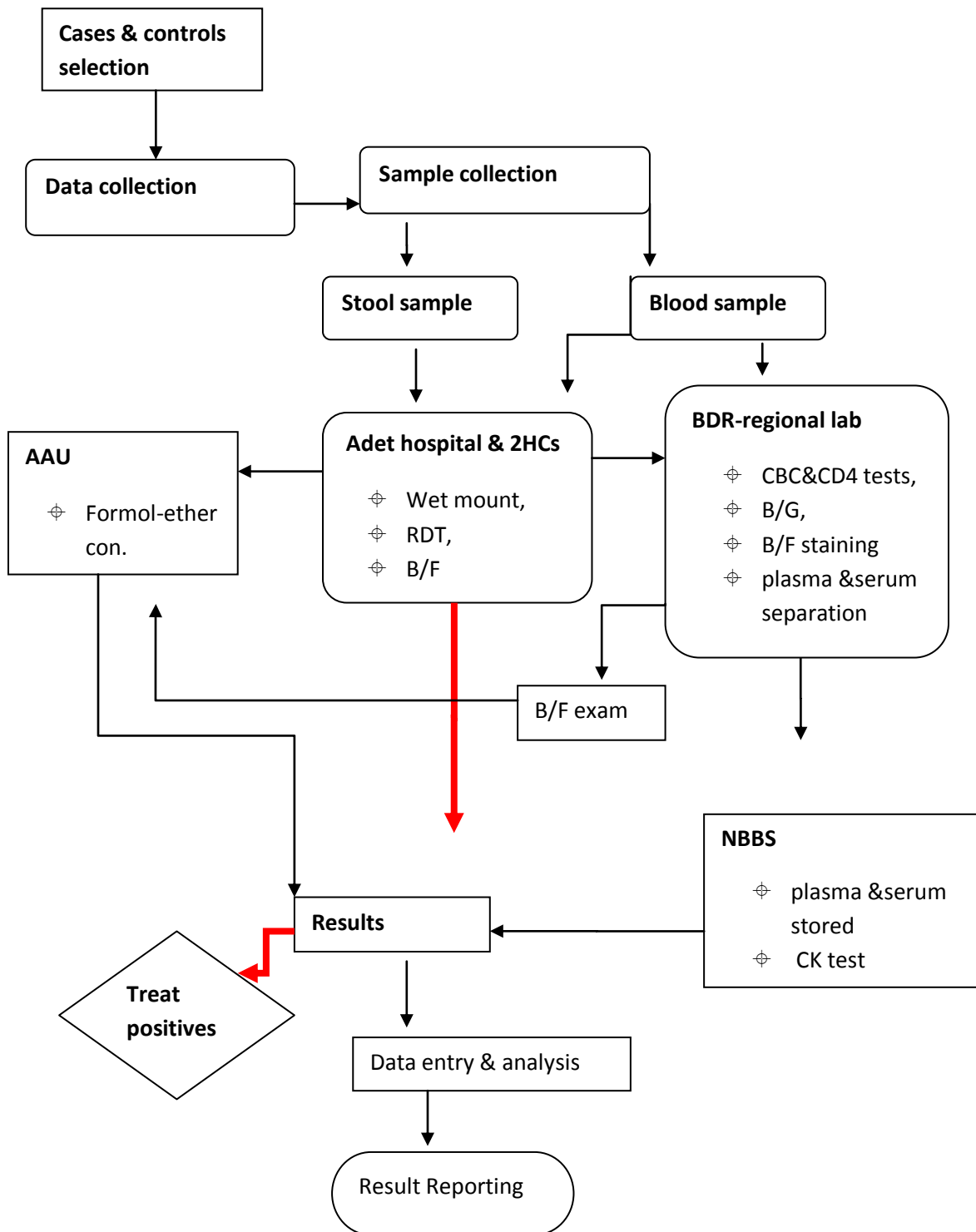


Figure 4: Flow chart for work flow

## **5.7. Data Quality Assurance**

### **Data collection tools:**

Pre-testing of structured translated questionnaire was done and questionnaire was redesigned based on the feedback obtained. Training was given for data collectors before the actual data collection begins. The consistency of data were evaluated and modified based on the feedbacks from study participants and checked by the researcher daily. Data were double entered, checked and authenticated to maintain its quality. The pre-analytical, analytical and post-analytical steps for data quality assurance were strictly followed.

### **Pre-analytical quality assurance**

The study participants were identified by their names and unique code number. Then specimens were collected aseptically, labeled with legible hand writing, transported and stored at the right temperature and time following SOPs.

### **Analytical quality assurance**

SOPs and manufacturer leaflets were strictly followed and daily quality control was run and checked for each test. Laboratory tests were performed at the right time and temperature, by the right reagent and calibrated instrument.

### **Post-analytical quality assurance:**

The laboratory results were interpreted based on reference range specified on the SOPs or manufacturer instruction. Data were documented, entered to statistical software (SPSS). and analyzed. The results of analyzed data were extracted to thesis writing and dissemination of findings will be done accordingly.

## **5.8. Data Analyses and Interpretation**

Data were entered, cleaned and analyzed using SPSS statistics version 20 software. The data were stratified according to the immunology-haematology profile of podoconiosis patients and apparently healthy subjects living in the same environment. Central tendency parameters like mean, median and measure of variation like; and standard deviation were calculated to see the distribution of data. Differences in Immunohematological parameters between cases and controls were determined using the Man Whitney U test, Independent sample t test and Univariate analysis. The results were presented using tables and figures. Statistical tests like chi-square, and

t-test were calculated using 95% confidence interval to determine significant associations between variables and  $p < 0.05$  was considered as statistically significant.

### **5.9. Ethical considerations:**

Ethical approval was obtained from Addis Ababa University, Department of Medical Laboratory Sciences, Research Ethical Review Committee and Amhara National Regional State Health Bureau. Appropriate support letters were obtained from responsible administrative bodies. Prior to recruitment, the aim, potential benefits and risks of the study was explained to potential participants of age  $\geq 18$  years and those who were agree to take part in the study signed a consent form. Participation was voluntary and confidentiality was maintained using identification codes. Names of participants were not mentioned on the result and those who had parasite infections were given deworming treatments during data collection.

### **5.10. Dissemination of the result:**

The result of this study was submitted to Department of Medical Laboratory Science and presented on thesis defense. The findings will also be submitted to Amhara health Bureau and presented at national and international conferences and will be sent to publication on peer reviewed journals and disseminated to the concerned bodies.

### **5. 11. Operational Definition**

**Immuno-hematological profile:** is a combination of immunological and hematological investigations including CBC, CD4, Cytokines levels and blood group.

**Case:** Lymphoedema of the lower limb present for more than one year in a resident of an endemic area, for which other causes of lymphoedema have been excluded (52, 62).

**Control:** an individual living in the household closest to the case, and clinically demonstrated not to have pododermatitis (52).

## 6. Results

### 6.1. Socio-demographic Characteristics

A total of 120 study participants (65.8% males and 34.2% females), 53 podocniosis cases and 67 controls were involved in the study. Of the cases 34 (64.2 %) were males whereas of the controls 45 (67.2%) were males giving a male to female ratio 1.8:1 in cases and 2:1 in controls. Majority of the study participants (73.5%) were found in the age group 25-54 years and the least age group was less than 24 years, 4 (3.3%). More than half of the cases (52.83%) were found in the age range 35-54 years, followed by  $\geq 55$  years (35.85%) and the least (11.32%) were found in the age group 20-34 years and age group was associated with podocniosis ( $p=0.03$ ). The mean age of podocniosis cases was  $49.9 \pm 13.9$  (SD) years, older than controls  $41.6 \pm 13.5$  (SD) years ( $p < 0.01$ ) (Table 1).

Most of the study participants (98.5%) were orthodox Christian. All the cases (100%) and 85.1% of the controls were farmers. All cases and 97% of the controls were residing in rural areas and 84.9 % of the cases and 64.2% of the controls were illiterate. Most of the cases (79.25 %) and controls (82.09%) were married. The mean monthly income of the cases was 208.09 birr with SE of 8.8 and controls 268.70 birr, SE=30.4, respectively. There was no statistically significant mean difference between monthly incomes of cases and controls ( $p=0.084$ ) (Table 1).

Of the cases and controls respectively, 32.1% and 34% had blood group A, 11.3% and 7.5% blood type AB, 30.2% and 25% B, 26.4% and 33% O blood group. Age group, length of stay in the kebele and educational status had statistically significant association with podocniosis ( $p=0.03$ ,  $p < 0.01$  and  $p=0.012$ ), respectively. The rest socio-demographic factors like sex, residence, occupational status, marital status, income and blood group were not statistically significant,  $p > 0.05$  (Table 1).

**Table 1: Socio-demographic characteristics of podoconiosis patients and controls in Yilmana Densa Woreda, West Gojjam zone Ethiopia, 2018.**

Variables		Cases		Controls		P-value
		N=53	%	N=67	%	
Sex	Male	34	64.2	45	67.2	<sup>1</sup> p = 0.73
	Female	19	35.8	22	32.8	
Age : Mean(SD)		49.9 (13.9)		41.6 (13.5)		<sup>2</sup> P<0.01
	<24	1	1.9	3	4.5	<sup>1</sup> p =0.03
	25-34	5	9.4	19	28.4	
	35-44	16	30.2	23	34.3	
	45-54	12	22.6	8	11.9	
	55+	19	35.8	14	20.9	
Residence	Rural	53	100	65	97	<sup>1</sup> p =0.205
	Urban	0	0	2	3	
Survival years in kebele(Mean/SD)		49.91 (13.93)		39.18 (15.23)		<sup>2</sup> P<0.01
Religion	Orthodox	53	100	66	98.5	<sup>1</sup> p=0.37
	Muslims	0	0	1	1.5	
Educational Status: Illiterate		45	84.9	43	64.2	<sup>1</sup> p =0.01
Literate		8	15.1	24	35.8	
Occupation	Farmer	53	100	57	85.1	<sup>1</sup> p =0.071
	Merchant	0	0	5	7.5	
	Gov't employee	0	0	1	1.5	
	Student	0	0	2	3.0	
	Unemployed	0	0	2	3.0	
Marital Status	Married	42	79.2	55	82.1	<sup>1</sup> p =0.182
	Single	8	15.1	5	7.5	
	Divorced	0	0.0	4	6.0	
	Widowed	3	5.7	3	4.5	
Monthly income: Mean/SE		208.1 (8.8)		268.70 (30.4)		<sup>2</sup> P=0.1
Blood group	A+/A-	17	32.1	23	34	<sup>1</sup> p=0.76
	AB+/AB-	6	11.3	5	7.5	
	B+/B-	16	30.2	17	25	
	O+/O-	14	26.4	22	33	

SD= Standard deviation, SE= Standard Error

<sup>1</sup>p value was calculated using Chi-square

<sup>2</sup>P value was calculated using student t test

## 6.2. Leg washing and Shoe wearing practice

Majority (66.6%) of the study participants (60.4% of the cases and 71.6% of the controls) had washed their legs daily. Leg washing frequency was statistically associated with podoconiosis ( $p=0.014$ ) (Table 2).

About 88.7% of the cases and 98.5% of the controls had worn shoes. Shoes wearing was statistically associated with podoconiosis ( $p=.023$ ). The mean age at which shoes wearing started was  $27.1\pm 15.85$  years (mean  $\pm$  SD) and  $27.06\pm 14.62$  for cases and controls, respectively. There was no statistically significant difference between the two means ( $p=0.988$ ). During interview, 45.3% of the cases and 82.1% of the controls were wearing shoes. However, 4.2% of the cases and 40% of the controls did not wear protective shoes. Shoes wearing status and protective shoes wearing at interview was strongly associated with podoconiosis ( $p<0.01$ ). About 56% of the study participants (49.1% of cases and 61.2% of the controls) had never worn shoes during soil contact activities. All the cases and 95.5% of the controls did not cover their home floor and floor covering was not statistically associated with podoconiosis,  $p>0.05$  (Table 2).

**Table 2: Shoes wearing and foot care practice of podoconiosis patients and controls in Yilmana Densa woreda, West Gojjam zone, 2018**

Variable	Cases		Controls		p-value
	N	%	N	%	
<b>Frequency of leg washing</b>					
More often than daily	13	24.5	4	6.0	0.014
Daily	32	60.4	48	71.6	
Several times per week	8	15.1	15	22.4	
<b>Shoes wearing status</b>					
Yes	47	88.7	66	98.5	0.023
No	6	11.3	1	1.5	
<b>Shoes wearing during interview</b>					
Yes	24	45.3	55	82.1	<0.01
No	29	54.7	12	17.9	
<b>Types of shoes worn during interview</b>					
Protective	23	95.8	33	60	<0.01
Non-protective	1	4.2	22	40	
<b>Shoes wearing during soil contact</b>					
Yes	27	50.9	26	38.8	0.184
No	26	49.1	41	61.2	
<b>Age of shoes wearing started</b>					
Mean(SD)	27.1(15.85)		27.06(14.62)		0.988
<b>House floor covering</b>					
Yes	0	0	3	4.5	0.119
No	53	100	64	95.5	

### **6.3. Clinical history and physical examination**

The mean age of leg swelling started was 22.6 (11.4=SD) years. The minimum and the maximum years were 5 and 55 years respectively. The swelling was seen below the knee and the average duration of swelling was 27.4 years, the minimum 5years and the maximum 62 years. The cases said the swelling usually aggravated by seasons and activities like spring (7.5%), winter(30.2%), summer (7.5%), waking (24.5%), barefoot (5.7%), fog (5.7%), plough (1.9 %), cold air (3.8%), and groin swelling (1.9%). and 9.4% unknown reasons. They also reported that it was improved in summer (39.6%), taking rest (30.2 %), unknown (7.5%), winter (7.5%), cold air, hot air and leg washing 1.9 % each (Table 3).

More than half (60.4%) of the cases had hard and fibrotic type of leg swelling where as 39.6% had soft and water bag (pitting ) type of swelling . Most of the cases (92.5%) responded that the site of leg swelling started were below the knee and the rest 7.5% above the knee.

There were four clinical stages of podoconiosis identified by trained professionals. Stage III (45.3%) was the most common followed by stage II (39.6%), stage I (9.4%) and stage IV (5.7%). There was no stage V found in this study.

About 68% of the Podoconiosis cases responded that they had no groin swelling related to leg swelling. Majority of the cases (83%) said that they had no symptoms of numbness of their affected toes. Of the cases, 39.6% had family history of leg swelling and the most affected family members were mother (33.3%) and father 38.1% followed by others(19.0%) , siblings (4.8%).and grand family members (4.8%). Of the cases who had family history 12/21(57.1%) were males and 9/21(42.9%) were females.

**Table 3: Leg swelling history and physical examination of podoconiosis patients in Yilmana Densa Woreda , West Gojjam Zone,2018.**

Variables	Frequency (N=53)	%
<b>Average onset of podoconiosis(years); mean( SD)</b>		
	22.6(11.4)	
<b>Average duration of the swelling(years); mean( SD)</b>		
	27.4(12.8)	
<b>Aggravating factors:</b>		
Barefoot	3	5.7
Cold air	2	3.8
fog seas	3	5.7
Groin swelling	1	1.9
Hot air	1	1.9
Plough	1	1.9
Spring	4	7.5
Summer	4	7.5
Unknown	5	9.4
Walking	13	24.5
Winter	16	30.2
<b>Improving factors:</b>		
Cold air	1	1.9
Hot air	1	1.9
Rapture	2	3.8
Rest	16	30.2
Shoe wear	2	3.8
Summer	21	39.6
Unknown	5	9.4
Washing	1	1.9
Winter	4	7.5
<b>Type of swelling:</b>		
Hard fibrotic	21	39.6
Soft and watery	32	60.4
<b>Site of swelling</b>		
Both legs and below the knee	49	92.5
One leg, above the knee	1	1.9

Both legs above the knee		3	5.7
<b>Stage</b>	<b>I</b>	5	9.4
	<b>II</b>	21	39.6
	<b>III</b>	24	45.3
	<b>IV</b>	3	5.7
<b>Groin swelling</b>			
	Yes	17	32.1
	No	36	67.9
<b>Numbness of the toes</b>			
	Yes	9	17.0
	No	44	83.0
<b>Family history</b>			
	Yes	21	39.6
	No	32	60.4
<b>Affected family</b>			
	Mother	7	5.8
	Father	8	6.7
	Grandparents	1	0.8
	Sibling	1	0.8
	Others	4	3.3

## 6.4. Immunohematological parameters

### 6.4.1. CD4 and the Complete Blood Cell Count (CBC)

The mean level of each CBC parameter was determined and comparison was done between cases and controls using independent sample t test.

The mean difference in leukocyte counts and percentage between cases and controls were statistically significant ( $p < 0.01$  for all leukocyte parameters except the manual eosinophil and basophil counts). The mean Lymphocyte percentage was significantly higher (64.16%) in cases as compared to the controls (37.17%) and it was above the normal range (18-54%). The mean MID % was also higher in cases compared to the controls, above the normal range (6.8-26.3%). However, the mean granulocyte percent were too much lower in cases than controls, 2.82% and 50.03%, respectively. The significant reduction in mean MNEUT% was also detected in cases using manual differential count(12.6%),below the normal range (55-75%) (Table 4).

Cases had lower RBC count , HGB(12.06g/dl), MCH (24.58pg) and MCHC(26.45g/dl) levels than normal controls, 14.14g/dl, 27.66pg and 30.03g/dl, respectively. The HGB, MCH, and MCHC of cases were below the normal reference range. The only erythrocyte parameters that had no any statistically significant association with podconiosis was RDW ( $p > 0.05$ ).The mean platelet count of cases and controls was  $248.66 \times 10^9/\text{ul}$  and  $261.48 \times 10^9/\text{ul}$  respectively. But the difference was not statistically significant ( $p = 0.448$ ). The other parameters of platelet like PDW,PCT and MPV also had no significant difference between cases and controls (Table 4).

As shown in Table 4, the mean CD4 level of cases was lower than controls, 673.58 cells / $\mu\text{l}$  and 681.13cells / $\mu\text{l}$  respectively. There was no statistically significant mean difference between cases and controls CD4 count ( $p = 0.86$ ). The mean CD4 % of cases and controls was 34.89% and 37.61% respectively and the difference was not statistically significant ( $p > 0.05$ ).

**Table 4: Mean difference in immuno-hematological parameters among podoconiosis patients as compared to controls.**

Parameter	Patients (N = 53)			Controls(N=67)			Mean Diff	95% CI for the difference	P-value
	Mean	SD	SE	Mean	SD	SE			
<b>Leukocytes</b>									
WBC ( $10^9/L$ )	5.86	2.00	0.27	7.39	2.63	0.32	-1.53	(-2.40, -0.67)	<0.01*
LYMP (#)	3.75	1.38	0.19	2.52	1.24	0.15	1.22	(0.75, 1.70)	<0.01*
LYMPP (%)	64.16	10.92	1.50	37.17	20.57	2.51	26.99	(20.81, 33.19)	<0.01*
MID (#)	2.15	1.62	0.22	1.00	1.08	0.13	1.15	(0.66,1.64)	<0.01*
MIDP (%)	33.02	9.41	1.29	12.67	7.94	0.97	20.35	(17.21,23.45)	<0.01*
GRAN (#)	0.17	0.13	0.02	4.12	2.92	0.36	-3.95	(-4.74,-3.15)	<0.01*
GRANP (%)	2.82	2.21	0.30	50.03	2.21	3.16	-47.21	(-54.27,40.16)	<0.01*
MLYMP (%)	56.09	12.77	1.75	25.82	10.37	1.27	30.27	(26.09,34.46)	<0.01*
MMON(%)	25.55	12.81	1.76	10.04	11.94	1.46	15.51	(11.01,19.99)	<0.01*
MNEUT(%)	12.60	13.16	1.81	56.69	15.62	1.91	-44.09	(-49.39,-38.77)	<0.01*
MEOS(%)	5.36	4.17	0.57	7.07	6.87	0.84	-1.71	(-3.84,0.41)	0.11
MBASO (%)	0.40	0.63	0.09	0.39	0.92	0.11	0.01	(-0.29,0.30)	0.96
MGRAN(%)	18.36	15.28	2.10	64.13	13.64	1.67	-45.77	(-51.01,-40.54)	<0.01*
CD4 (#)	673.58	224.46	30.83	681.13	246.78	30.15	-7.55	(-93.90, 78.80)	0.86
CD4P (%)	34.89	7.54	1.04	37.61	8.56	1.05	-2.72	(-5.68,0.24)	0.07
<b>Erythrocytes</b>									
RBC ( $10^{12}/L$ )	4.85	0.39	0.05	5.12	0.5	0.06	-0.27	(-0.44, -0.10)	<0.01*
HGB* (g/dL)	12.06	0.94	0.01	14.14	1.59	0.19	-2.08	(-2.57, -1.59)	<0.01*
HCT (%)	45.59	3.44	0.47	47.06	4.14	0.51	-1.47	(-2.87,-0.07)	0.04*
MCV (fL)	93.99	3.48	0.48	92.18	4.32	0.53	1.80	(0.54, 3.24)	0.015*
MCH (pg)	24.85	0.97	0.13	27.66	1.86	0.23	-2.81	(-3.37,-2.26)	<0.01*
MCHC (g/L)	26.45	0.38	0.05	30.03	2.03	0.25	-3.58	(-4.14,-3.02)	<0.01*
RDW (%)	13.89	1.03	0.14	13.48	1.33	0.16	0.41	(-0.03, 0.85)	0.07
<b>Thrombocytes</b>									
PLT ( $10^9/L$ )	248.66	97.62	13.41	261.8	91.2	11.14	-13.14	(-47.42, 21.07)	0.45
MPV* (fL)	13.44	13.9	0.05	11.34	2.21	0.20	2.10	(-1.64, 5.18)	0.31
PDW (fL)	16.55	0.93	0.13	16.59	0.98	0.11	-0.04	(-0.49, 0.21)	0.18
PCT (%)	0.56	0.09	0.28	0.31	.09	0.01	0.25	(-0.24, 0.34)	0.425

\* p-value <0.05 taken as statistically significant

The correlation between manual differential count and automation was strong and significant for MLYMP versus LYMP( $r=0.606, P<0.01$ ), MMONO versus MIDP ( $r=0.638, P<0.01$ ), and MGRAN versus GRANP ( $r=0.814, P<0.01$ ) respectively. The correlation was positive for these pairs but MGRAP was negatively correlated with MIDP ( $r= -0.703, p<0.01$ ) and LYMP(  $r= -0.722, p<0.01$ ). The manual neutrophil percent (MNEUT) was strongly and negatively correlated with MIDP( $r=-0.720, P<0.01$ ) and LYMP( $r= -0.698, P<0.01$ ) but positively correlated with GRANP( $r=0.809, P<0.01$ ) (Table 5).

**Table 5: Correlation between manual differential count and 3 part differential of cell dyne 1800**

Automation	Correlation	Manual differential WBC count					
		MLYMP	MMONO	MGRAN	MNEUT	MEOS	MBASO
<b>LYMP</b>	R	0.606	0.514	-0.703	-0.698	-0.094	-0.007
	P-value	<0.01	<0.01	<0.01	<0.01	0.308	0.943
<b>MIDP</b>	R	0.539	0.638	-0.722	-0.720	-0.093	0.083
	P-value	<0.01	<0.01	<0.01	<0.01	0.312	0.367
<b>GRANP</b>	R	-0.665	-0.642	0.814	0.809	0.110	-0.030
	P-value	<0.01	<0.01	<0.01	<0.01	0.233	0.741

P-value < 0.05 taken as statistically significant

#### 6.4.2. Cytokines levels of podocniosis cases and healthy controls

The data generated on cytokines level were not normally distributed. As a result, the non-parametric test Mann Whitney U test was applied. The minimum detectable dose of cytokines assayed was given by ABclonal Technology, USA. The minimum detectable dose of IL-4, TNF- $\alpha$ , IL-17 and IFN gamma was 4pg/ml for each. However, the minimum detectable dose of IL-6 and IL-10 was 0.7pg/ml and 15pg/ml respectively. The mean IL-17 levels of cases (1.25pg/ml) and controls (0.63pg/ml) was under the lower limit of detection whereas IL-4, IL-6, TNF- $\alpha$ , and IL-10 levels were above the limit of detection. But the mean concentration of IL-17 in cases was almost two times higher than the controls. The concentration of IFN gamma of cases was lower than the controls and below the minimum detectable dose. The median concentration was zero pg/ml for IL-4, TNF- $\alpha$ , IL-17, IFN gamma and IL-10 for both cases and controls. However, the median concentration of IL-6 was 1.13pg/ml for cases and 0.911pg/ml for controls. The Mann Whitney U test showed that only the distribution of TNF alpha concentration was statistically significantly different between cases and controls ( $p < 0.05$ ) (Table 6). Therefore, we rejected the null hypothesis at the level of  $\alpha = 0.05$ . The rest five of the six cytokines had the same distributions across cases and controls. As a result, the null hypotheses were accepted at the level of  $\alpha = 0.05$ . The detailed distributions of the data are shown in the box plots (Figure 5).

**Table 6: The mean/Median level of selected cytokines among podocniosis cases and controls, in Yilmana Densa Woreda, West Gojjam 2018.**

Cytokines	Cases(N=53)			Controls (N=67)			p-value
	Mean Rank	Mean	Median	Mean rank	Mean	Median	
<b>IL-4</b>	61.40	9.97	0	59.79	9.10	0	0.715
<b>TNF <math>\alpha</math></b>	53.72	4.43	0	65.87	4.79	0	0.019
<b>IL-6</b>	64.62	2.74	1.13	57.24	2.64	0.911	0.247
<b>IL-17</b>	58.85	1.25	0	61.82	0.63	0	0.416
<b>IL-10</b>	60.75	38.25	0	60.31	39.06	0	0.458
<b>IFN <math>\gamma</math></b>	58.23	3.72	0	62.30	4.79	0	0.825

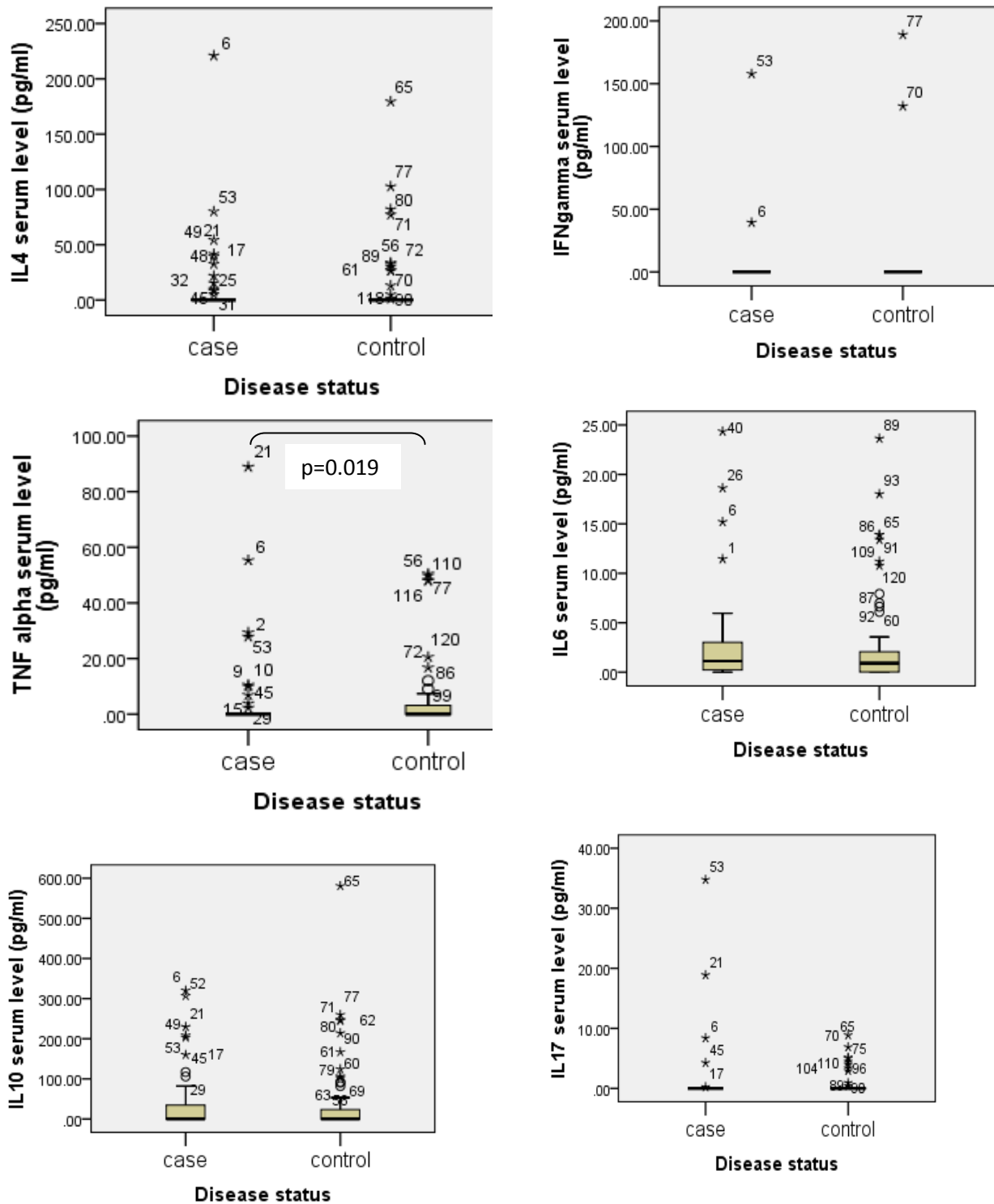
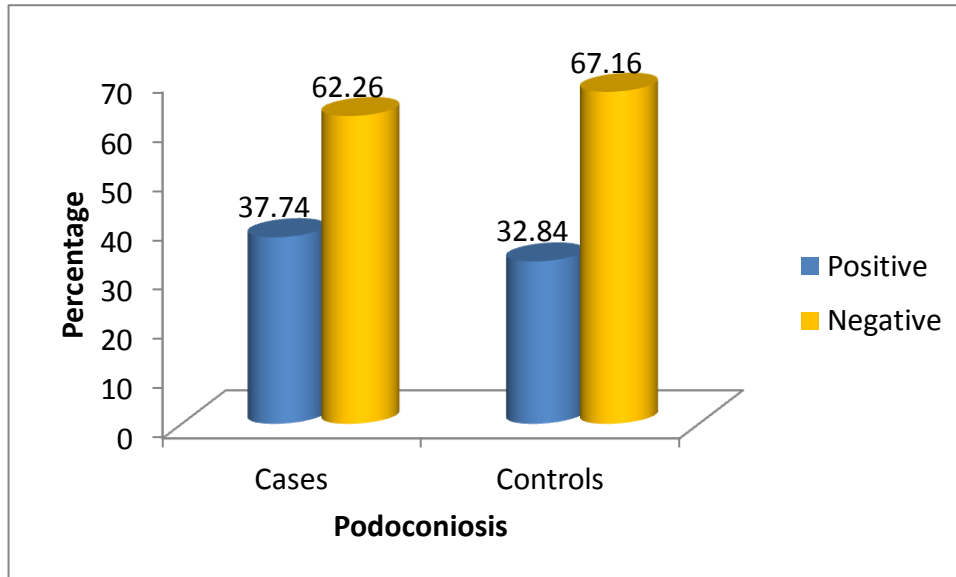


Figure 5: Box plots of IL-4, IFN gamma, TNF alpha, IL-6 , IL-17 and IL-10 levels among cases and controls in Yilmana Densa woreda, West Gojjam, 2018 .

#### 6.4.4. Magnitude of Intestinal parasite and malaria infection

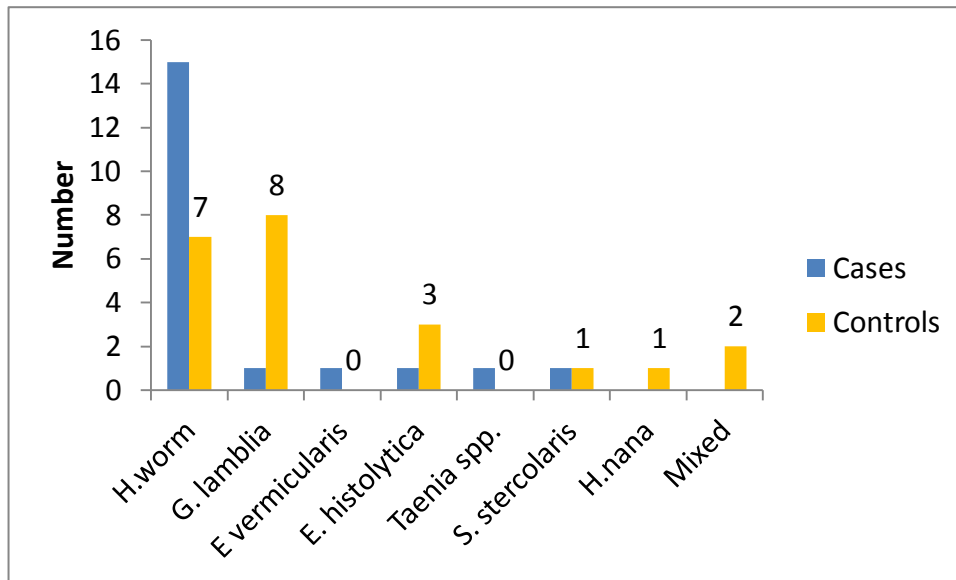
##### 6.4.4.1. Magnitude of Intestinal parasite

From the total study participants, 16.67 % (20/120) were cases and 18.33% (22/120) were controls having intestinal parasitic infection, giving the overall prevalence of 35% (42/120). Of the cases, 37.74% and of the controls 32.84 % had parasitic infection (Figure 6). Intestinal parasite infection had no association with the disease podoconiosis ( $X^2=0.312(1)$ ,  $p=0.576$ ).



**Figure 6: Burden of intestinal parasite among Podoconiosis cases and controls in Yilmana Densa woreda, West Gojjam Zone June 2018.**

Hookworm was the most prevalent intestinal parasite in podoconiosis patients and controls, 15/42(35.7%) and 7/42(16.67%), respectively. The other parasite were *G.lambli*(1,8), *E.histolytica*(1,3), *E.vermicularis*(1,0), *S.stercoralis*(1,1), and *Tanea species* (1,0 ) in cases and *H.nana* (0,2) in the order of cases and controls (Figure 7).



**Figure 7: The species of intestinal parasites identified from faecal samples of cases and controls in Yilmana Densa woreda, West Gojjam, 2018.**

#### **6.4.5. Hemoglobin levels among podoconiosis Cases and Controls by hookworm status**

The univariate analysis of HGB using Podo disease status and Hookworm infection status as factors indicated that there was no statistically significant variation on HGB level between Hookworm infected and uninfected individuals ( $F=1.39$ ,  $p=0.166$ ). However, disease status had statistically significant variation on HGB level ( $F=42.33$ ,  $p<0.0001$ ). The mean HGB level of Podoconiosis-Hookworm patients and podoconiosis-Hook worm negative patients was 11.69g/dl (0.96) and 12.21g/dl (0.90), respectively and the variation was not statistically significant ( $F=0.061$ ,  $p=0.805$ ). The mean HGB level of Podo cases (12.06g/dl) was lower than the mean HGB level of controls (14.14g/dl) and the difference was statistically significant ( $F=43.32$ ,  $p<0.001$ ) (Table 7).

**Table 7: Univariate analysis of Hemoglobin level among cases and controls at risk of Hookworm infection in Yilmana Densa woreda, West Gojjam, 2018.**

<b>Disease status</b>	<b>H.worm</b>	<b>HGB level Mean (SD)</b>	<b>F test</b>	<b>p- value</b>
<b>Cases</b>	Yes (15)	11.69(0.96)	0.061(Disease status*H.worm*HGB)	0.805
	No (38)	12.21(0.90)		
	Total (53)	12.06(0.94)	43.32(Disease status*HGB)	< 0.001
<b>Controls</b>	Yes (9)	13.82(1.55)		
	No (58)	14.18(1.61)		
	Total (67)	14.14(1.59)		
<b>Total</b>	Yes(24)	12.49 (1.58)	1.94 (H.worm*HGB)	0.166
	No(96)	13.40(1.68)		
	Total	13.22(1.69)		

#### **6.4.4.2. Malaria infection**

Malaria was not detected in all study participants using both RDTs and microscopic examinations of blood films.

## 7. Discussion

### 7.1. Socio-demographic characteristics

This case control study aimed at assessing hematological and cytokine profiles of individuals with podoconiosis as compared to healthy controls. The study also assessed the magnitude of intestinal parasites with special emphasis to hookworm, as shoe wearing habit is a common risk factor. Podoconiosis being a neglected tropical disease which gets attention globally as well as nationally only in recent years, published researches focus on epidemiology, associated risk factors, psychosocial, behavioral factors and not on Immunopathogenesis. Thus, the discussion will include comparing and explaining the socio-demographic, behavioral and clinical data then followed by immuno-hematological profiles, though published researches in the later is very limiting.

Most of the cases, 64.2% were males and 35.8% were female with a male to female ratio of 1.8:1 which was similar to male to female cases in Midakegn district, central Ethiopia and Debre Elias Northern Ethiopia, 2012 found 1.2:1 (36, 37). However, most studies showed that the male to female ratio was almost equal to 1:1, Southern and Northern Ethiopia (35, 37, 49) and in contrast to the current study a nationwide study found 0.7:1(36). This difference might be due to the study design difference, our study was case control where as these studies were survey and cross sectional studies. Being male or female was not statistically associated with podoconiosis, similar to a study done in Southern Ethiopia in 2010 ( $p=0.487$ ) (49). However, a nationwide study in Ethiopia and a case control study in Northern Ethiopia showed that female sex was associated with increased risk of podoconiosis (35, 38).

Majority of the study participants (73.5%) were found in the age group 25-54 years, similar with the study conducted in rural Southern Ethiopia (73.7%) (49). More than half of the cases (52.83%) were found in the age range 35-54 years similar to 52.88% in Wolayita, Southern Ethiopia, and age group was associated with podoconiosis (49). The mean (SD) age of podoconiosis cases and controls was 49.9(13.9) and 41.6(13.5) years respectively and statistically significant ( $p<0.01$ ). The mean age of cases was slightly higher than Midkagn district, Central Ethiopia, 43.4±(15.9) years in 2012 (36) and Southern Ethiopia, 2010 cases 39.9 (13.0) and controls 35.3 (11.7) years, respectively (49). The difference might be due to the variation in study population (49). All the cases were farmers as in other studies in Ethiopia (37) residing in rural areas.

## 7.2. Leg washing and Shoe wearing practice

Majority (66.6%) of the study participants had washed their legs on daily bases, agreeing with a study done in Ethiopia (69.5%) in 2015 (35). Leg washing difference between cases and controls was statistically significantly associated with podoconiosis ( $p=0.014$ ). It agrees with the study done in West and East Gojjam, 2017(39). But a study done in Northern Ethiopia, 2013 found no association ( $p=0.78$ ) (38). The difference might be more controls were literate than cases and literacy may increase awareness on foot hygiene in our study.

The study further consolidates studies reporting strong association between shoes wearing status with podoconiosis. One such an example is a study from Northern Ethiopia, in 2017 which demonstrated a three times more likely risk of podoconiosis in those not wearing shoes (39).

The mean age at first shoes wearing of cases and controls was  $27.1\pm 15.847$  years and  $27.06\pm 14.62$ , respectively and the mean difference was not statistically significant ( $p=0.988$ ). The mean age at first shoes wearing of cases and controls in a study from East and West Gojam was 38.68 years and  $28.84 \pm 13.1$ , respectively which is higher than the current study (38). The difference might be due to increased awareness of shoes wearing in our study area and more awareness about podoconiosis and NTDs in general between 2013 and 2018 could partly explain the difference. However, our study agrees with 25.2 years for cases in Dembecha and Debre Eliyas, Northern Ethiopia, 2012 (37). Large proportion (54.7%) of the cases as compared to 17.9% of the controls, were barefoot at the time of interview. The findings in Podo cases was, higher than the 23.6% in East and West Gojjam, Northern Ethiopia, 2012(37).

Almost half of the cases (49.1%) and 61.2% of the controls had never worn shoes during soil contact activities. 52.8% (28/53) of the cases and 70.1% (47/67) of the controls had never worn protective shoes during soil contact activities. The difference between the cases and controls in wearing protective shoes during soil contact might be due to cases wore protective shoes to prevent the pain of the swelling induced by contact with hard soil texture.

### **7.3. Leg swelling history and physical examination**

The mean age of leg swelling started was 22.6 years and comparable with 25.8 years in Midakegn district, central Ethiopia, 2012 (36). This implies that the disease Podoconiosis was developed before people started wearing shoes. The swelling usually aggravated by seasonal factors like winter season is one of the common factors and physical activities like walking barefoot. The reason suggested by most participants was in winter the soil became hard to plough and damage the swelling and cause laceration. The damaged wound became painful when it was exposed to cold air and their groin swells up. On the other hand, when they had long distance walk through barefoot, their leg became swell up and painful. However, in most cases the disease was improved in summer and taking rest and about 10% of the cases did not know the improving and aggravating factors of leg swelling. The possible reason for improving factors is that during summer, the soil texture became soft mud which could not damage their leg swelling while working on barefoot. However, rain fall plays an important role in soil formation and increase exposure to mineral particles linked to podoconiosis, by producing sticky and slippery mud that can be easily absorbed in the skin (63).

More than half (60.38%) of the cases had hard and fibrotic type of leg swelling where as 39.62% had soft and water bag (pitting ) type of swelling . All of the cases responded that the site of leg swelling started were below the knee and 98.1% had bilateral swelling similar to 94.3% Midakegn, Central Ethiopia, 2012(36).

There were four stages identified by trained professional. Stage III (45.3%) was the most common followed by stage II (39.6%), stage I (9.4%) and stage IV (5.7%). It was different from a nationwide study in Ethiopia, 2015 in which the commonest stage was stage II(48.8%), followed by stage III(26.6%), stage I (16.7%), stage IV(5.9%) and stage V was the least(2.1%) (35). The difference might be due to our small sample size and our study was not a survey.

Twenty one cases (39.6%) had family history of podoconiosis and 17(32.1)% of cases had at least one first degree relative (father, mother, sibling, parents) that had podoconiosis. This agrees with report of 30.1% of cases that had at least one first degree relative in Central Ethiopia, 2012 (36).

## 7.4. Immunohematological Profiles

The mean of each CBC parameter measurement was determined and comparison was done between cases and controls using independent sample t test.

### 7.4.1. CBC and CD4 parameters of Cases and Controls

The mean leukocyte count for Podo cases ( $5.86 \times 10^9/L$ ) was significantly lower ( $p < 0.01$ ) than that of controls ( $7.39 \times 10^9/L$ ). The lower WBC counts in cases may be related to silica exposure which may induce autoantibody production and results in destruction of WBCs (64, 65) or toxic mineral exposure may cause increased apoptosis (66). On the other hand, the mean absolute lymphocyte count of cases was higher than controls ( $3.75 \times 10^9/L$  versus  $2.52 \times 10^9/L$ , respectively) and the difference was statistically significant ( $p < 0.01$ ). The lymphocyte count was also found to be higher in the affected tissue (26). This probably could be due to marked influx of lymphocytes to swelling (67) but in the circulation it could mainly be due to increased proliferation and differentiation signals of cytokines released from macrophages during inflammation. Cases had a mean absolute MID count of  $2.15 \times 10^9/L$  and controls  $1.0 \times 10^9/L$  and the difference was significant ( $p < 0.01$ ). In animal models, there were accumulation of lymphocytes and macrophages after pulmonary inflammation induced by silica particles (47).

The mean absolute granulocyte count of cases and controls were  $0.17 \times 10^9/L$  and  $4.12 \times 10^9/L$ , respectively and the mean percentage of granulocytes was remarkably decreased in cases. The mean difference between the two was statistically significant. The marked reduction in granulocytes might be due to destruction of granulocytes by circulating autoantibodies in cases (64).

The finding of higher mean Lymphocyte percentage (64.16%) in cases as compared to the controls (37.17%) which was above the normal range (18-54%) supports the preferential loss of granulocytes in Podo cases. The mean MID % was also higher in cases compared to the controls, above the normal range (6.8-26.3%).

The mean CD4 count of cases and controls were 673.58/ $\mu l$  and 681.13/ $\mu l$ , respectively whereas the mean CD4% was 34.89% for cases and 37.61% for controls. The mean difference in CD4 count and CD4% between cases and controls was not statistically significant ( $p > 0.05$ ). These reflected that toxic minerals might not have significant effect on the CD4 count and CD4 % of

people because both the cases and controls were exposed to these toxic particles; lymph node sample analysis revealed that both patients and healthy controls had microparticles and macrophages in their lymph node (68). However, genetic susceptibility has been reported to play a role (23).

The mean erythrocyte count for cases and controls was  $4.85 \times 10^{12}/L$  and  $5.1 \times 10^{12}/L$  respectively and the mean difference was statistically associated with podoconiosis ( $p < 0.01$ ). In addition, most erythrocyte indices had significant association with podoconiosis. Cases had lower mean HGB (12.06g/dl), MCH (24.58pg) and MCHC (26.45g/dl) levels than normal controls, 14.14g/dl, 27.66pg and 30.03g/dl respectively. The lower HGB, MCH and MCHC in cases indicated the existence of anemia, consistent with findings from Wolayita (49).

#### **7.4.2. Cytokines among cases and controls**

The mean IL-17 levels of cases (1.25pg/ml) was twice the controls (0.63pg/ml) but under the lower limit of detection whereas IL-4, IL-6, TNF- $\alpha$ , and IL-10 levels were above the limit of detection in both. The concentration of IFN gamma of cases was lower than the controls and below the minimum detectable dose. The median concentration was below the limit of detection for IL-4, TNF- $\alpha$ , IL-17, IFN gamma and IL-10. This might be due to the level of released cytokines were below the minimum detectable dose (69). However, the median concentration of IL-6 was 1.13 pg/ml in cases and 0.911pg/ml in controls. The Mann Whitney U test showed that except TNF alpha concentration, the five cytokines had the same distribution across the categories of disease status ( $p > 0.05$ ). Increased in pro-inflammatory cytokines (IL-6 and TNF- $\alpha$ ) might be related to increased pro-inflammatory response to toxic minerals in the soil. TNF $\alpha$  triggers the vascular endothelial cell expression of lipid mediators that promote tissue oedema, as well as leukocyte adhesion molecules that stimulate immune cell infiltration (70). It was decreased in cases in contrast to silica dust exposed individuals and more higher in patients with silicosis (71).

### **7.4.3. Intestinal Parasites among cases and controls**

The overall prevalence of intestinal parasite infections prevalence was 35% (42/120). Of the cases, 37.7% and of the controls 32.8 % had intestinal parasitic infection. However, intestinal parasite infection had no association with the disease podoconiosis ( $X^2=0.312$ ,  $df=1$ ,  $p=0.576$ ).

Hookworm was the most prevalent intestinal parasite in podoconiosis patients and controls, 15/53(28.30%) and 7/67(10.45%), respectively. Of the total parasite detected and identified, Hookworm accounts 15/42(35.7%) in cases and 7/42(16.67%) in controls. The finding was lower than 43.8% total prevalence (47.6% of patients and 33.1% of controls) in Southern Ethiopia, 2010. The prevalence of Hookworm was also lower in cases and controls in our study compared to 40.9% in cases and 27.5% of the controls (49).

### **7.4.5. Hemoglobin levels among Cases and Controls**

Univariate analysis of HGB as an outcome (dependent variable) and disease status and Hook worm infection status as factors indicated that Hookworm infection did not bring statistically significant variation on HGB level of study participants ( $F=1.39$ ,  $p=0.166$ ). However, HGB level varies significantly by Podo status ( $F=42.33$ ,  $p<0.0001$ ). This means that the mean HGB level of cases were significantly lowered from that of controls. The mean HGB level of Podoconiosis-Hook worm patients (double burdened) and podoconiosis-Hookworm negative patients was 11.69g/dl (0.96) and 12.21g/dl (0.90), respectively and the variation was not statistically significant ( $F=0.061$ ,  $p=0.805$ ). This agrees with a study conducted in Southern Ethiopia, 2010 (49). The mean HGB level of cases (12.06g/dl) was lower than the mean HGB level of controls (14.14g/dl) and the difference was statistically significant ( $F=43.32$ ,  $p<0.001$ ). The mean HGB level of controls infected with Hook worm was 13.82g/dl which was in the normal reference range (12-18gm/dl for females, 13.6-19.8gm/dl), therefore, the anemia developed in case might not be due to Hook worm infection, rather it could be due to podoconiosis.

## **8. Strength and Limitation of the study**

### **8.1. Strength of the study**

The strength of this study is that it investigated CBC parameters, CD4 count and percent and cytokines level in podoconiosis patients and controls. As far as the researchers knowledge go, there were no published information on these blood components and cell signaling molecules.

### **8.2. Limitation of the study**

The greatest limitation of this study was lack of related literatures because it is original work.

## **9. Conclusion and Recommendation**

### **9.1. Conclusion**

The study revealed that podo cases were predominantly males, start wearing shoe after onset of disease, had bilateral swelling which is aggravated during winter season and after long walk barefooted which in most cases improved in summer and after taking rest. Family history of podoconiosis had played a role in the susceptibility of individuals to this disease. Podo cases had lower WBC and granulocyte count but much higher lymphocyte and MID count (%) than controls. Cases had lower mean HGB, MCH and MCHC levels than controls. However, Platelet parameters (PLT count, MPV, PCT and PDW) had no association with podoconiosis. The cytokines IL-4, IL-6, IL-10, IL-17 and IFN $\gamma$  had no association with podoconiosis but TNF $\alpha$ .

The burden of intestinal parasite infections was higher in cases and in controls. Hookworm was the most prevalent intestinal parasite in podo cases and controls.

### **9.2. Recommendation**

The recommendation is forwarded to the Ethiopian Federal Ministry of Health and Amhara National Regional Health Bureau to incorporate analysis of hematological parameters for early diagnosis and management of hematological abnormalities related to Podoconiosis. Additionally, early diagnosis of intestinal parasite infection and provision of deworming treatment is needed. We also recommend stakeholders like IOCC, Ethiopia for expanding its activities and ensuring mechanisms for sustainable shoes and hygiene materials availability. Finally, we recommend researchers on this area, should further investigate the immunological and molecular level of the disease.

## References

1. Deribe K, Tomczyk S, Tekola-Ayele F. Ten years of podoconiosis research in Ethiopia. *PLoS neglected tropical diseases*. 2013;7(10):e2301.
2. Davey G, Tekola F, Newport MJ. Podoconiosis: non-infectious geochemical elephantiasis. *Trans R Soc Trop Med Hyg*. 2007;101(12):1175-80.
3. Molla YB, Wardrop NA, Le Blond JS, Baxter P, Newport MJ, Atkinson PM, et al. Modelling environmental factors correlated with podoconiosis: a geospatial study of non-filarial elephantiasis. *International Journal of Health Geographics*. 2014;13(1):24.
4. Fuller LC. Podoconiosis: endemic nonfilarial elephantiasis. *Current opinion in infectious diseases*. 2005;18.
5. Deribe K, Brooker SJ, Pullan RL, Hailu A, Enquselassie F, Reithinger R, et al. Spatial distribution of podoconiosis in relation to environmental factors in Ethiopia: a historical review. *PloS one*. 2013;8(7):e68330.
6. Onapa AW, Simonsen PE, Pedersen EM. Non-filarial elephantiasis in the Mt. Elgon area (Kapchorwa District) of Uganda. *Acta Trop*. 2001;78(2):171-6.
7. De Lalla F, Zanoni P, Lunetta Q, Moltrasio G. Endemic non-filarial elephantiasis in Iringa District, Tanzania: a study of 30 patients. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 1988;82(6):895-7.
8. Crivelli PE. Non-filarial elephantiasis in Nyambene range: a geochemical disease. *East African medical journal*. 1986;63(3):191-4.
9. Price EW, Bailey D. Environmental factors in the etiology of endemic elephantiasis of the lower legs in tropical Africa. *Trop Geogr Med*. 1984;36.
10. Corachan M, Tura JM, Campo E, Soley M, Traveria A. Podoconiosis in Aequatorial Guinea. Report of two cases from different geological environments. *Trop Geogr Med*. 1988;40(4):359-64.
11. Ruiz L, Campo E, Corachan M. Elephantiasis in Sao Tome and Principe. *Acta Trop*. 1994;57(1):29-34.
12. Tada MS, Marsden PD. Probable podoconiosis in Brasilia. *Revista da Sociedade Brasileira de Medicina Tropical*. 1993;26(4):255.
13. WHO. Accelerating work to overcome the global impact of neglected tropical diseases: A roadmap for implementation. ; . Geneva; 2012.

14. WHO. Country leadership and collaboration on neglected tropical diseases: Third progress report of the London Declaration. 2015.
15. Davey G, Burridge E. Community-based control of a neglected tropical disease: the mossy foot treatment and prevention association. *PLoS neglected tropical diseases*. 2009;3(5):e424.
16. Price EW. The management of endemic (non-filarial) elephantiasis of the lower legs. *Tropical doctor*. 1975;5(2):70-5.
17. WHO. The global atlas of podoconiosis *The lancet*. 2017;5:3.
18. Korevaar D, Visser B. Podoconiosis, a neglected tropical disease. *Neth J Med*. 2012;70:210-4.
19. Yimer M, Hailu T, Mulu W, Abera B. Epidemiology of elephantiasis with special emphasis on podoconiosis in Ethiopia: a literature review. *Journal of vector borne diseases*. 2015;52(2):111.
20. Deribe K, Cano J, Newport MJ, Golding N, Pullan RL, Sime H, et al. Mapping and modelling the geographical distribution and environmental limits of podoconiosis in Ethiopia. *PLoS neglected tropical diseases*. 2015;9(7):e0003946.
21. Destas K, Ashine M, Davey G. Prevalence of podoconiosis (endemic non-filarial elephantiasis) in Wolaitta, Southern Ethiopia. *Tropical doctor*. 2003;33(4):217-20.
22. Tekola F, Mariam DH, Davey G. Economic costs of endemic non-filarial elephantiasis in Wolaita Zone, Ethiopia. *Tropical medicine & international health : TM & IH*. 2006;11(7):1136-44.
23. Tekola Ayele F, Adeyemo A, Finan C, Hailu E, Sinnott P, Burlinson ND, et al. HLA class II locus and susceptibility to podoconiosis. *N Engl J Med*. 2012;366(13):1200-8.
24. GebreHanna E. The social burden of podoconiosis and familial occurrence in its development. Addis Ababa: Addis Ababa University. 2005.
25. Mousley E, Deribe K, Tamiru A, Tomczyk S, Hanlon C, Davey G. Mental distress and podoconiosis in Northern Ethiopia: a comparative cross-sectional study. *International health*. 2014;7(1):16-25.
26. Wendemagegn E, Tirumalae R, Böer-Auer A. Histopathological and immunohistochemical features of nodular podoconiosis. *Journal of Cutaneous Pathology*. 2015;42(3):173-81.
27. Wanji S, Kengne-Ouafu JA, Datchoua-Poutcheu FR, Njouendou AJ, Tayong DB, Sofeu-Feugaing DD, et al. Detecting and staging podoconiosis cases in North West Cameroon:

- positive predictive value of clinical screening of patients by community health workers and researchers. *BMC public health*. 2016;16(1):997.
28. Price E. A possible genetic factor in non-filarial elephantiasis of the lower legs. *Ethiopian medical journal*. 1972;10(3):87-93.
  29. Price E. Non-filarial elephantiasis of the lower legs in Ethiopia. A simple method for rapid survey by school enquiry. *Tropical and geographical medicine*. 1973;25(1):23.
  30. WHO. Investing to overcome the global impact of neglected tropical diseases: Third WHO report on neglected tropical diseases. 2015.
  31. Davey G, Bockarie M, Wanji S, Addiss D, Fuller C, Fox L, et al. Launch of the international podoconiosis initiative. *The Lancet*. 2012;379(9820):1004.
  32. Deribe K, Cano J, Trueba ML, Newport MJ, Davey G. Global epidemiology of podoconiosis: A systematic review. *PLoS neglected tropical diseases*. 2018;12(3):e0006324.
  33. Muli J, Gachohi J, Kagai J. Soil iron and aluminium concentrations and feet hygiene as possible predictors of Podoconiosis occurrence in Kenya. *PLoS neglected tropical diseases*. 2017;11(8):e0005864.
  34. Davey G. Recent advances in podoconiosis. *Annals of tropical medicine and parasitology*. 2009;103(5):377-82.
  35. Deribe K, Brooker SJ, Pullan RL, Sime H, Gebretsadik A, Assefa A, et al. Epidemiology and individual, household and geographical risk factors of podoconiosis in Ethiopia: results from the first nationwide mapping. *The American journal of tropical medicine and hygiene*. 2015;92(1):148-58.
  36. Geshere Oli G, Tekola Ayele F, Petros B. Parasitological, serological and clinical evidence for high prevalence of podoconiosis (non-filarial elephantiasis) in Midakegn district, central Ethiopia. *Tropical Medicine & International Health*. 2012;17(6):722-6.
  37. Molla YB, Tomczyk S, Amberbir T, Tamiru A, Davey G. Podoconiosis in East and west gojam zones, northern ethiopia. *PLoS neglected tropical diseases*. 2012;6(7):e1744.
  38. Molla YB, Le Blond JS, Wardrop N, Baxter P, Atkinson PM, Newport MJ, et al. Individual correlates of podoconiosis in areas of varying endemicity: a case-control study. *PLoS neglected tropical diseases*. 2013;7(12):e2554.
  39. Feleke BE. Determinants of podoconiosis, a case control study. *Ethiopian journal of health sciences*. 2017;27(5):501-6.

40. Tekola-Ayele F, Embiale W. Podoconiosis: tropical lymphedema of the lower legs. *Dermatology and Allergology-Principles and Practice* 1st ed Hong Kong: iConcept Press Ltd. 2014.
41. Prieto-Pérez L, Cea JJS, Hernández-Mora MG. Podoconiosis, a society and medical community neglected disease. *Medicina Clínica (English Edition)*. 2015;145(10):446-51.
42. Davey G, Gebrehanna E, Adeyemo A, Rotimi C, Newport M, Desta K. Podoconiosis: a tropical model for gene-environment interactions? *Trans R Soc Trop Med Hyg*. 2007;101(1):91-6.
43. Pollard KM. Silica, Silicosis, and Autoimmunity. *Frontiers in immunology*. 2016;7:97.
44. Famakinde DO, Adenusi AA. Involvement of Hookworm Co-Infection in the Pathogenesis and Progression of Podoconiosis: Possible Immunological Mechanism. *Tropical Medicine and Infectious Disease*. 2018;3(2):37.
45. Kolahian S, Fernandez IE, Eickelberg O, Hartl D. Immune Mechanisms in Pulmonary Fibrosis. *American journal of respiratory cell and molecular biology*. 2016;55(3):309-22.
46. Addisu S, El-Metwally T, Davey G, Worku Y, Titheradge M. The role of transforming growth factor- $\beta$ 1 and oxidative stress in podoconiosis pathogenesis. *British Journal of Dermatology*. 2010;162(5):998-1003.
47. Barbarin V, Nihoul A, Misson P, Arras M, Delos M, Leclercq I, et al. The role of pro-and anti-inflammatory responses in silica-induced lung fibrosis. *Respiratory research*. 2005;6(1):112.
48. Gan WQ, Man SFP, Senthilselvan A, Sin DD. Association between chronic obstructive pulmonary disease and systemic inflammation: a systematic review and a meta-analysis. *Thorax*. 2004;59(7):574-80.
49. Taye B, Alemayehu B, Birhanu A, Desta K, Addisu S, Petros B, et al. Podoconiosis and soil-transmitted helminths (STHs): double burden of neglected tropical diseases in Wolaita zone, rural Southern Ethiopia. *PLoS neglected tropical diseases*. 2013;7(3):e2128.
50. Nenoff P, Simon JC, Muylowa GK, Davey G. Podoconiosis–non-filarial geochemical elephantiasis—a neglected tropical disease? *JDDG: Journal der Deutschen Dermatologischen Gesellschaft*. 2010;8(1):7-13.
51. Mapping of Lymphatic Filariasis and Podoconiosis in Ethiopia Field Protocol. Addis Ababa, Ethiopia: Federal Ministry of Health; 2013.

52. Tekola F, Ayele Z, Mariam DH, Fuller C, Davey G. Development and testing of a de novo clinical staging system for podoconiosis (endemic non-filarial elephantiasis). *Tropical Medicine & International Health*. 2008;13(10):1277-83.
53. Alemayehu B, Alemayehu M. Factors Associated with Lymphoedema Management Success Among Podoconiosis Patients in Wolaita Zone, Southern Ethiopia. *Science Journal of Public Health*. 2017;5(5):392.
54. Sikorski C, Ashine M, Zeleke Z, Davey G. Effectiveness of a simple lymphoedema treatment regimen in podoconiosis management in southern Ethiopia: one year follow-up. *PLoS neglected tropical diseases*. 2010;4(11):e902.
55. Ethiopia Demographic and Health Survey. Addis Ababa, Ethiopia: Central Statistical Agency; 2016.
56. Population projection of Ethiopia for all regions at wereda level from 2014 – 2017. Addis Ababa, Ethiopia: Central Statistical Agency 2013.
57. Desta K, Ashine M, Davey G. Predictive value of clinical assessment of patients with podoconiosis in an endemic community setting. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 2007;101(6):621-3.
58. Cheesbrough M. *District laboratory practice in tropical countries*: Cambridge university press; 2006.
59. Humasis malaria P.f/Pan antigen test ;high sensitive differential diagnosis of malaria infection. Korea.
60. BD FACS count TM CD4; Enumerating absolute counts and determining percentages of CD4 T lymphocytes in unlysed whole blood, Becton Dickinson and company BD Biosciences, USA.
61. ABclonal. ELISA KITS Leader in Biomolecular Solutions For Immune Science. Human IL-10 ,IL-4,IL-6 , IL-17, TNF alpha ,IFN gamma ELISA Kit. USA.
62. Deribe K, Wanji S, Shafi O, Tukahebwa EM, Umulisa I, Molyneux DH, et al. The feasibility of eliminating podoconiosis. *Bulletin of the World Health Organization*. 2015;93(10):712-8.
63. Price E. The relationship between endemic elephantiasis of the lower legs and the local soils and climate. *Tropical and geographical medicine*. 1974;26(3):225-30.

64. Tervaert J, Mulder L, Stegeman C, Elema J, Huitema M, The H, et al. Occurrence of autoantibodies to human leucocyte elastase in Wegener's granulomatosis and other inflammatory disorders. *Annals of the rheumatic diseases*. 1993;52(2):115.
65. Solomon G, editor A clinical and laboratory profile of symptomatic women with silicone breast implants. *Seminars in arthritis and rheumatism*; 1994: Elsevier.
66. Borges VM, Lopes MF, Falcão H, Leite-Júnior JH, Rocco PR, Davidson WF, et al. Apoptosis underlies immunopathogenic mechanisms in acute silicosis. *American journal of respiratory cell and molecular biology*. 2002;27(1):78-84.
67. Kumar R, Li W, O'grady R. Activation of lymphocytes in the pulmonary inflammatory response to silica. *Immunological investigations*. 1990;19(4):363-72.
68. Price E, Henderson W. The elemental content of lymphatic tissues of barefooted people in Ethiopia, with reference to endemic elephantiasis of the lower legs. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 1978;72(2):132-6.
69. Kleiner G, Marcuzzi A, Zanin V, Monasta L, Zauli G. Cytokine levels in the serum of healthy subjects. *Mediators of inflammation*. 2013;2013.
70. Turner MD, Nedjai B, Hurst T, Pennington DJ. Cytokines and chemokines: at the crossroads of cell signalling and inflammatory disease. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*. 2014;1843(11):2563-82.
71. Jiang P, Cao Z, Qiu Z, Pan J, Zhang N, Wu Y. Plasma levels of TNF- $\alpha$  and MMP-9 in patients with silicosis. *European review for medical and pharmacological sciences*. 2015;19(9):1716-20.

## **Annexes**

### **Annex- I: Participant Information Sheet**

**Name of Investigator:** Ayteneu Atnaf

**Institution Name:** Addis Ababa University

**Title of Project:** Immuno-hematological profiles of individuals with podoconiosis in Yilmana Densa Woreda, West Gojjam

#### **Introduction:**

Podoconiosis (endemic non-filarial elephantiasis) is a geochemical, non-infectious and neglected tropical disease resulting in swelling of the lower legs in individuals with long-term exposure to irritant alkaline red clay soil. We are conducting a research on it and we need your voluntary participation. Before you decide whether to take part, it is important for you to understand why we are collecting this information and what it will involve. Please take time to hear this paper carefully and discuss it with friends and relatives if you wish to. Ask us if there is anything that is not clear or if you would like more information.

#### **Purpose of the study:**

The study aimed at assessing the immuno-hematological profiles on podoconiosis patients compared to individuals without podoconiosis. This study only includes adult individuals of age 18 and above. Through this study we will identify the socio-demographic factors affecting the disease and relationship with hematological and immunological parameters. We hope that this will improve the prevention and treatment of podoconiosis.

#### **Procedure:**

If you decide to participate in the study, you will be asked a series of questions about you and your family, the way you live and work, and in particular, the contact you have with the red soil. If you have podoconiosis, we will also ask questions related to the disease and finally we would like to take a sample of blood and stool. The blood samples will be analysed in Bahirdar Regional Laboratory.

**Benefits:** The immediate benefit of the study will be you can get free treatment for parasitic infections detected in stool examination. The future benefits to the study will be better understanding of the disease process and potential therapeutic options.

**Harms:** There may be slight pain on drawing blood, but experienced phlebotomists are available

**Confidentiality:** All information which is collected about you during the course of the research will be kept on strictly confidential. Any information about you which leaves the research unit will have your name and address removed so that you cannot be recognized from it.

**Autonomy:** Even if all the information you give us are very important to the study, you have the right to withdraw from the study at any time you want. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide not to participate, the treatment you or your family receives in future at government or NGO treatment sites will not be affected.

If a problem arises, you can report it to one of the project staff, your kebele head, or the study coordinators at the address given below.

Phone number

Contact Address: Aytnew Atnaf or \_\_\_\_\_

Cell Phone: 0924474452\_\_\_\_\_

Thank you in advance for considering taking part in this study!

**የተሳታፊዎች መረጃ መስጫ ቅጽ**

**የተመራማሪው ስም:** አይተነው አጥናፍ

**የመ/ቤቱ ስም:** አዲስ አበባ ዩኒቨርሲቲ

**የጥናቱ ርዕስ:** የኢሜዎ-ሄማቶሎጂካል ሁኔታ የዝሆኔ በሽታ ባለባቸው የይልማዥ ደንሳ ወረዳ ሰዎች፣ ምራብ ጎጃም

**መግቢያ: -**

ዝሆኔ ከሰው ወደ ሰው የማይተላለፍ ትኩረት ያላገኘ የትሮፒካል በሽታ ሲሆን ለረጅም ጊዜ ከቤዛማ ቀይ አፈር ጋር ግንኙነት ያላቸውን ሰዎች የእግር እብጠት ያስከትላል፡፡ እኛ በዚህ በሽታ ላይ ምርምር ጀምረናልና የእናንተን በጎ ፈቃድ ተሳትፎ ልናገኝ ይገባናል፡፡ ለዚህ በጥናቱ ከመሳተፍዎ አስቀድሞ ለምን መረጃው እንዳስፈለገን ከዚህ በታች በተዘረዘሩት ነጥቦች ማዳመጥና መረዳት ይችላሉ፤ ግልጽ ካልሆነ ለዎት ከማቅረብ ሰው ወይ ጓደኛ ጋር መጠቀስ ይችላሉ፡፡

**የጥናቱ ዓላማ -**

የዚህ ጥናት ዓላማ የኢሜዎ-ሄማቶሎጂካል ሁኔታ ዝሆኔ በሽታ ባለባቸው እና በሽታው በሌለባቸው ሰዎች ላይ ያለውን ልዩነት ዳሰሳ ማድረግ ነው፡፡ ጥናቱ የሚካተተው 18 ዓመትና ከዚያ በላይ የሆኑትን ሰዎች ነው፡፡ ይህ ጥናት የዝሆኔን በሽታን ስርጭት የሚወስኑ አካባቢያዊና ማህበራዊ መካኒዎችን ያመለክታል፤ እነኚህ ምክንያቶችና ከደም ጋር የተያያዙ የመከላከል አቅም ችግር ያላቸውን ግንኙነት ያመለክታል፡፡ የዚህም ጥናት ወጠት የዝሆኔ በሽታን ለመከላከልና ለመክምን በሚደረገው ጥረት ጉልህ ድርሻ ይኖረዋል፡፡

**የጥናቱ ሂደት:** - በጥናቱ ለመሳተፍ ከወሰኑ: - የተወሰኑ ጥያቄዎችን ስለቤተሰብዎ፣ ስለኑሮዎ ሁኔታ ከቀይ አፈር ጋር ያለዎትን ግንኙነት እንጠይቀዎታለን፡፡ የዝሆኔ በሽታ ካለብዎት ደግሞ ከበሽታው ጋር የተያያዙ ጥቂት ጥያቄዎችን እንጠይቀዎታለን፡፡ በመጨረሻም የደምና የሰገራ ናሙና ይሰጣሉ፡፡ የደም ናሙናው ምርመራ የሚከሄደው በባህርዳር ሪጅናል ላቦራቶሪ ነው፡፡

**የጥናቱ ትቅም:** - በጥናቱ ወቅት ከሰገራ ምርመራ የአንጀት ትላትል ከተገኘብዎት ነፃ የሚህኒት አገልግሎት ይሰጠዎታል፡፡ የጥናቱ የወደፊት ጥቅም የሽታውን የመተላለፍ ሂደት ለማወቅና ለመከላከል ጉልህ ማድ ይኖረዋል፡፡

**የጥናቱ ጉዳት:** - ደም በሚሰጠዎት ጊዜ ትንሽ የህመም ስሜት ሊኖር ይኖራል፤ ነገር ግን ልምድ ያላቸው ባለሙያዎች ስላሉን አይጨቁ፡፡

**የሚከፈልዎት አጠባበቅ:** - ማንኛውም ስለእርስዎ በጥናቱ የሚሰጡት መረጃ ሚኒስቴር በተጠበቀ መልኩ ይቀመጣል፡፡ ማንኛውም እርስዎን የሚጠላኩት መረጃ ከጥናት ክፍል ሲወጣ ስምዎ አድራሻዎ እንዳይኖረው ይደረጋል፡፡

**በራስ መወሰን:** - በማንኛውም ጊዜ ቃለመጠይቅ ላለማድረግና የደምና ሰገራ ናሙና ላለመስጠት ከፈለጉ ማቋረጥ ይችላሉ ነገር ግን የሚሰጡት መረጃ ለጥናቱ በጣም ጠቃሚ መሆኑን አይዘነጉ፤ በጥናቱ ለመሳተፍና ስለመሳተፍ



**Annex-II: Informed Consent form**

**Code No -----**

I have read the information above, or it has been read to me. I have been given the opportunity to ask questions and my questions have been answered to my satisfaction. I voluntarily consent that I would participate in this study.

To give stoolsample of tea spoon full

To give 10ml blood samples for this study  and

I understand that I have the right to withdraw from the study at any time

Participant's name \_\_\_\_\_ Sign. \_\_\_\_\_ Date \_\_\_\_\_

Participant's name \_\_\_\_\_ Fingerprint \_\_\_\_\_ Date \_\_\_\_\_

(For those who cannot write)

Researcher's name \_\_\_\_\_ Sign. \_\_\_\_\_ Date \_\_\_\_\_

የተሳታፊዎች የስምምነት ቅፅ

የምስጢር ቁጥር -----

ከላይ የተጠቀሰውን መረጃ በአጥኚው ወይም ተወካዮች አስፈላጊውን ገለፃና ማበራሪያ ተደርገዋል፡፡  
ማንኛውንም ግልፅ ያልሆነ ጥያቄ የመጠየቅና መልስ የማገኘት ዕድል ተሰጥቶኛል፡፡

በመሉ ፈቃድ በጥናቱ ለመስተፍ ወስኘኛለሁ፡ -

1 ማክሲም የሰገራ ናሙና ለመስጠት

10 ሙሉሊትር የደም ናሙና ለመስጠት

በማንኛውም ጊዜ ከጥናቱ ራሴን ማገለል እንደምችል ተረድቻለሁ

የተሳታፊ ስም \_\_\_\_\_ ፊርማ \_\_\_\_\_ ቀን \_\_\_\_\_

የተሳታፊ ስም \_\_\_\_\_ የእጣት አሻራ \_\_\_\_\_ ቀን \_\_\_\_\_

(መግፍ ለማይችሉ)

የተመራመራው ስም \_\_\_\_\_ ፊርማ \_\_\_\_\_ ቀን \_\_\_\_\_

## Annex-III: Questionnaires

### English version

	<b>Date:</b>	<b>Interviewer:</b>	
	<b>Participant ID</b>	<b>Signature</b>	
<b>Section I: Demographic and Socioeconomic Information</b>			
<b>S</b>	<b>Questions and filters</b>	<b>Response &amp; Coding Categories</b>	<b>Skip</b>
<b>N</b>			
1	Sex	<input type="checkbox"/> 1 = Male <input type="checkbox"/> 2 = Female	
2	How old are you?(>=18years)		
3	Kebele		
4	Where is your permanent residence?	<input type="checkbox"/> 1 = Rural <input type="checkbox"/> 2 = Urban	
5	How long you lived in the current location?	_____	
6	What is your Religion?	<input type="checkbox"/> 1 = Christian <input type="checkbox"/> 3 =protestant <input type="checkbox"/> 2 = Muslim <input type="checkbox"/> 4= Other	
7	What is your educational status at this time?	1= Illiterate 2=literate <input type="checkbox"/> 1=Primary school <input type="checkbox"/> 2=Secondary school <input type="checkbox"/> 3=More than secondary	
8	What is your major occupation currently?	<input type="checkbox"/> 1 = Farmer <input type="checkbox"/> 5 = Student <input type="checkbox"/> 2 = merchant <input type="checkbox"/> 6 = Daily laborer <input type="checkbox"/> 3 = Employed <input type="checkbox"/> 7 = Have no Job <input type="checkbox"/> 4 = Housewife <input type="checkbox"/> 8 = Other specify	
9	What is your marital status?	<input type="checkbox"/> 1=Married <input type="checkbox"/> 3=Divorced <input type="checkbox"/> 2=Single <input type="checkbox"/> 4=Widowed	
10	What is your monthly income in Birr(on average)?		
<b>Section II: Shoe wearing and foot care practice</b>			
11	How often do you wash your legs?	<input type="checkbox"/> 1= More often than daily <input type="checkbox"/> 2= Daily <input type="checkbox"/> 3= several times per week	

12	Have you ever worn shoes?	<input type="checkbox"/> 1=Yes <input type="checkbox"/> =2 No	
13	How old were you when you first worn shoes?		
14	Is the person wearing shoes at the time of the interview?	<input type="checkbox"/> 1=Yes <input type="checkbox"/> =2 No	
15	What type of shoes is the person wearing during interview?	<input type="checkbox"/> 1= Hard plastic <input type="checkbox"/> 3= Open sandal <input type="checkbox"/> 2= Leather <input type="checkbox"/> 4= Shera <input type="checkbox"/> 5= other	
16	When do you prefer to wear shoes?( multiple answers possible)	<input type="checkbox"/> 1= On market days & on Sunday <input type="checkbox"/> 2= During rainy season <input type="checkbox"/> 3= At home <input type="checkbox"/> 4= On the field <input type="checkbox"/> 5= When walking far	
17	Do you always wear shoes when you perform activities that make soil contact?	<input type="checkbox"/> 1=Yes <input type="checkbox"/> =2 No	
18	What type of shoe do you wear when you perform activities related to soil like plowing, digging, etc?	<input type="checkbox"/> 1= Hard plastic <input type="checkbox"/> 3= Open sandal <input type="checkbox"/> 2= "bush " <input type="checkbox"/> 4= Shera <input type="checkbox"/> 5= other	
19	Does your house's floor is covered?	<input type="checkbox"/> 1=Yes <input type="checkbox"/> =2 No	
<b>Section III: Leg swelling history &amp; physical examination</b>			
20	When did you have leg swelling ?		
21	How old were you when you first noticed this swollen leg?		
22	Where did the swelling start from?	<input type="checkbox"/> 1=above the knee <input type="checkbox"/> 2=below the knee(foot)	
23	How many years you lived with leg swelling?		
24	When will the swelling be exacerbated and relief?	Exacerbated: _____ Relieved: _____	
25	What type of Leg swelling does he/she had?	<input type="checkbox"/> 1=Hard & fibrotic <input type="checkbox"/> =2 soft and	

		watery	
26	Where is the site of leg swelling?	<input type="checkbox"/> 1= bilateral and below the knee <input type="checkbox"/> 2= unilateral and below the knee <input type="checkbox"/> 3= unilateral and above the knee <input type="checkbox"/> 4= bilateral and above the knee	
27	Do you have swelling of the groin?	<input type="checkbox"/> 1=Yes <input type="checkbox"/> =2 No	
28	Is there preservation of sensation in the toes?	<input type="checkbox"/> 1=Yes <input type="checkbox"/> =2 No	
29	Are you diagnosed as a leprosy patient?	<input type="checkbox"/> 1=Yes <input type="checkbox"/> =2 No	
30	Do you have any family member (living or dead) with history of leg swelling?	<input type="checkbox"/> 1=Yes <input type="checkbox"/> =2 No	
31	If you say "yes" for question 31, which family member was affected?	<input type="checkbox"/> 1= Mother <input type="checkbox"/> 2= Father <input type="checkbox"/> 3= Grand parents <input type="checkbox"/> 4= siblings <input type="checkbox"/> 5=others	
32	The stage of podoconiosis	<input type="checkbox"/> 1= Stage1 <input type="checkbox"/> 2= Stage 2 <input type="checkbox"/> 3= Stage 3 <input type="checkbox"/> 4= Stage4 <input type="checkbox"/> 5= Stage 5	

**Thank you!**

ቃለ መጠይቅ ( Amharic version)		
	ቀን	የጠቅላይ ሰዎች
	የተሳታፊ ሙያ ቁጥር	ፊርማ
<b>ክፍል 1: የግለሰብ አካላዊ፣ ማህበራዊ እና አካላዊ መረጃ</b>		
ተ.ቁ	ጥያቄዎች እና ማጠቃለያዎች	መልስ መስጫ ኮድና ክፍፍሎች
1	የታ	<input type="checkbox"/> 1 = ወንድ <input type="checkbox"/> 2 = ሴት
2	እድሜዎ ስንት ዓመት ነው? (>=18ዓመት )	
3	ቀበሌ	
4	በቋሚነት የሚኖሩት የት ነው?	<input type="checkbox"/> 1 = ገጠር <input type="checkbox"/> 2 = ከተማ
5	ከዚህ ቦታ ምን ያህል ዓመት ኖረዋል?	_____
6	ኃይማኖትዎ ምንድን ነው?	<input type="checkbox"/> 1 = ኦርቶዶክስ <input type="checkbox"/> 3 = ፕሮቴስታንት <input type="checkbox"/> 2 = እስላም <input type="checkbox"/> 4 = ሌላ
7	የትምህርት ደረጃዎ ምንድን ነው ?	1= ያልተማረ 2=የተማረ
8	ዋና መተዳደሪያ ስራዎ ምንድን ነው?	<input type="checkbox"/> 1 = ግብርና <input type="checkbox"/> 5 = ተማሪ <input type="checkbox"/> 2 = ነጋዴ <input type="checkbox"/> 6 = የቀን ስራተኛ <input type="checkbox"/> 3 = ተቀማጭ <input type="checkbox"/> 7 = ስራ አጥ <input type="checkbox"/> 4 = የቤት አመጪት <input type="checkbox"/> 8 = ሌላ
9	የትዳር ሁኔታዎ እንዴት ነው ?	<input type="checkbox"/> 1=ያገባ/ች <input type="checkbox"/> 3=የተፋታ/ች <input type="checkbox"/> 2=ያላገባ/ች <input type="checkbox"/> 4=የትዳር ጓደኛ የሞተባት
10	ወርሃዊ ገቢዎ በአማካይ ምን ያህል ነው?	
<b>ክፍል 2: የጭን መልበስና የእግር ንፅህና አያያዝ ልምድ</b>		
11	እግርዎትን ምን ያህል ጊዜ ይታጠብሉ?	<input type="checkbox"/> 1= በቀን ሁለት ጊዜና ከዚያ በላይ <input type="checkbox"/> 2= በየቀኑ <input type="checkbox"/> 3= በሳምንት ከ2ቀን በላይ <input type="checkbox"/> 4=ከሳምንት አንድ ቀንና በታች
12	ጭን ለብሰው ያውቃሉ?	<input type="checkbox"/> 1=አዎ <input type="checkbox"/> =2 አላውቅም
13	ለመጀመሪያ ጊዜ ጭን ለብሰው እድሜዎ ስንት ነበር?	
14	በቃለ-መጠይቁ ሰዓት ተጠያቂው/ዋ ጭን ለብሰዋል?	<input type="checkbox"/> 1=አዎ <input type="checkbox"/> =2 አለብሰም
15	ምን አይነት ጭን ነበር የለብሰዎት?	<input type="checkbox"/> 1= ሸፍን <input type="checkbox"/> 3= ነጠላ/ክፍት ጭን
16	መቼ ጭን መልበስ ይመርጣሉ? ( ከአንድ በላይ መሟረጥ ይቻላል)	<input type="checkbox"/> 1= በገበያ ቀን እና አሁን <input type="checkbox"/> 2= በዝናብ ወቅት <input type="checkbox"/> 3= ቤት ሲሆን <input type="checkbox"/> 4= በስራ ቦታ <input type="checkbox"/> 5= ረጅም መንገድ ስሄድ
17	ከአፈር ጋር የሚገናኙ ስራዎችን ሲሰሩ ሁልጊዜ ጭን ይለብሳሉ?	<input type="checkbox"/> 1=አዎ <input type="checkbox"/> =2 አለብሰም → 19

18	ከአፈር ጋር የመጥናት ስራ ሰሰሩ ምን ዓይነት ጭጭ ይለብሳሉ?	<input type="checkbox"/> 1= ጠንካራ ጥላሰቲክ <input type="checkbox"/> 3= ነጠላ ጭጭ <input type="checkbox"/> 2= በሽ <input type="checkbox"/> 4= ሽራ <input type="checkbox"/> 5= ሌላ	
19	የቤትዎ ሳሎን ምንጣፍ አለው?	<input type="checkbox"/> 1=አዎ <input type="checkbox"/> =2 የለውም	

**ክፍል 3: ስለእግር እብጠት መጀምሪያ አካላዊ ምርመራ**

**Section IV: Laboratory Investigation report form**

SN	Tests types	Results	Comments
20	መቼ ነበር የእግር እብጠቱ የጀመረው?		
21	እብጠቱ እንደጀመረው እድሜ ስንት ነበር??		
22	እብጠቱ ከየት ነበር የጀመረው?	<input type="checkbox"/> 1=ከጉልበት በላይ <input type="checkbox"/> 2=ከጉልበት በታች (ከእግር ጭቅካባቢ)	
23	የእግር እብጠቱ ከጀመረው ስንት ዓመት ሆነው?		
24	እብጠቱ የሚገባሰብዎትና የሚሸገልዎት መቼ ነው?	የሚገባሰብኝ: _____ የሚሸገልኝ: _____	
25	የእግር እብጠቱ ምን ዓይነት ነው?	<input type="checkbox"/> 1=ጠንካራና ደረቅ <input type="checkbox"/> =2 ለስላሳና ወህማ	
26	የእግር እብጠቱ በየት በክል ነው?	<input type="checkbox"/> 1= በሁለቱም ጎን እና ከጉልበት በታች <input type="checkbox"/> 2= በአንድ ጎን ከጉልበት በታች <input type="checkbox"/> 3= በአንድ ጎን ከጉልበት በላይ <input type="checkbox"/> 4= በሁለቱም ጎን እና ከጉልበት በላይ	
27	የብሽሽት ማጠጥ አለ??	<input type="checkbox"/> 1=አዎ <input type="checkbox"/> =2 የለም	
28	የእግር ጣትዎ አካባቢ የሚደንዘዝ ስሜት ይሰማዎታል?	<input type="checkbox"/> 1=አዎ <input type="checkbox"/> =2 አይሰማኝም	
29	ከዚህ በፊት የስጋ ደዌ በሽታ አለብዎት ተብሎ በጠፍ ባለመቻ ተነግሮዎት ነበር?	<input type="checkbox"/> 1=አዎ <input type="checkbox"/> =2 አልተነገረኝም	
30	በቤተሰባችሁ ወይም ዘመድ (በህይወትያለ/የሌለ) እግሩ ያበጠበት ሰው አለ/ነበረ?	<input type="checkbox"/> 1=አለ <input type="checkbox"/> =2 የለም	ጭ 32
31	አለ ካለ፣ ከቤተሰባችሁ የማን እግር ነበር ያበጠመ?	<input type="checkbox"/> 1= የእናት <input type="checkbox"/> 3= የቅድመ አያት <input type="checkbox"/> 2= የአባት <input type="checkbox"/> 4= የልጅ <input type="checkbox"/> 5=ሌላ -- ---	
32	የእግር እብጠቱ ደረጃ	<input type="checkbox"/> 1= ደረጃ1 <input type="checkbox"/> 4= ደረጃ4 <input type="checkbox"/> 2= ደረጃ 2 <input type="checkbox"/> 5= ደረጃ 5 <input type="checkbox"/> 3= ደረጃ 3	

አመሰግናለሁ!!!

**Annex -IV: Data extraction formats**

01	Complete Blood Cell count(CBC)		RBC_____	
			WBC_____	
			Hgb_____	
			Hct_____	
			MCV_____	
			MCH_____	
			MCHC_____	
			RDW_____	
			PLT_____	
			PCT_____	
			MPV_____	
			PDW_____	
	WBC differential		Gran_____ /ul ,	
			_____ %	
			Lymp_____ /ul	
			, _____ %	
			Mid_____ /ul	
			, _____ %	
	Manual Diff count(%)		Neutrophils_____	
			Lymphocyte_____	
			Monocytes_____	
			Eosinophils_____	
			Basophils_____	
02	CD4/CD8 level		CD4 count _____	
			CD8 count _____	
			CD4/CD8 ratio _____	
03	Malaria	1. RDTs:	a) Positive: <input type="checkbox"/> P.v <input type="checkbox"/> P.f <input type="checkbox"/> mixed	
			b) Negative	
		2. Blood film	a) positive <input type="checkbox"/> P.v <input type="checkbox"/> P.f <input type="checkbox"/> mixed	
			b) Negative	
04	Stool Examination:	1. Wet mount:	a)positive;	

			Species _____ b) Negative	
		2. Concentration technique:	a) _____ Positive; species _____ b) Negative	
05	Cytokine levels		IL-4 _____ IL-6 _____ TNF- $\alpha$ _____ IL-10 _____ IFN $\gamma$ _____ IL-17	
06	ABO blood group and Rh			

## **Annex-V: SOP for Stool sample collection, processing & examination procedure**

### **1. Stool sample collection**

A stool should be collected in a tightly covered, clean (not necessarily sterile) container and must not be contaminated with urine. The label on the specimen must include the time of collection of the stool as well as appropriate patient identification information.

1. Label the specimen container with study participant's name and code
2. Give the container to them and instruct how to bring fresh stool sample
3. Receive the sample by checking their name and code
4. Prepare wet mount and preserve the remaining sample with 10% formalin

### **2. Direct wet mount preparation for stool examination**

1. Add a drop of physiological saline (0.9%) on a clean glass slide
2. Add small quantity (about 2 mg) of faeces in a drop of saline.
3. Emulsify the stool using applicator stick
4. Cover it with cover slip
5. Examine the smear under microscope using 10x, then 40x objective

### **3. Formol- ether concentration Technique**

**Principle:** In the Ridley modified method, faeces are emulsified in formol water, the suspension is strained to remove large faecal particles, ether or ethyl acetate is added, and the mixed suspension is centrifuged. Cysts, oocysts, eggs, and larvae are fixed and sedimented and the faecal debris is separated in a layer between the ether and the formol water. Faecal fat is dissolved in the ether.

1. Using a rod or stick, emulsify an estimated 1 g (pea-size) of faeces in about 4 ml of 10% formol water contained in a screw-cap bottle or tube.
2. Add a further 3–4 ml of 10% v/v formol water, cap the bottle, and mix well by shaking.
3. Sieve the emulsified faeces, collecting the sieved suspension in a beaker.
4. Transfer the suspension to a conical (centrifuge) tube made of strong glass, copolymer, or polypropylene. Add 3–4 ml of diethyl ether or ethyl acetate.
5. Stopper the tube and mix for 1 minute. If using a Vortex mixer leave the tube unstoppered and mix for about 15 seconds (it is best to use a boiling tube).
6. With a tissue or piece of cloth wrapped around the top of the tube, loosen the stopper (considerable pressure will have built up inside the tube).

7. Centrifuge immediately at approx. 3000 rpm for 1 minute. After centrifuging, the parasites will have settled to the bottom of the tube and the faecal debris will have collected in a layer between the ether and formol water.
8. Using a stick or the stem of a plastic bulb pipette, loosen the layer of faecal debris from the side of the tube and invert the tube to discard the ether, faecal debris, and formol water. The sediment will remain.
9. Return the tube to its upright position and allow the fluid from the side of the tube to drain to the bottom. Tap the bottom of the tube to resuspend and mix the sediment. Transfer the sediment to a slide, and cover with a cover glass.
10. Examine the preparation microscopically using the 10X objective with the condenser iris closed sufficiently to give good contrast. Use the 40x objective to examine small cysts and eggs.
11. If required, count the number of each species of egg in the entire preparation. This will give the approximate number per gram of faeces.

## **Annex -VI: SOP for Blood collection**

**1. Purpose:** To perform complete blood cell count, CD4 count, malaria microscopy and RDTs, Blood group and to test CRP and Cytokine levels.

**2. Principle:** Venous blood is collected through the pressure created by the needle and it can be drawn by putting the needle in the same direction to the flow of blood in the vessels.

**3. Specimen:** blood

### **4. Materials and Reagents:**

- Gloves
- vacutainer tube
- vacutainer tube holder
- Two-way needle
- tourniquet
- gauze pads or cotton,
- test tubes with and without anticoagulant
- Sharp container
- test tube racks

### **5. Method:** Venous blood collection

#### 5.1. Procedures:

1. Assemble the necessary materials and equipment
2. Thread the short end of the double-pointed needle into the holder
3. Identify the right patient and allow him/her to sit
4. Apply the tourniquet.
5. Prepare the arm by swabbing the antecubital fossa with a gauze pad
6. Grasp the back of the patient's arm and insert the needle properly into the vein;
7. Then the point of the needle is advanced 0.5-1.0cm into the subcutaneous tissue (at an angle of 45<sup>0</sup>) and is pushed forward at a lesser angle to pierce the vein wall
8. Push the vacuum tube into the needle holder so that the blood flows into the tube under vacuum.
9. The tourniquet should be released the moment blood starts entering the vacuum tube
10. Apply a ball of cotton to the puncture site and gently withdraw the needle.
11. Instruct the patient to press on the cotton
12. Remove the tube from the vacutainer holder and if the tube is with anticoagulant, gently invert several times
13. Label the tubes with clientt's name, code number

## **Annex -VII: SOP for Cell dyne 1800 blood cell counter**

**1. Purpose:** To enumerate complete blood cells and parameters

**2. Principle:** The Cell-Dyne 1800 Hematology Analyzer performs a Complete Blood Count (CBC), Platelet Count, and a three-Part Differential. Whole blood is aspirated, diluted, and then divided into two samples. One sample is used to analyze the red blood cells and platelets while the second sample is used to analyze the white blood cells and hemoglobin. Electrical impedance is used to count the white blood cells, red blood cells, and platelets as they pass through an aperture. As each cell is drawn through the aperture, a change in electrical resistance occurs generating a voltage pulse. The number of pulses during a cycle corresponds to the number of cells counted. The amplitude of each pulse is directly proportional to the cell volume.

**3. Specimen requirements:** Whole blood collected in an EDTA tube. The instrument aspirates 30 µl of patient sample.

### **Equipment & Items Required:**

1. Cell-Dyn Diluent, Cell-Dyn Lytic Agent, Cell-Dyn Detergent: stable at room temperature
2. Enzymatic Cleaner: Stable at 2-8°C until the expiration date on the container.

### **Procedures:**

1. Check operation of the machine, ensuring it is clean and that all required supplies are present in sufficient quantities.
2. Switch the instrument on by pressing the ON/OFF switch, located on the back of the instrument.
3. Press MAIN to return to the MAIN MENU. At the MAIN MENU, enter in the operator ID and press RUN, next press SPECIMEN TYPE. The results shall be within the following specifications. Background counts for  $WBC \leq 0.3(k/mm^3)$ ,  $RBC \leq 0.05(M/mm^3)$ ,  $HGB \leq 0.1(g/dl)$ ,  $PLT \leq 5(k/mm^3)$
4. If the Open Mode Background count results are acceptable, proceed to Step 4.
5. If the Open Mode Background count results fail, press CLEAR ORIFICE to clear the orifice. Press MAIN then SPECIAL PROTOCOL then AUTO CLEAN and put enzymatic cleaner in tube and place the sample probe in the tube and press RUN. When cleaning is complete, press NORMAL BACKGROUND and press the Plate.
6. To perform patient testing:

- A. Press MAIN to return to the MAIN MENU screen. Enter in the Operator ID and press RUN. Press SPECIMEN TYPE then press PATIENT SPECIMEN. Verify that RUN Ready is displayed in the Status Box.
- B. Mix the patient sample well and remove the cap.
- C. Place the sample probe in the tube so that the end is immersed in the sample but not resting on the bottom of the tube.
- D. Press the Touch Plate to start the run. The Status Box on the RUN menu indicates the stage of the run.
- E. When Remove Specimen is displayed in the Status Box and the probe has moved up through the wash block, remove the sample tube and replace the tube cap. A beep will indicate that the probe cleaning cycle has begun.
- F. After the probe cleaning cycle is complete, the probe will move down into position for the next sample and the results will be displayed on the screen.
- G. If needed, press PRINT REPORT for a hardcopy of the report.
- H. Dilute the sample if White blood cell counts  $\geq 100,000$  /mm<sup>3</sup> and platelet counts  $\geq 1,000,000$  /mm<sup>3</sup> are outside the linearity specifications of the instrument.

**Quality control procedure:**

1. At the beginning of each work shift, all parameters are tested with blood control.
2. The 3 levels include: Abnormal Low, Normal, Abnormal High
3. Controls are stored at 2-8°C and brought to room temperature on a roller mixer before use .
4. Controls are gently inverted many times according to the manufacturer's instruction before use.
5. From the RUN screen, press [SPECIMEN TYPE].
6. Use the arrow key on the keyboard to move the cursor to the appropriate QC file (i.e., low, normal or high) and press the [QC SPECIMEN] key.
7. Control values must be within three standard deviations, otherwise the measurement has to be repeated. if the control still out of range:
  - a. Check operation of the machine, ensuring it is clean and that all required supplies are present in sufficient quantities.

b. Check reagents for expiration dates and lot numbers. Ensure that all machine lines are in appropriate receptacle where applicable. If this does not solve the problem:

- ✓ Prepare new control(s) and try again.
- ✓ If the controls are still out, inform your supervisor to check the operator's manual, or recalibrate instrument and If controls are still out,. Contact Medical Maintenance where applicable, or servicing engineer.

**Interpretation of the result:** Certain disease states are defined by an absolute increase or decrease in the number of a particular type of cell in the bloodstream and many types of anemia.

**Reporting result:** According to lab policy.( automated printing or computerized)

## **Annex -VIII: SOP for Manual differential white blood cell count**

Modified Wright Stain Procedure: Thin blood films (only) – Rack Method

1. Lay air dried slides on staining rack and flood with stain; stain for 10 to 15 seconds (double the staining time for bone marrow smears).
2. Add an equal volume of deionized/distilled water and stain for 10 seconds.
3. Rinse the slide by dipping in deionized/distilled water for 30 seconds. The slide may also be rinsed by swishing or washing with deionized/distilled water.
4. air dry the slide and examine at 100x oil immersion

**1. Purpose:** To determine the relative number of each type of white cell present in the blood by performing differential cell counts on five relatively normal blood smears and five sets of abnormal blood smears within a  $\pm 15\%$  accuracy of the instructor's values.

### **2. Principle**

A stained smear is examined in order to determine the percentage of each type of leukocyte present and assess the erythrocyte and platelet morphology. Increases in any of the normal leukocyte types or the presence of immature leukocytes or erythrocytes in peripheral blood are important diagnostically in a wide variety of inflammatory disorders and leukemia. Erythrocyte abnormalities are clinically important in various anemias. Platelet size irregularities are suggestive of particular thrombocyte disorders.

### **3. Specimen**

Peripheral blood smear made from EDTA-anticoagulated blood. Smears should be made within 1 hour of blood collection from EDTA specimens stored at room temperature to avoid distortion of cell morphology. Unstained smears can be stored for indefinite periods in a dry environment, but stained smears gradually fade unless cover slipped.

### **4. Reagents, Supplies and Equipment**

- Manual cell counter designed for differential counts
- Microscope, immersion oil and lens paper

## 5. Quality Control

Training and experience in examining immature and abnormal cell morphology are essential. A set of reference slides with established parameters should be established to assess the competence of an individual to perform differential and morphological identification of leukocytes and erythrocytes. Participation in a quality assurance program continues to document the expertise of the hematologist in microscopy. Questionable or abnormal smears should be referred to a supervisor or pathologist for verification.

### Procedure

1. Focus the microscope on the 10X objective (low power). Scan the smear to check for cell distribution, clumping, and abnormal cells
2. Examine the peripheral edge of the smear. If there are an increased number of white cells in this area, the differential count is inaccurate. Most of the cells at the edge of the smear are the large white cells, namely neutrophils and monocytes.
3. If the smear is acceptable, estimate the white cell count by counting the number of WBC in each of 5 or 6 low power fields. Average the numbers. Multiply the average by 1000 and divide by 4. This number should be within  $\pm 20\%$  of the actual white cell count. If it is not within this range, the white cell count and the estimation should be repeated.

$$\frac{\text{(Average \# WBC per 5 fields)} \times 1000}{4}$$

4

4. To perform the differential, choose the portion of the smear where there is close proximity but little overlapping of the red cells.
5. Begin the count in the thin area of the slide using modified battlement method

Count each white cell seen and record on a differential cell counter, until 100 white cells have been counted. If any nucleated red cells (NRBCs) are seen during the differential count, enumerate them on a separate counter. They are not to be included in the 100-cell

differential count. They are reported as #NRBC/100 WBCs and the WBC count must be corrected if there are  $\geq 10$  NRBCs / 100 WBC. The following formula is used:

$$(\text{WBC in thousands} \times 100) / (100 + \text{NRBC})$$

6. Results are expressed as a percentage of the total leukocytes counted.

It is also helpful to know the actual number of each white cell type per  $\mu\text{L}$  of blood. This is referred to as the absolute count and is calculated as follows:

$$\text{Absolute number of cells}/\mu\text{l} = \% \text{ of cell type in differential} \times \text{white cell count}$$

## **Annex-IX: SOP for Enumeration of CD4+ T lymphocyte (FACS count Flow cytometry)**

### **Clinical significance:**

The test is mainly used for baseline assessment and monitoring response to treatment.

### **Principle:**

A single test requires one ready-to-use reagent tube. When whole blood is added to the reagent tube, fluorochrome-labeled antibodies in the reagents bind specifically to white blood cell surface antigens, and a fluorescent nuclear dye binds to the nucleated blood cells. After a fixative solution is added, the sample is run on the instrument. During sample acquisition, the cells pass through the laser light, which causes the labeled cells to fluoresce. This fluorescent light provides the information necessary for the instrument to identify and count the lymphocytes and CD4 T lymphocytes. In addition, the reagent tubes contain a known number of fluorescent reference beads to which a precise volume of whole blood is added. The software automatically identifies the lymphocyte populations of interest and calculates the CD4 counts (cells/ $\mu$ L) by comparing cellular events to bead events. Results include CD4 counts and CD4 percentages.

### **Reagents or Materials required but not provided**

- BD Vacutainer® EDTA blood collection tubes or equivalent
- Disposable pipet tips
- Vortex mixer
- BD FACSCFlow™ sheath fluid
- BD FACSCCount™ controls
- BD FACSCCount system

### **Equipment:**

- BD FACS count instrument
- Automatic electronic pipette and tips
- Vortex mixer
- Coring station
- Cleaning tubes
- FACS count workstation
- Disposable clothing
- Biohazard waste container or bag

## **Reagents Provided, Sufficient for 50 Tests**

The following are provided:

- 50 reagent tubes containing CD4 PE/ CD14 PE-Cy<sup>TM</sup>5\*/CD15 PE-Cy5, fluorescent nuclear dye, and reference beads
- 65 reagent tube caps
- One 5-mL vial of 5% formaldehyde in phosphate-buffered saline (PBS), used as fixative solution

### **Quality control**

Perform a control run using BD FACS Count controls to check system accuracy and linearity. Run controls each day before you run patient samples or whenever you open a new reagent lot.

### **Procedures:**

#### **Preparing Tubes**

Note: We recommend that you prepare no more than 15 reagent tubes at one

1. Label the tab of each reagent tube with the patient accession number or number that identifies the tube of blood.
2. Vortex each tube upside down for 6 seconds and upright for 6 seconds. NOTE Set the vortex speed to a setting that causes the liquid to rise to the top of the tube.
3. Open each reagent tube with the coring station.

#### **Adding blood**

1. Invert the EDTA tube 5 to 10 times to make sure that the whole blood is adequately mixed.
2. Pipette 50  $\mu$ L of whole blood into the reagent tube labeled with the corresponding patient accession number.
3. Cap the tube and vortex upright for 6 seconds.
4. Repeat steps 1 through 3 to prepare a sample tube for each patient specimen.
5. Incubate the tubes for 30 minutes at room temperature (20°C–25°C) in the workstation. Close the cover to protect the reagents from light. NOTE Correct incubation time is critical and must be at least 30 minutes but no longer than 40 minutes for each sample tube.

#### **Adding Fixative**

1. Uncap each sample tube and pipette 50  $\mu$ L of fixative solution into each tube.

2. Recap each tube and vortex upright for 6 seconds. Run the sample tubes on the BD FACS Count instrument within 48 hours of adding fixative. Store samples at room temperature, protected from light, until they are run on the instrument.

### **Running Patient Samples**

Make sure you enter the patient accession number in the software before you begin.

1. Vortex the CD4 tube upright for 6 seconds. Warning: Inadequate suspension of white blood cells can result in inaccurate results.
2. Uncap the tube and set the cap aside.
3. Place the sample tube in the sample holder and press Run. A software message will indicate when the analysis is complete.
4. Remove the sample tube and recap it. Discard the sample tube in an appropriate biohazard container.
5. Repeat steps 1 through 4 for the remaining samples.

### **Expected Results:**

Important immunological evaluation includes:

- CD4 absolute count
- %CD4
- ✓ The reference ranges for BD FACS Count CD4 reagents for healthy adults between the ages of 18 and 65 years is given below.
- ✓ Absolute CD4 (cells/ul)=380-1704
- ✓ %CD4= 30.13–60.23

### **Interpretation of Results:**

- The lower the CD4 count, the more the disease has progressed.

## **Annex -X: SOP for Giemsa Staining Of Malaria Blood Films and Microscopy**

### **1. Purpose**

To describe the procedure for properly staining malaria blood films with Giemsa stain.

### **2. Introduction**

A properly stained blood film is critical for malaria diagnosis, especially for precise identification of malaria species. Use of Giemsa stain is the recommended and most reliable procedure for staining thick and thin blood films.

**3. Principle:** Giemsa solution is composed of eosin and methylene blue (azure). The eosin component stains the parasite nucleus red, while the methylene blue component stains the cytoplasm blue. The thin film is fixed with methanol. De-haemoglobinization of the thick film and staining take place at the same time. The ideal pH for demonstrating stippling of the parasites to allow proper species identification is 7.2.

### **Methods of staining**

#### **The rapid (10% stain working solution) method**

This is the commonest method for staining 1–15 slides at a time. It is used in outpatient clinics and busy laboratories where a quick diagnosis is essential for patient care. The method is efficient but requires more stain. The need for speed justifies the additional cost.

### **3. Supplies and Materials**

- Giemsa stain (10% solution) (See MM-SOP-04 for method of preparation);
- a small container or beaker for Giemsa working stain;
- absolute methanol, acetone-free;
- Pasteur pipette with a rubber teat;
- small container or beaker for methanol;
- staining rack;
- timer;
- slide-drying rack;
- protective latex gloves, powder-free, disposable and
- Distilled or deionized water buffered in PH 7.2.

## 5. Procedure

Prepare a 10% Giemsa working solution and place it in a small container.

- 1) Fix only the thin film with methanol.
- 2) Allow the blood film to dry in air on a drying rack or tray.
- 3) Place the slides facing up in a staining rack.
- 4) Pour stain slowly on the slides. Do not pour it directly onto the thick films.
- 5) Set the timer to 8-10 minutes and stain the blood film.
- 6) Remove each slide individually. Gently flush the stain from the slide by adding drops of buffered water until all the stain has been washed away.
- 7) Gently pour off the remaining stain, and rinse with clean water.
- 8) Carefully remove the slides, and allow them to dry in vertical position.
- 9) Discard the remaining 10% Giemsa solution..

## **Annex -XI: SOP for Malaria RDTs**

**Purpose:** To detect Plasmodium parasites which are responsible for malaria infections in human

### **Principle of the test**

It is based on an immunochromatographic technique. As the test sample flows through the membrane assembly, after addition of the clearing buffer, the colored colloidal gold conjugates Monoclonal anti-P.falciparum (HRP II specific) and monoclonal anti-pan(pLDH specific) complexes the HRP II/pLDH in the lysed sample. This complex moves further on the membrane to the region where it is immobilized by the monoclonal anti HRP II and monoclonal pLDH specific antibody coated on the membrane leading to formation of pink purple colored band, which confirms a positive result. Absence of colored band in the test region indicates a negative test result.

### **Materials and reagents**

- P.f/pan antigen test kit
- Specimen collection inverted cup & buffer

Specimen: Whole blood

### **Test procedure:**

1. Pull out the specimen and device, leave it on room temperature for 15 minutes before the test
2. Open the sealed pouch and take out the test device
3. Take 5ul of whole blood by inverted cup and drop the specimen in specimen insertion hole
4. Add 4 drops of buffer (approximately 120ul) and start the timer
5. Wait for 15-30 minutes and then read the results. Do not interpret the test result after 30 minutes.

### **Interpretation of Results**

1. Negative: a colored band is visible only in the control region
2. positive :
  - a) Positive for p.f: two colored bands are visible in the p.f region and control region
  - b) positive for pan: two colored bands are visible in the pan region and control region
  - c) Positive for p.f and pan: three colored bands are visible in the p.f region, pan region and control region. If there is no color in the control region, the result is invalid.

## **Annex-XII: SOP for ABO Blood group & Rh**

### **1. Purpose**

To determine the correct ABO and Rh group of an individual and ensure the reliability of the result

**2. Principle:** ABO system is the only system in which there is a reciprocal relationship between the antigens on the red cell and the naturally occurring antibodies in the serum. Routine grouping of donor and patient must therefore include both cell and serum tests, each serving as check on the other. The procedure is based on the principle of agglutination of antigen positive red cells in the presence of antibody directed towards the antigen.

### **3. Materials and Equipments Required:**

- Refrigerator to store samples and reagents at 2 – 8 °C
- Microscope
- Blood samples of donor/ patient.
- Anti-A, anti-B, anti-D
- Plastic pipette
- Glass slides for microscopic reading

### **4. Procedure:**

1. Label the slides with code number
2. Add 1 drop of well mixed hole blood on the 3 areas of the slide
3. Add 1 drops of anti-A, anti-B and anti-D reagent on the labeled slide containing 3 drop of blood respectively.
4. Gently mix the contents
5. Examine for agglutination within 2 minutes
6. Interpret & record test results

## **Annex -XIII: SOP for Cytokine measurements**

### **Introduction**

Cytokine sandwich ELISA is sensitive enzyme immunoassays that can specifically detect and quantitate the concentration of soluble cytokine and chemokine proteins.

### **Principle:**

The basic cytokine sandwich ELISA method makes use of highly-purified anti-cytokine antibodies (capture antibodies) which are noncovalently adsorbed ("coated"—primarily as a result of hydrophobic interactions) onto plastic microwell plates. After plate washings, the immobilized antibodies serve to specifically capture soluble cytokine proteins present in samples which were applied to the plate. After washing away unbound material, the captured cytokine proteins are detected by biotin-conjugated anti-cytokine antibodies (detection antibodies) followed by an enzyme-labeled avidin or streptavidin stage. Following the addition of a chromogenic substrate, the level of colored product generated by the bound, enzyme-linked detection reagents can be conveniently measured spectrophotometrically using an ELISA-plate reader at an appropriate optical density (OD). Data storage and reanalysis are greatly simplified when the plate reader is connected to a computer.

### **Quality control:**

A standard curve is incorporated into a sandwich ELISA assay by making serial dilutions of a standard cytokine protein solution of known concentration. Standard curves (also known as "calibration curves") are generally plotted as the standard cytokine protein concentration (typically nanogram or picogram of cytokine/ml) versus the corresponding mean OD value of replicates. The concentrations of the putative cytokine-containing samples can be interpolated from the standard curve. This process is made easier by using an ELISA computer software program. Generally, it is useful to perform a dilution series of the unknown samples to be assured that the OD will fall within the linear portion of the standard curve.

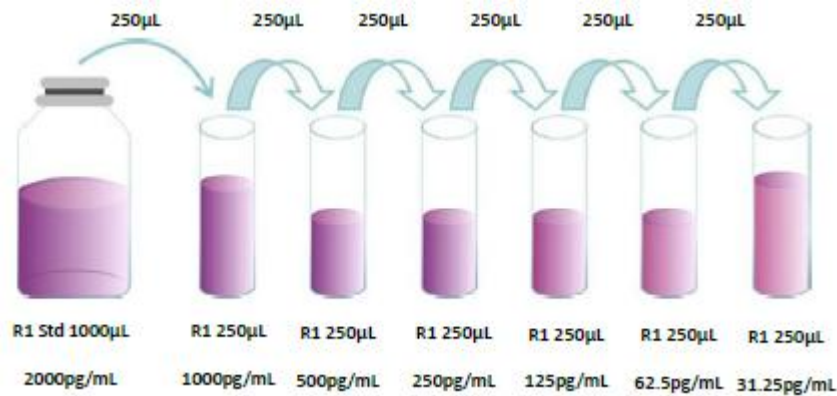
### **Materials:**

1. ELISA Reader
2. Pipettes and pipette tips: 0.5-10, 2-20, 20-200, 200-1000  $\mu$ L

3. Microplate washer, Squirt bottle
4. Micro-oscillator
5. Deionized or double distilled water, graduated cylinder
6. Polypropylene Test tubes for dilution
7. Troughs
8. Paper towel

Reagent Preparation:

- 1) Bring all reagents to room temperature before use. If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved.
- 2) Wash buffer: 1:20 diluted with double distilled or deionized water before use.
- 3) Biotin-Conjugate antibody: 1:100 diluted with the Biotin-Conjugate antibody Diluent (R2) before use, and the diluted solution should be used up within 30 min. (depends on the number of strips being used, example for 12 strips mix 120ul of biotin-conjugate concentrate with 11,880ul of R2, for 8 strips 80ul of concentrate with 7920ul of diluent(R2)).
- 4) Streptavidin-HRP: 1:100 diluted with the Streptavidin-HRP Diluent (R3) before use, and the diluted solution should be used up within 30 min. (depends on the number of strips being used, example for 12 strips mix 120ul of biotin-conjugate concentrate with 11,880ul of R2, for 8 strips 80ul of concentrate with 7920ul of diluent (R3)).
- 5) Standard: Add standard/sample dilution (R1) 1mL into freeze-dried standard, sit for a minimum of 15 minutes with gentle agitation prior to making dilutions (2000 pg/mL, 500pg/ml, 100pg/ml or 1000pg/ml), Prepare EP tubes containing Standard Diluent/sample dilution R1) , and produce a dilution series according to the picture shown below (the standard concentration may be different for different cytokine kits)



### Assay Procedure :

The procedure for all assayed cytokines (IL-4, IL-6, IL-17, TNF $\alpha$  and IFN $\gamma$ ) was the same.

- 1) Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
- 2) Add wash buffer 300  $\mu$ L/well; aspirate each well after holding 30 seconds, repeating the process three times for a total of four washes. Then use enzyme-marked plate in a short time, do not let it dry.
- 3) Add 100 $\mu$ L Standard /Sample Diluent (R1) in blank well.
- 4) Apart from blank well, add 100  $\mu$ l different concentrations of standard and sample in other wells, cover with the adhesive strip provided. Incubate for 2 hours at room temperature (20 to 25°C)
- 5) Wash the plate 5 times as in step 2.
- 6) Prepare the Biotin-Conjugate antibody Working Solution 20 minutes early.
- 7) Add Biotin-Conjugate antibody diluent (R2) in blank well and Biotin-Conjugate antibody Working Solution in other wells (100  $\mu$ L/well), cover with adhesive strip provided, shake with Micro-oscillator (100 r/min). Incubate for 1 hours at room temperature (20 to 25°C)
- 8) Prepare the Streptavidin-HRP Working Solution 20 minutes early, place away from light at room temperature.
- 9) Wash the plate 5 times as in step 2.
- 10) Aspirate Streptavidin-HRP diluent (R3) in blank well and aspirate Streptavidin-HRP Working Solution in other wells (100  $\mu$ L/well), cover with adhesive strip provided, shake with Micro-oscillator (100 r/min). Incubate for 30 minutes at room temperature (20 to 25°C)

- 11) Warm-up the ELIASA.
- 12) Wash the plate 5 times.
  
- 13) Aspirate substrate Solution (100  $\mu\text{L}$ /well). Incubate for 20 minutes at room temperature under dark.
- 14) Aspirate Stop Solution (100  $\mu\text{L}$ /well), mix, determine the optical density of each well within 5 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 570 nm or 630 nm. If wavelength correction is not available, subtract readings at 570 nm or 630 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

## Declaration

I, the undersigned, declare that this M.Sc. 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 thesis have been duly acknowledged.

**M.Sc. candidate: Aytenuw Atnaf (B.Sc.)**

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Date of submission: \_\_\_\_\_

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