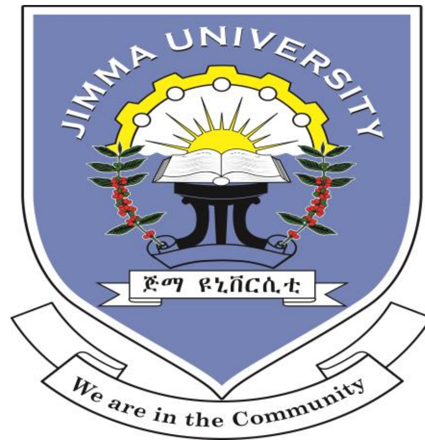
HEMATOLOGICAL PARAMETERS AND HEMOZOIN-CONTAINING LEUKOCYTES IN CHILDREN WITH MALARIA: ASSOCIATION WITH DISEASE SEVERITY AT PAWE GENERAL HOSPITAL, NORTH WEST ETHIOPIA



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ABSTRACT

Background: *Hematological changes are some of the most common complications in malaria. Severe malarial anemia is the most prominent one particularly in children and is associated with higher prevalence of hemozoin-containing monocytes.*

Objective:*To determine the hematological parameters and hemozoin-containing leukocytes in children with malaria and their association with disease severity at Pawe General Hospital, Northwest Ethiopia.*

Methods:*A facility-based across sectional study was conducted at Pawe General Hospital from July 30 to December 31, 2014. A total of 377 malaria infected children aged from 1 to 15 years were recruited for the study. Demographic and clinical data were collected using structured questionnaire. 4 ml venous blood was collected from each patient for complete blood count and blood film preparation. Thick blood film was stained by 10% Giemsa for malaria parasite detection and to determine parasitemia whereas intraleukocytic malaria pigment was detected and enumerated from thin films. The complete blood count was analyzed using CELL-DYNE 1800® (Abott diag, USA). In addition children were screened for HIV-1/2 status and intestinal parasites. Statistical analysis of the data (student's t-test, chi square, ANOVA and Pearson correlation) was done using SPSS V-20 statistical software.*

Results:*Anemia and thrombocytopenia were found in 40.3% and 56.8% of malaria infected children whereas leukocytosis and leukopenia in 15.4% and 10.3% of malaria infected children, respectively. Hemozoin containing -monocytes and -neutrophils were found in 80.1% and 58.9% of the study subjects, respectively. Anemia (x^2=17.8, p<0.001), leukocytosis (x^2=11.0, p=0.001), HCM (x^2=19.72, p<0.001) and HCN (x^2=7.91, p=0.005) were significantly associated with severity of malaria. However, thrombocytopenia (x^2=0.2, p=0.623), severe thrombocytopenia (x^2=0.035, p=0.851) and leukopenia (x^2=3.0, p=0.09) were not associated with severity of malaria. The mean percentage of monocytes-containing hemozoin was significantly associated with the severity of malarial anemia (p<0.001). Parasitaemia was negatively correlated with hemoglobin (r=-0.117, p=0.023) and positively with white blood cell count (r=0.21, p<0.001), mean hemozoin containing–monocytes (r=0.343, p<0.001) and hemozoin containing–neutrophils (r=0.414, p<0.001), but was not correlated with platelet count (r=-0.050, p=0.335).*

Conclusion: *Anemia, thrombocytopenia, hemozoin-containing monocytes and hemozoin-containing neutrophils were the most common hematological abnormalities in children infected with malaria. Anemia, leukocytosis, hemozoin-containing monocytes and hemozoin-containing neutrophils were significantly associated with severe malaria. Furthermore, mean percentage of HCMs ≥16.2 and HCNs ≥2.7 may be used as indicators of severe malaria. Beside parasite density; findings of anemia, leukocytosis, the presence and quantities of hemozoin-containing monocytes and hemozoin-containing neutrophils were hematological indictors suggestive of severe malaria in children residing in malaria endemic areas.*

Key words: *Hematological parameters, hemozoin, malaria, children, Ethiopia*

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ABBREVIATIONS AND ACRONYMS

CI: Confidence interval

CM: Cerebral malaria

Hb: Hemoglobin

HCM: Hemozoin-containing monocyte

HCN: Hemozoin-containing neutrophil

Hct: Hematocrit

Hz: Hemozoin

MA: Malarial anemia

OR: Odds ratio

PCV: Packed cell volume

PLT: Platelet

PRBC: Parasitized red blood cell

RBC: Red blood cell

SD: Standard deviation

SM: Severe malaria

SMA: Severe malarial anemia

UM: Uncomplicated malaria

WBC: White blood cell

OPERATIONAL DEFINITIONS

Anemia: Hemoglobin value below 11g/dl (1).

Children: - Individuals aged one to fifteen years.

Fever: -Axillary temperature ≥ 37.5^0C (1).

Hematological abnormalities: - Refers to anemia, thrombocytopenia, leukopenia, leukocytosis, hemozoin-containing monocytes (HCMs), and/or hemozoin-containing neutrophils (HCNs).

Hematological parameters: Refers to red blood cell (RBC) count, hemoglobin (Hb), hematocrit (Hct), platelet (PLT), and white blood cell (WBC) counts.

Hemozoin-containing leukocytes: - Leukocytes (neutrophils and/or monocytes)-containing amber to black-brown solid looking granules of malaria pigment in Giemsa-stained thin blood films.

Leukopenia: -WBC count less than 4.0 x 10^9/L (2).

Leukocytosis: -WBC count> 11.0 x 10^9/L (2).

Mild anemia: - Hemoglobin value between 8 and 10.9g/dl (1).

Mild thrombocytopenia: -PLT count between 100 and 149 x 10^9/L (2).

Moderate anemia: - Hemoglobin values between 5 and 7.9g/dl (1).

Moderate thrombocytopenia: - PLT count between 50 and 99 x 10^9/L (2).

Severe anemia: -Hemoglobin value less than 5g/dl (1).

Severe thrombocytopenia: - Platelet count below 50 x10^9/L (2).

Thrombocytopenia: - Platelet count less than 150 x10^9/L (2).

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CHAPTER ONE: INTRODUCTION

1.1. Background

Blood is a fluid connective tissue that transports oxygen from lungs to all part of the body and carbon dioxide back to the lungs. It contains three types of cells: the red blood cells (RBCs), white blood cells (WBCs) and platelets (PLTs) (3). Hematological parameters are those measurable indices of the blood that serve as a marker for disease diagnosis. Changes in hematological parameters can be caused by any disease condition which affects the hematopoietic physiology at any level. This occurs with an endemic disease such as malaria (4). Malaria is both an acute and/or chronic infection of the blood stream caused by protozoan parasites of the genus *Plasmodium* and transmitted by the female *Anopheles* mosquito. Five *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*) are known to cause malaria in human (5).

The clinical symptoms of malarial infection are primarily due to schizont rupture and destruction of erythrocytes that results in the release of merozoites and parasite products into the circulation which stimulate phagocytes and other cells to produce cytokines and other soluble factors which act to produce fever, chills, headache and other symptoms that characterize the disease and probably influence other severe pathophysiologic conditions associated with malaria (6). The pathogenesis of malaria is multifactorial. Serious sequel can result from three primary pathophysiological events; RBC destruction, the adhesion of parasitized RBCs (PRBCs) to vascular endothelium of various organs, and an excessive proinflammatory response. Severe malarial anemia (SMA) and cerebral malaria (CM) are the most common complications of malaria in children (7, 8).

Hematological changes are some of the most common complications in malaria and they play a major role in the outcomes of the disease. These changes involve the major cell lines: the RBCs, WBCs and PLTs. Hematological changes such as anemia, thrombocytopenia, and leukocytosis or leukopenia have been well recognized in patients infected with malaria. The extent of these alterations varies with the level of malaria endemicity, background hemoglobinopathy, nutritional status, demographic factors, and malaria immunity (9, 10).

Anemia, which is a reduction in RBC mass or number below the normal for age, sex, environment or pregnancy status, is an inevitable outcome of malarial infection as parasitized RBCs are destroyed during schizgony. Malarial anemia (MA) is the most common cause of morbidity and mortality in malaria infection, particularly in pregnant women and in children. The rate of development and degree of MA depend on the severity and duration of parasitemia. The potential mechanisms contributing to MA can be divided into two categories: increased destruction of parasitized and un-parasitized erythrocytes (immune-mediated lysis, phagocytosis, splenic sequestration) and decrease of RBC production (dyserythropoiesis and bone marrow suppression, inadequate reticulocyte production, effects of inflammatory cytokines, and effects of parasite factors) (11, 12).

MA is the most prominent hematological abnormality in malarial infection particularly in children and pregnant women. In malaria endemic areas, the prevalence and severity of MA are usually determined by a number of interacting factors. These include the parasite species, level of parasitemia, age of host, host genetic factors [e.g., coexisting RBC polymorphisms like hemoglobinopathies, glucose-6-phosphate dehydrogenase deficiency (G6PD)], and nonmalarial causes of anemia such as infections [e.g., bacteremia, human immunodeficiency virus (HIV), hookworm] and malnutrition (13).

SMA is the primary clinical manifestation of severe childhood malaria. It is a life-threatening complication of falciparum and vivax malaria in areas of high transmission and is highly prevalent in non-immune infants and young children. It may result from repeated infections, in which case the asexual parasitemia is generally low but there is abundant malaria pigment in monocytes and other phagocytic cells, reflecting recent or resolving infection. In holoendemic transmission areas chronic MA is more common and dyserythropoeitic changes in the bone marrow are prominent. SMA also develops rapidly after infections with high density parasites. In these cases, acute destruction of parasitized RBCs (PRBCs) is responsible for the anemia (14, 15). In children, anemia can negatively affect cognitive development, school performance, physical growth, and immunity (16).

Hemozoin (Hz), also known as malaria pigment, is the end product of hemoglobin (Hb) digestion by *Plasmodia*, during which a potentially toxic heme molecule is transformed into an insoluble polymer, called Hz and is released into host circulation during schizogony. Both circulating and resident phagocytes acquire Hz through phagocytosis of PRBCs or free Hz crystals released after schizont rupture. Several studies have demonstrated that the acquisition of Hz by circulating monocytes and neutrophils is significantly associated with malarial severity (17−19).

Given that the typical half-life of a neutrophil is 6–8 hours and that of a monocyte is several days, the quantity and distribution of engulfed pigment within these phagocytic cells may reflect the chronology of a patient's infection. As such, the presence of pigmented neutrophils, with the rapid turnover of neutrophils, may indicate a recent heavy parasitic burden and provide prognostic indications of disease, while longer-lived pigmented monocytes with longer clearance rates may reflect a more protracted infection or repeated infections (20, 21).

An important feature of MA in humans and experimental animals is low reticulocytosis, indicating reduced RBC output. One important cause of impaired erythroid responses in children with SMA is dysregulation in innate immune response. Phagocytosis of Hz by monocytes, macrophages and neutrophils is a central factor for promoting dysregulation in innate inflammatory mediators. The Hz-monocyte complex has been associated with SMA, immunosuppresssion, and cytokine dysregulation. Hz directly promotes SMA through enhancing increased destruction of PRBCs and non-PRBCs, and through inhibition of erythropoiesis. It catalyzes the production of free radicals, reduces the deformability of both PRBCS and non-PRBCs and their life-span. The prevalence of Hz-containing monocytes (HCMs) is thus one of the strongest predictors of risk for developing SMA (22−24).

Thrombocytopenia is one of the most common hematological complications in malaria. Malaria is strongly associated with various degrees of PLT counts, such as mild, moderate and severe thrombocytopenia (25).Thrombocytopenia in malaria is associated with high parasitemia levels, lower age, low Hb levels, increased mean PLT volume (MPV) and PLT aggregate flag (9). Thrombocytopenia is thus a common finding in malaria and the severity of thrombocytopenia is also an indicator of both disease severity and prognosis in childhood malaria (26−28).

Both non-immunological as well as immunological destruction of PLTs have been implicated in causing malarial thrombocytopenia. The speculated mechanisms are PLT activation and aggregations, sequestration in spleen, antibody mediated PLT destruction, bone marrow alterations and the role of PLTs as cofactors in triggering severe malaria (SM). PRBCs can also bind to PLTs (via CD36) and PLTs can mediate the adhesion of PRBCs to the endothelium of critical microvasculature (29).

Changes in blood cell counts are a well-known feature of malaria and leukocytic changes in malaria are in general variable and depend on many factors such as acuteness of infection, parasitemia, disease severity, state of the host immunity to malaria, and concurrent infections (30). The leukocyte count is usually low (mostly due to low lymphocytes and eosinophils) to normal, but it may be increased (especially neutrophils and monocytes) in severe infections. In fact leukocytosis is strongly found to be associated with younger age, deep breathing, severe anemia, thrombocytopenia and death – irrespective of bacteremia (31). In addition, significant dysfunctions of leukocytes have been observed during both human and murine malaria. Monocytic cell dysfunctions such as inhibition of oxidative burst, inability to repeatedly phagocytose Hz, and abrogation of the ability to kill ingested bacteria that typically start 4-6 hours after completion of phagocytosis of Hz and which may persist for several days has also been demonstrated (32).

Detection of Hz within phagocytic cells such as monocytes and neutrophils is observed to be an important tool for the diagnosis of malaria and considered as one of the signs of severity (18−20, 33). It is easily visible by light microscopy, appearing as a retractile black-brown or amber pigment (20). Intraleukocytic Hz can also be detected by more advanced techniques such as flow cytometry, spectrophotometer and polarizing microscope which are more expensive and time-consuming (34). As such, the detection and enumeration of intraleukocytic malaria pigment by light microscopy is a rapid, simple and practical prognostic method for determining malaria severity and disease outcomes (33).

1.2. Statement of the problem

SMA is a significant cause of morbidity and mortality in children below five years of age. It accounts for between 26% and 62% of SM admissions in malaria endemic countries and up to 29% of total hospital admissions as reported in Nigeria and Kenya. Hospital based data of deaths from MA ranges between 11.2% in Sierra Leone and 14% in Kenya for children below 5years (35). MA is also a major public health problem in Ethiopia. Ethiopia National Malaria Indicator Survey (2011) reported that MA affects 43.4% of under-five children, with moderate and severe anemia affecting 8.6% and 0.9% of the children, respectively (36). Reported malaria in-patient admissions (/10,000) and deaths (/100,000) with SMA were 0.720 and 0.387, respectively (37). Health facility based studies reported that the prevalence of SMA in children with SM ranges from 13.3% in Kersa and Halaba Kulito (38) to 25.7% in Awash, Metehara and Ziway (39) and 37.8% in Gambella (40).

Acute malaria is often associated with mild or moderate thrombocytopenia in non-immune adults and in children from malaria-endemic areas and is a sensitive but non-specific indicator of infection with malaria parasites (41).The extent of the thrombocytopenia is also a predictor of both outcome and severity in children (42). The association of thrombocytopenia with malaria infection is well recognized, but prevalence varies with levels of malarial endemicity and immunity, age, malarial species and malarial severity. In non-endemic countries such as United Kingdom, France, Sweden, studies done showed a varying prevalence rate of thrombocytopenia in malaria to be between 64% - 87.3%. In a study in Dakar, Senegal where malarial transmission is hypo-endemic, a prevalence of 56.2% was seen. In holoendemic countries like Pakistan, India, Kenya, Cameroon, and Nigeria, reported prevalence rates ranged between 13% and 90% (43).

Malaria also induces both numerical and functional changes in the WBCs. The changes in WBCs are less dramatic than other blood cell series, but they remain a significant index of disease progression and ultimate prognosis (44). A large study in Kenyan children with moderately severe or severe *falciparum* malaria reported that the prevalence of leukopenia and leukocytosis was ~10% and 20%, respectively (45) while another large study in Burkina Faso reported 15.4% prevalence of leukocytosis with higher prevalence in those with SM (19.0%) than in those with uncomplicated malaria (UM) which was 12.7% (46).

Hz is a product of Hb digestion by *Plasmodia* and the acquisition of Hz by circulating monocytes and neutrophils is significantly associated with malarial severity (23).A large multicenter study conducted in African children hospitalized with SM reported that the prevalence of mean HCMs and Hz-containing neutrophils (HCNs) was 4 % and 2%, respectively (47). Similarly, study conducted in Gabon (48), Nigeria (18), and Mali (20) reported that the prevalence of mean HCMs and HCNs in children with UM and in those with SM were (15.3% vs. 25.4%) and (7.5% vs. 15.7%), (17% vs. 53%) and (9% vs. 27%), and (5% vs.14%) and (2% vs. 4%), respectively.

Hematological abnormalities are considered a hallmark of malaria bearing an impact on final outcome and representing indices of prognostic and follow-up values (49). Leukocytosis (WBC $>12\times10^9$/L); severe anemia [hematocrit (Hct) < 15%]; thrombocytopenia (PLT $<50\times10^9$/L); hyperparasitemia (>100,000/µL (high mortality > 500,000/µL)); >20% of parasites are pigment-containing trophozoites and schizonts; and > 5% of neutrophils contain visible malaria pigment are identified as hematological indictors of poor prognosis in patients with SM (50).

Although there are several factors that increase mortality due to *falciparum* malaria in Ethiopia, the absence of reliable diagnosis and management of severe malaria is the most important. In almost all of the health centers in Ethiopia, severe malaria has been diagnosed by counting the number of *Plasmodium* parasites found in peripheral blood of the patients alone (39). However, peripheral parasitemia alone is a poor indicator of disease severity and in many cases the severity of MA does not directly correlate with the level of circulating parasitemia (51).

Nevertheless, in children with malaria, the prevalence of HCM is significantly associated with SMA (23).In addition; thrombocytopenia (27, 28) and leukocytosis (28, 45−46) have been found to be associated with SM. However, some studies reported the lack of association of PLT (48, 52) and WBC (52, 53) counts with malaria severity. Moreover, the presence and extent of hematological abnormalities in children with malaria and their association with disease severity were not well studied in Ethiopia. Hence this study was sought to determine the hematological parameters and hemozoin containing leukocytes in children with malaria and their association with disease severity at Pawe general hospital.

1.3. Significance of the study

The findings can assist in early recognition of patients at risk of severe disease. These could be useful in early management of malarial complications and for timely institution of specific therapy. These could be further useful for reducing morbidity and mortalities resulting from malaria, particularly in the most vulnerable group, children. In addition, the findings could bridge the knowledge gap on hematological parameters and hemozoin containing leukocytes in children infected with malaria in Ethiopia. Besides the findings will be used as a base line for those who are interested in the area for further research.

CHAPTER TWO: LITERATURE REVIEWS

Malaria is one of the most important public health problems in the world, with about 3.4 billion of the world's population at risk of the disease in 2013. It caused an estimated 627,000 deaths and 207 million cases in 2012; of which 90% of the deaths and 80% of the cases occurred in the African Region, mainly in children under five years of age. An estimated 482,000 malaria deaths occurred in children less than 5 years of age in 2012, of which 97% of the deaths occurred in the African Region. Most of the deaths were due to *P. falciparum*; however, *P. vivax*is increasingly recognized as a cause of severe malaria and death (54).

Malaria is a major public health problem in Ethiopia, where 68% of the population is at risk. It is one of the three leading causes of morbidity and mortality. In 2009/2010, malaria was the leading cause of outpatient visits and health facility admissions, accounting for 14% and 9%, respectively. An estimated 5 to 10 million clinical malaria cases occurs each year. *P. falciparum* and *P. vivax* are the dominant species in Ethiopia, in respective order (55). Approximately, an estimated 47,507 malaria deaths occurred in Ethiopia in 2010 in the general population, of which 22,165 were among children under-five years of age (56). According to Integrated Disease Survillance and Response (IDSR) report of Ethiopia for July 2004 to June 2009, the highest annual incidence of total outpatient malaria cases (clinical and confirmed) by reporting unit (zone) was reported in Pawe woreda which was 535.2 per 1000 per year (37).

Hematological changes, which are the most complications in malaria, play a major role in fatal complications of the disease. They include anemia, thrombocytopenia, leukocytic changes followed by the induction of cytokines and splenomegaly (13). In general, the extent and nature of these changes depends on the level of malaria endemicity, parasite species, disease severity and the immune status of the individual (30).

A retrospective study conducted to determine the epidemiology and clinical features associated with acquisition of SM in children in Houston Texas from January 1994 to December 2007 showed that children with SM (n=21) had significantly higher median parasitemia (11.2% vs.1.5%, $p<0.001$), and lower Hb (6.5g/dl vs. 9.5 g/dl, $p \leq 0.001$) and PLT count (36×10^3/µl vs. 97×10^3/µl, $p< 0.001$) than children with UM (n=83). The median age of the children was 7.2 years (57).

Another clinico-epidemological study conducted on anemia and thrombocytopenia in children hospitalized with *P. vivax* malaria at Santos Anibal Domincci hospital in Venezuela between January 2000 and December 2002 showed that anemia was found in 94.87% of the children; out of which 10.26% had severe anemia (Hb< 5 g/dl). 58.97% of the children had also thrombocytopenia, out of which 24.36% had severe thrombocytopenia (PLT < 60,000/µl). 25.64% of the children had malnutrition and 10.26% had intestinal parasitosis ($p < 0.05$). There was no difference in the occurrence of anemia and thrombocytopenia in relation to age. The mean age of the children was 3.97±3.54 years (58).

A prospective study conducted to assess thrombocytopenia during acute *P. falciparum* malaria in children aged 6 months to 15 years living in areas with different exposures (64 traveler children from France, 85 from Senegal with intermittent exposure and 81 from Gabon with perennial exposure) between 1999 and 2000 showed that 45.6% and 8.8% of the children in Paris, 43.6% and 14.1% of the children in Dakar, and 58% and 14.8% in Libreville had thrombocytopenia (PLT count <150 000/µl) and severe thrombocytopenia(PLT count <50 000/µl), respectively. There was no association between thrombocytopenia and disease severity. There was no difference in splenomegaly rate between children with and without thrombocytopenia (59).

A retrospective study conducted to assess the occurrence and severity of thrombocytopenia in children infected with malaria in India from January 2010 to June 2011 showed that 66% (70/106) of the children with *P. vivax* malaria had thrombocytopenia; out of which 22%, 31% and 13% had mild, moderate and severe thrombocytopenia, respectively. Similarly 80% (21/26) of the children with *P. falciparum* malaria had thrombocytopenia; out of which 15%, 27% and 39% had mild, moderate and severe thrombocytopenia, respectively. 81% (22/27) of the children with mixed malaria had thrombocytopenia: out of which 30%, 33% and 19% had mild, moderate and severe thrombocytopenia, respectively. There was no association between thrombocytopenia and age. The mean age of the children was 9±4 years (60).

A cross-sectional study conducted to determine the clinical and hematological patterns of *P. vivax* malaria in children aged 5 to 14 years in Pakistan from April to October 2010 showed that 91% (137/150) of the children had thrombocytopenia, out of which 73% had PLT count $<100 \times 10^9$/L and 27% had $<50 \times 10^9$/L. Only 4 patients had PLT count $<20 \times 10^9$/L.

Anemia (Hb< 10g/dl) was found in 73% of the children, out of which 12% had Hb value <5g/dl and 18% had Hb value between 5–7 g/dl. Leukopenia (WBC<4 x10^9/L) was found in 39% of the children (61).

Data on age, parasitemia, hematological parameters and treatment outcomes were extracted from a database of seven randomized control trials (RCTs) conducted in sub-Saharan Africa. Analyses were restricted to children less than five years of age with uncomplicated *P. falciparum* malaria and the result showed that at presentation, leukocyte and neutrophil counts were higher in children with higher parasitemias (r = 0.20 for WBC and r = 0.30 for neutrophil, p = 0.001), and there was no significant interaction between age and parasitemia for both leukocytes (p= 0.057) and neutrophils (p= 0.72). Age, parasitemia, and their interaction were not significantly related to Hb or PLT counts (62).

A study conducted in Ghana to investigate the deregulated TNF and IL-10 production in malaria and their role in the pathogenesis of SMA in children aged 1 to 12 years with non-overlapping SMA (n = 108), CM (n = 144) or UM (n = 80) syndromes from July to August in 2001 and 2003 showed that age, gender, parasitemia, and leukocyte counts did not differ between groups (p > 0.05). The median age (years) of the children with SMA, CM and UM was (3.5 vs. 3.1 vs. 3.8), respectively (53).

A study conducted to determine the impacts of *P. falciparum* malaria on hematological parameters in children in western Kenya from March to December 2005 showed that the prevalence of anemia (Hb< 10g/dl) and SMA in malaria infected children was 60% and 3%, respectively. There was poor correlation between parasitemia and Hb (r=–0.08, p=0.78). Thrombocytopenia and severe thrombocytopenia (PLT<50,000/µl) was present in 49% and 5% of malaria-infected children, respectively. Thrombocytopenia was associated with parasitemia (r=–0.44, p<0.0001), age (r= 0.14, p=0.0073), and anemia (r=0.2, p=0.0003). 53% of children with parasitemia above 10% had a PLT count <50,000/µl. The prevalence of leukocytosis in malaria infected children was 8%. WBC count was not associated with parasite density. A total of 961 children (523 malaria-infected and 438 uninfected controls) that were participated in vaccine trial were studied. The mean age of the children was 32 (5-48) months (9).

Another study conducted to determine the changes in WBC and PLT counts in children with *falciparum* malaria at Kilifi district hospital Kenya between January and December 2000 showed that leukocytosis (WBC > 16.5 $x10^9$/L) was occurred in 20.1% of the children with malaria and was associated with prostration, coma, deep breathing, hyperparasitemia, severe anemia and death; p<0.0001 for all variables, but was not associated with bacteremia (p=0.09). Leukopenia (WBC< $6.1x10^9$/L) occurred in 10.2% of children with malaria and was associated with thrombocytopenia (x^2= 55.4, p < 0.0001), absence of hyperparasitemia (x^2= 11.9, p =0.001) and absence of deep breathing (x^2=4.36, p = 0.037), but not with mortality (x^2= 1.26,p = 0.26). Thrombocytopenia was found in 56.7% of the children with malaria and 11.6% of the children had PLT count <$20x10^9$/L. Thrombocytopenia was positively associated with age (x^2 = 28.2, p < 0.0001) and parasite density (x^2 = 113.3, p < 0.0001), but it was not associated with severe anemia, bleeding problems or mortality (45).

A prospective cross sectional study conducted in Nigeria from October 2010 to March 2011 to determine the prevalence of thrombocytopenia in children aged 6 months to 15 years with *falciparum* malaria infection showed that 12.8% of the children had PLT counts < 150 x 10^9/L with significant higher prevalence in those with SM (54.2%) than in those with UM (6.4%), p < 0.001. Only one child (0.6%) had PLT counts < 50 x 10^9/L. A total of 180 children with malaria were studied (43).

A similar study conducted in Nigeria on age as risk factor for thrombocytopenia and anemia in 695 children aged 6months to 14 years with acute uncomplicated *P. falciparum* malaria showed that anemia (Hct<30%) occurred in 43.8% of the patients with 58% in children <5 yr old and 21% in those >5 yr. There was no difference in Hct value between both sexes. Hct values correlated with age, r = 0.4, p <0.0001; but not with parasite density (r = –0.01, p = 0.73) or temperature (r =0.05935, p = 0.1556). Leukocytosis (WBC >$11x10^9$/L) was more frequently seen than leukopenia (WBC<$4x10^9$/L) (9.5% *vs.* 3%, respectively). The WBC count was not affected by age or gender. Leukocyte count showed no correlation with age, parasite density or temperature. Thrombocytopenia was present in 59.3% of subjects; of whom 1.9% had PLT count <50 x 10^9/L. PLT count showed no correlation with age, parasite density or temperature (2).

A prospective study conducted to investigate the prognostic significance of thrombocytopenia in 288 African children with *falciparum* malaria (215 SM and 73 UM cases) living in Dakar, Senegal showed that thrombocytopenia (PLT<150 x 10^3/μl) was significantly higher in children with SM (68.4%) than in those with UM (56.2%), (p<0.02). The median PLT count was significantly lower in children with SM than in those with UM (p<0.02), and in those who died than in those who recovered (p<0.002). Parasitemia had negative (p < 0.0001) and age had positive association (p < 0.01) with thrombocytopenia. In those with CM, thrombocytopenia was more frequent (71.4% vs. 37.5%; p<0.003) and pronounced (median PLT count = 79,000/μl vs. 128,000/μl; p<0.02) among children who died than in those who recovered. Logistic regression identified thrombocytopenia (PLT<100 x 10^3/μl) as an independent predictor of death (OR=13.3, 95% CI (3.2–55.1). The effect of thrombocytopenia on fatality was not affected by gender, age, parasitemia, or presumed iron deficiency. The median age of the children was 7.5 years (27).

A prospective study conducted in Uganda to assess the clinical description of SM in1,933 children aged 6 months to 12 years showed that children with SM (n = 855) had significantly lower median PLT and higher WBC counts than children with UM (n = 1078); p < 0.0001. Severe thrombocytopenia and leukocytosis were found in 10% and 27% of UM and in 19% and 75% of the SM cases, respectively, with significant differences (p < 0.0001). SMA was observed in 65%of children with SM and was associated with younger age, leukocytosis and monocyte count (p < 0.0001). Children with CM were significantly older, had a higher median Hb and a lower PLT count than children without CM (p <0.0001). CM was associated with death and severe thrombocytopenia whereas hyperparasitemia was associated with lactic acidosis and severe thrombocytopenia. Logistic regression identified five factors independently associated with death: CM, hypoxia, severe thrombocytopenia, leukocytosis, and lactic acidosis (28).

A longitudinal study conducted on anemia in children with *Plasmodium falciparum* infection in the Mount Cameroon region and its prevalence, risk factors and perceptions by caregivers from January to November 2006 showed that anemia (Hb<11g/dl) was found in 80.3% (n=282) of the children; out of which 22.7% (n=64), 65.2% (n=184) and 12.1%(n=34) had mild, moderate and severe anemia, respectively. Children ≤5 years had a significantly (p< 0.01) higher prevalence of anemia when compared with those greater >5 years. Similarly the prevalence of anemia in

children with enlarged spleens was significantly ($p< 0.01$) higher than those with normal spleen. Although not significant ($p>0.05$) children who were malnourished had a higher prevalence of anemia than their counter parts.Hb was negatively correlated with parasite density (rs= -0.155, p=0.002) and WBC count (rs= -0.192, p=0.0001). The mean age of the children was 6.45 $\pm$ 7.9 years (63).

A large epidemiological study conducted in Burkina Faso to assess leukocytosis as a risk factor for mortality in various types of SM in 1719 children with malaria (736 had SM and 983 had UM)showed that the overall prevalence of leukocytosis was 15.4% with significant differences between SM (19.0%) and UM (12.7%); p = 0.0004. Those with leukocytosis had lower mean age than those without in each group (3.79vs. 4.52 years in SM, p = 0.0002; & 3.43 vs. 4.30in UM, p = 0.0001); no association was observed between leukocytosis and *P. falciparum* parasite density. Among SM patients, after age stratification, leukocytosis was associated with severe anemia (p < 0.00l), hypoglycemia (p < 0.00l) and with a 3.5-fold increase in mortality [34.2% (40/117) vs. 9.7% (50/514); Relative Risk = 3.51; p < 0.00l] (46).

A study conducted on the association of severe malaria outcomes with PLT- mediated clumping and adhesion to a novel host receptor in children less than 5 years of age residing in a malaria-endemic area of Mozambique showed that SM cases (n=46) had significantly lower Hct (p=0.019) and higher parasitemia (p=0.007) than UM controls (n=46). However, the median PLT count (p=0.164), WBC count (p=0.059), age (p=0.871) and axillary temperature (p=0.880) was not significantly different between the two groups (52).

A study conducted at Kersa and Halaba Kulito districts in southern Ethiopia to assess the prevalence of SM complications related to *P. vivax* malaria in children aged ≤ 10 years showed that the prevalence of severe *P. vivax* malaria was 13.67% (19/139). The prevalence of anemia was 43.2% (n=60), out of which 60%, 26.7% and 13.3% were mild, moderate, and severe, respectively, and majority of SMA cases (7/8) were occurred in children < 3 years. In terms of sex, female children had significantly higher risk of anemia (OR = 1.91). Pearson correlation analysis revealed that age of children was negatively correlated to parasite count ($r = -0.2358$, $p < 0.05$) but had positive correlation to Hb levels ($r = 0.31$, $p< 0.001$). However, there was no

significant differences ($r = 0.057$, $p > 0.05$) between Hb level and parasite count. Parasite counts of all children with severe anemia were found in the range of 1000–9999 parasites/µl. The median age of children was 4.25 ± 2.95 years (38).

Intraleukocytic malaria pigment is a useful direct diagnostic marker with the amount of phagocytosed pigment being a good indirect measure of the sequestered parasite burden, recent schizogony, and disease severity (64). The prevalence of HCM in the circulation is one of the strongest predictors of risk for developing SMA and the presence of HCM is associated with ineffective erythropoiesss and/or suppression of erythropoiesis in children with malaria (16−17, 22). Moreover, in young children with malaria, assessment of Hz-containing leukocytes serves as a prognostic marker for disease severity and progression (34).

A multicenter study conducted in the Severe Malaria in African Children (SMAC) network to assess the prognostic value of circulating pigmented cells in 26,296 African children aged one-to-180months hospitalized with *P. falciparum* malaria at six sites in five countries showed that overall 37% and 63% of subjects had HCN and HCM, respectively, with median percentage of cells with HCN and HCM were 2% and 4%, respectively. HCN and HCM were each correlated with parasitemia (rs: 0.25–0.44 and 0.17–0.57, respectively), with the weakest correlations observed in Blantyre. The percentage of PM was negatively correlated with Hct (rs: –0.16 to –0.40).There was a significant association between increased HCN (> 5%) and fatal outcome, which was significant across all sites except for Kilifi ($p = 0.14$) when assessed by crude ORs of 13.6 for Lambaréné, 12.0for Blantyre, 3.0 for Kumasi, 2.8 for Banjul and 2.8 for Libreville; $p < 0.01$. Adjusted ORs maintained this association only in Blantyre, Kumasi and Lambaréné (47).

A study conducted in Lambaréné, Gabon, in 2003 and 2004 to investigate the full blood count and Hz-containing leukocytes in children with malaria showed that 65% of the children had thrombocytopenia. In addition, 91.3% and 100%of the patients with UM and SM had HCM whereas 56.1% and 91.7% had HCN, respectively. 80% of SMA and 100% of CM patients had HCN. Although children with SM (25.4) had higher number of mean HCM than those with UM (19.3), the difference was not significant ($p = 0.14$). In contrast to this, HCN was significantly higher in the SM (15.7) than in UM (7.5) group; $p <0.0001$. Children with SMA (39.9) had higher numbers of HCM than those with CM (23.3) or hyperparasitemia (14.8) whereas HCN

was marginally higher in CM (18.0) than in SMA (15.3) or in hyperparasitemia (16.3).HCM and HCN showed an accuracy of 0.62 and 0.75 for diagnosing SM, respectively. A total of 152 children with falciparum malaria (104 with UM and 48 with SM) were studied and their mean age was 3.7 years (48).

A study conducted in Kenya to assess impact of Hz on hematological outcomes and inflammatory mediators in infants and young children (0-3yrs) with varying degrees of MA showed that 48.8% of the children had HCM with 19.8% and 29% had low (≤10%) and high (>10%) mean HCMs. Children with high HCM had the lowest Hb, Hct, and RBC counts and highest RDW ($p < 0.0001$). In addition, the percent of study participants diagnosed with SMA was significantly different among HCM categories ($p< 0.0001$), and the greatest proportion with SMA (61.49%) were in the > 10% HCM group. Parasitemia was significantly associated ($p = 0.002$) with % of HCM and increased with increasing % HCM. The clinical groups were healthy control (n=63), UM (n=36), mild MA (n=146), moderate MA (n=167), and SMA (n=236) (65).

Another study conducted in western Kenya to assess the role of HCM in suppression of macrophage MIF in 357 children (HC=39; UM=26; mild MA=75; moderate MA=98; and SMA=119) aged from 3 to 31 months showed that the mean HCM (%) and absolute HCM (10^3/μl) of the children with UM, mild MA, moderate MA and SMA were (0.8 & 0.01), (3.1 & 0.04), (7.6 & 0.09) and (15.2 & 0.23), respectively, with significant difference between the groups ($p < 0.001$). However, parasitemia ($p = 0.117$) and HDP (0.656) were not associated with disease severity. In addition, 53% of the children had no HCM (0%) while 20% had low (≤10%) and 27% had high (>10%) HCMs. Parasite density increase with increasing HCM ($p = 0.34$). The mean Hb value significantly decreases with increasing HCM ($p < 0.001$) and the greatest proportion of children with SMA (65%) were in the > 10% HCM group ($p < 0.001$). Multivariate logistic regression after controlling for age, gender, and parasitemia showed that, the odds of having SMA was significantly higher in the >10 group than in the ≤ 10% group ($p < 0.001$): OR and 95%CI of 3.4 (1.8–6.6) and 7.5 (4.1–14.0), respectively (17).

A study conducted on intraleukocytic malaria pigment and clinical severity of malaria in children aged 6months to 14 years in a holoendemic area in Nigeria showed that100% of the patients with CM had HCM and HCN whereas 95% of those with UM had HCM and HCN. The median

percentage of HCMs and HCNs in patients with CM was significantly higher than those with UM (p<0.0001): HCMs53% in CM and 17% in UM whereas HCNs 27% in CM and 9% in UM. A total of 43 children each CM and UM were studied (18).

A case-control study of SM conducted in Uganda to investigate the association of pigmented leukocytes with measures of disease and clinical immunity in 195 under-five year children (99 SM & 96 UM cases) showed that pigmented leukocytes, predominated by monocytes, were significantly greater in number in SM than in UM (p <0.0001). In SM and UM blood smears, the total number of HCM/µland HCN/µl was 32 (0-640) and 0 (0-272) for HCM whereas 0 (0-80) and 0(0-16) for HCN, respectively.HCM was observed in 67.7% and 36.5% of the children with SM and UM, respectively, while HCN in 16.2% and 1.0%, respectively. SM children with no HCM had significantly higher median Hb (p = 0.004) and PCV (p = 0.01) than those with HCM, and those with high numbers of HCM (≥150/µl) had significantly lower Hb (p=0.004) and PCV (p=0.01) than those with low numbers of HCM (<150/µl). These differences were not observed in UM except for age in which those without HCMs were significantly older. In SM children, there was a highly significant negative correlation between numbers of HCM on one hand, and Hb and PCV (rs=−0.46and −0.49, respectively). A significant inverse correlation between numbers of HCN and Hb (rs= −0.23; p= 0.02) as well as PCV (rs= −0.28; p= 0.006) in SM (19).

A case control study conducted to determine the association of pigmented leukocytes with malaria severity and prognosis in children in Bandiagara Mali from July 2000 to December 2001showed that 85.9%and 54% of the children with SM and UM had HCN with mean pigmented cells of 4.4% and 1.7%, respectively. Similarly, 85.2% and 69.9% of SM and UM had HCM with mean pigmented cells of 14.4% and 5.4%, respectively. An increased amount of Hz in phagocytic cells was more likely in SM cases compared with UM cases (HCN: OR =5.6, 95% CI=2.83−12.31, p< 0.0001, HCM: OR =2.85, 95% CI =1.38−5.85, p =0.003). Within the SM group, subjects in the CM group (448 vs. 260, p = 0.018) had significantly more HCN than in those without CM, but no such association was found for HCM (226 vs. 208, respectively; p = 0.80). A higher concentration of HCM was noted in children with SM who were not hyperparasitemic than who were (143 vs. 290, p= 0.04) and in those with SMA than without (522 vs. 129, p = 0.0002) but HCN did not show such association with SMA (516 vs. 316,

respectively; p = 0.066). In multivariate regression analysis no significant association was found between both HCM (p = 0.11) and HCN (p = 0.68) with parasite density.SM cases who were died had higher HCN than survived (635 vs. 320, respectively, p = 0.02), but such association was not noted for HCM (407 vs. 198, respectively; p =0.09). Age and sex matched 172 children aged from 3 months to 14 years each with UM and SM was studied (20).

Hematological changes are some of the most common complications in malaria as the changes involve the major cell lines such as RBCs, WBCs and PLTs. Hematological abnormalities such as severe anemia, thrombocytopenia, leukocytosis and pigmented leukocytes have been identified as markers of severe malaria. Except very few literatures on anemia, there was no published study on other hematological abnormalities in children with malaria and their associations with disease severity in Ethiopia. Therefore, this study was intended to determine the hematological parameters and hemozoin containing leukocytes in children infected with malaria and their association with disease severity.

CHAPTER THREE: OBJECTIVES

3.1. General objective

To determine the hematological parameters and hemozoin-containing leukocytes in children infected with malaria and their association with disease severity at Pawe General Hospital, Northwest Ethiopia.

3.2. Specific objectives

- To determine the hematological parameters in children infected with malaria
- To determine hemozoin-containing leukocytes in blood film of children infected with malaria
- To determine the association between peripheral malaria parasite densities and hematological parameters
- To determine the association between hematological parameters and malarial severity
- To determine the association between hemozoin-containing leukocytes and malaria severity

CHAPTER FOUR: MATERIALS AND METHODS

4.1. Study area and period

The study was conducted at Pawe general hospital from July 30 to December 31, 2014.Pawe general hospital was one of the two hospitals found in Benishangul-Gumuz Regional State (BGRS). Geographically, it is located in Pawe woreda, 557 Km northwest of Addis Ababa. The woreda had one hospital, 21private and governmental clinics and 12 health posts. Pawe woreda is located at 11019'59.47"N latitude and 36025'00.66'' E longitude with an altitude of 1050-1250meters above sea level. The climate was 'Kolla', with an annual rainfall of 1150mm and average temperature of 32^0C. The area has been endemic for malaria. The total population of the woreda was about 42,443 of which 21,588 are males and 20,855 are females. The dominant groups were individuals under the age 18 years (26,539). Pawe woreda had a total of 20 villages of which 3 are urban and the rest 17 were rural villages with an estimated 10,610 households. Each village had an average family size of 4 persons per household. Pawe general hospital was the only hospital in Metekel zone of BGRS and provides services for the catchment population of about 288,880 people with bed capacity of 180 (from hospital record).

4.2. Study design

A facility based cross sectional study was conducted at Pawe general hospital from July 31 to December 30, 2014.

4.3. Population

4.3.1. Source population

All children presenting at Pawe general hospital with symptoms suggestive of malaria were the source population.

4.3.2. Study population

The study populations were children aged one to fifteen years seen at outpatient or admitted to pediatrics ward of Pawe general hospital with microscopy confirmed malaria.

4.4. Inclusion and exclusion criteria

Inclusion criteria

Children of both sexes aged between one and fifteen years with microscopy confirmed malaria and with parental or guardian consent and participants assent was obtained.

Exclusion criteria

- ❖ Children with concomitant intestinal parasitic infection.
- ❖ Children with clinical features or history suggestive of chronic illness.
- ❖ Critically ill children who are unable to give blood sample.
- ❖ Children with history of antimalarial treatment within the last 48 hours.

4.5. Sample size

A total of 377 children with microscopy confirmed malaria parasitemia were included in this study. The sample size was calculated by using a general statistical formula for single population proportion.

$$\mathbf{n} = \frac{(z_{\alpha/2})^2 \times p(1-p)}{d^2}$$

$$\mathbf{n} = \frac{(1.96)^2 \times 0.43(1-0.43)}{(0.05)^2}$$

n=376.6~377

Where:

n = samples size

$\mathbf{Z_{\alpha/2}}$=value of standard normal deviate at level of significance, 1.96 at 95% CI, α=0.05

d = the absolute precision and it was taken as 0.05.

P= the prevalence of MA in children. In this study 43% prevalence was used which was taken from a study conducted in Kersa and Halaba Kulito districts in southern Ethiopia (38).

4.6. Sampling technique

All children presenting to the hospital with symptoms suggestive of malaria and having microscopy confirmed asexual parasitemia during the study period were included consecutively as a study subjects.

4.7. Variables

4.7.1 Dependent variable

- Hematological abnormalities

4.7.2. Independent variables

- Age
- Sex
- Fever
- Splenomegaly
- Parasite density
- Parasite species
- Nutritional status
- Disease severity

4.8. Datacollection

Patient demographic and clinical information was collected using structured questionnaire. The clinical symptoms, anthropometric measurements and physical findings were recorded by a public health officer. Anthropometric measurements such as weight for children under-five years and mid-upper arm circumference (MUAC) for children aged five years and above were recorded to assess their nutritional status. The nutritional status of children was determined using the International Reference Population defined by the U.S National Center for Health Statistics (NCHS) and Centers for Disease Control and Prevention (66). Weight-for-age (WAZ) Z-scores was calculated based on this recommendation. Children were classified as malnourished when WAZ Z-score was <-2SD or when MUAC was below the reference range.

4.9. Laboratory sample collection and processing

A volume of 4 ml venous blood was collected in ethylene diamine tetra acetic acid (EDTA) containing vacutainer tube from every child with microscopy confirmed malaria for laboratory investigations prior to initiation of supportive care or other treatment interventions. The blood sample was used for hematological analysis [complete blood count (CBC)], for blood film examination and for HIV screening. The specimen was analyzed using CELLDYNE 1800® (Abott Laboratories Diagnostics Division, USA) for complete blood count. HIV-1/2 status was screened by rapid antibody tests based on the National test algorithm. The National rapid test algorithm uses three rapid HIV test kits using the serial method. Pre- and post-test HIV counseling was provided to the parents/guardians of all participating children. In addition stool specimen was collected from each child and was prepared by formalin-ether concentration technique to detect intestinal parasites. Those found to be positive for HIV and with intestinal parasites were excluded from further investigations. (Annex I, V, VI).

Drops of blood from EDTA test tube was dispensed immediately on clean and frosted slides to prepare two thin and thick blood smears per patient for microscopic detection and enumeration of malaria parasites and Hz-containing leukocytes. The slides were stained by 10% Giemsa for 10 minutes. Microscopic detection and enumeration of malaria parasites and pigmented leukocytes was performed by two expert laboratory technologists. The thick film was used for the detection of parasites and to quantify peripheral parasitemia, while the thin film was used to identify *Plasmodium* species as well as to detect and quantify pigmented leukocytes. Annex (II–IV).

4.10. Quality assurance

To avoid language barrier and ambiguities, the questionnaire was translated to the local language and written in Amharic and 5% of the questionnaire was pre-tested at Felege Selam health center, the only health center in Pawe district. Training was given for the data collectors to minimize technical and observer biases. To assure the quality of the data generated during the study, standard operating procedure was followed during specimen collection and other laboratory procedures. All reagents used were checked for their expiry date and prepared according to the manufacturer's instruction if in home preparation is needed (e.g., Giemsa).

Control reagents were used for the hematology analyzers to check the reproducibility of the results. The CELL-DYN 1800 analyzer can detect and flagged any abnormalities in the sample such as platelet aggregation and cold agglutinins. When such abnormalities were recognized, manual cell count and peripheral morphology was done to identify and correct the problem. To ensure accurate laboratory results, any discrepancy in parasite density and hemozoin-containing leukocytes readings between the two expert laboratory technologists were averaged. The laboratory technologists were also blinded to clinical presentation and outcome.

4.11. Data processing and analysis

Data were edited, cleaned and checked for its completeness before entering to SPSS. Descriptive statistical tests were used for analysis of some clinical, demographic and laboratory data. The means of normally distributed continuous data were compared using unpaired student's t-test Categorical variables were compared using chi-square (x^2) test. The Chi-square test was used to determine association of hematological abnormalities with disease severity. Pearson correlation analysis was used to determine the correlation of hematological parameters and hemozoin-containing leukocytes with peripheral parasite density. A level of statistical significance (two sided) was set at $p < 0.05$.All statistical analyses were done using SPSS V-20.0(SPSS Inc., Chicago, IL) statistical software.

Based on the clinical and laboratory findings, children with at least one or more symptoms of severe malaria complications set by World Health Organization (WHO) (67) were classified as severe malaria cases otherwise considered as uncomplicated cases. Based on the blood film findings, the degree of parasitemia was graded as mild, moderate, and severe, when a count is between 1–999 parasite/μl, 1000-9999/μl, and >10000/μl, respectively, following method described by Cheesbrough (68). Hyperparasitemia was defined as > 100 000 asexual forms per microlitre of blood as per WHO definition (67).

4.12. Ethical considerations

Ethical clearance was obtained from Jimma University, Ethical Review Board and supporting letter was obtained from Regional Health Bureau. Written consent for children under seven years and as well as assent for children aged seven years and above were obtained from the parents or guardians before the enrollment of the children and blood collection. The purpose of the study was clearly described to the children parents or guardians including the benefits and risks of the study. Any information concerning the patients was kept confidential and the specimen collected from the patients was only analyzed for the intended purposes. Pre- and- post HIV counseling was given to all study participants and relevant confidentiality was maintained for every participant. All children were treated as per the recommendation of National Malaria Treatment Guideline (35).

4.13. Plan for dissemination of the result

The results will be presented to Jimma university scientific community in thesis defense. After that the final report will be disseminated to theBenishangul-Gumuz Regional Health Bureau (BGRHB) and Pawe general hospital so that the findings of this study will be used by policy makers, clinicians and other health professionals for management, prevention and control of malaria and its complications. Furthermore, great effort will be made to send paper to different journals for publication both internally and externally.

CHAPTER FIVE: RESULT

5.1. Demographic, clinical and parasitological data of the study participants

A total of 377 malaria infected children aged 1 to 15 years attending Pawe general hospital were recruited for this study. Of these, 57.8% (n=218) were males and 42.2% (n=159) were females with mean age of 8.5 ± 4.91 years. The highest numbers of participants were within the age group of 11-15 with 37.1% (n=140). At the time of presentation, 63.9% (n=241) of the children had fever and 13.8% (n=52) had splenomegaly. The majority of malaria cases were caused by *P. falciparum* infection which accounted for 75.1% (n=283) of the total cases. Among the total malaria infected children, 27.1% (n=102) had SM. Of those with SM, 4.9% (n=5) had SMA (Table 1).

Table 1. Demographic, nutritional, clinical and parasitological data of malaria infected children attending Pawe General Hospital from July 30 to December 301, 2014

Variables	Frequency	Percent (%)
Age (years)		
1–5	127	**33.7**
6–10	110	**29.2**
11–15	140	**37.1**
Sex		
Male	218	**57.8**
Female	159	**42.2**
Fever		
Yes	241	**63.9**
No	136	**36.1**
Splenomegaly		
Yes	52	**13.8**
No	325	**86.2**

Nutritional status		
Normal	149	**39.5**
Acute malnutrition	228	**60.5**
***Plasmodium* species**		
P. falciparum	283	**75.1**
P. vivax	57	**15.1**
Mixed	37	**9.1**
Malaria severity		
Uncomplicated malaria	275	**72.9**
Severe malaria	102	**27.1**
Total	**377**	
Severe malaria categories		
Severe malarial anemia	5	**4.9**
Hyperparasitemia	37	**36.3**
Prostration	60	**58.8**
Total	102	

5.2. Hematological parameters data

The mean Hb (g/dl), Hct (%), RBC ($x10^{12}$/L), PLT ($x10^9$/L) and WBC ($x10^9$/L) count of the study subjects was (11.7 $\pm$ 2.18), (32.9 $\pm$ 5.6), (4.42 $\pm$ 0.6974), (153 $\pm$ 91.45), and (7.7 $\pm$ 3.7), respectively (Table 2).

Table 2. The mean value of hematological parameters of malaria infected children attending Pawe General Hospital from July 30 to December 31, 2014

Parameters	Mean value	95%CI
WBC ($x10^9$/L)	7.691	7.316 – 8.066
RBC ($x10^{12}$/L)	4.4203	4.3469 – 4.4909
Hb (g/dl)	11.697	11.476 – 11.918
Hct (%)	32.944	32.376 – 33.511
PLT($x10^9$/L)	153.01	143.75 – 162.27

Key: 95%CI- 95% confidence interval, Hb- hemoglobin, Hct- hematocrit, PLT- platelet, RBC- red blood cell, WBC- white blood cell

Among the total malaria infected children, 40.3% (n=152) had anemia; out of which 3.3% (n=5) had severe anemia. Thrombocytopenia was found in 56.8% (n=214) of the study participants; out of which 13.1% (n=28) had severe thrombocytopenia. Leukocytosis and leukopenia were found in 15.4% (n=58) and 10.3% (n=39) of the study subjects, respectively (Table 3).

Table 3. The prevalence of hematological abnormalities and their severity in children infected with malaria attending Pawe General Hospital from July 30 to December 31, 2014

Hematological abnormalities	**Frequency (n)**	**Percent (%)**
Anemia(Hb<11 g/dl)	152	**40.3**
Mild anemia(Hb: 8.0−10.9 g/dl)	133	**87.5**
Moderate anemia(Hb: 5.0−7.9 g/dl)	14	**9.2**
Severe anemia(Hb<5 g/dl)	5	**3.3**
Total	**152**	**100**
Thrombocytopenia (PLT<150 x 10^9/L)	214	**56.8**
Mild thrombocytopenia(PLT:100−149 x10^9/L)	89	**41.6**
Moderate thrombocytopenia(PLT:50−99 x10^9/L)	97	**45.3**
Severe thrombocytopenia(PLT <50 x 10^9/L)	28	**13.1**
Total	**214**	**100**
Leukopenia(WBC<4.0 x 10^9/L)	39	**10.3**
Leukocytosis(WBC>11.0 x 10^9/L)	58	**15.4**

5.3. Hemozoin-containing leukocytes data

Hz-containing monocytes and Hz-containing neutrophils were found in thin blood films of 80.1% (n=302) and 54.4% (n=205) of malaria infected children, respectively. The mean percentage of monocytes and neutrophils that contain Hz were 9.7 and 1.7, respectively. The total quantity of HCMs and HCNs per microliter (/µl) of blood of the study subjects were 80.6 $\pm$ 134.3 and 85.8 $\pm$ 137.1, respectively (Table 4).

Table 4. Hemozoin-containing leukocytes data of malaria infected children attending Pawe General Hospital from July 30 to December 31, 2014

Hz-containing leukocyte	Patients with Hz-containing leukocytes No (%)	Percent of leukocytes-containing Hz Mean (SD)	Quantity of Hz-containing leukocytes per microliter Mean (SD)
Monocyte	302 (80.1)	9.7 (9.2)	80.6 (134.3)
Neutrophil	222 (58.9)	1.7 (2.2)	85.8 (134.3)

Key:Hz- hemozoin, No- number, %-percentage, SD- standard deviation

Malaria pigment (hemozoin) was recognizable in cytoplasm of leukocytes (monocytes or neutrophils) in Giemsa stained thin blood films by their amber to black-brown (resembling cola) and solid appearance (Figure 1).

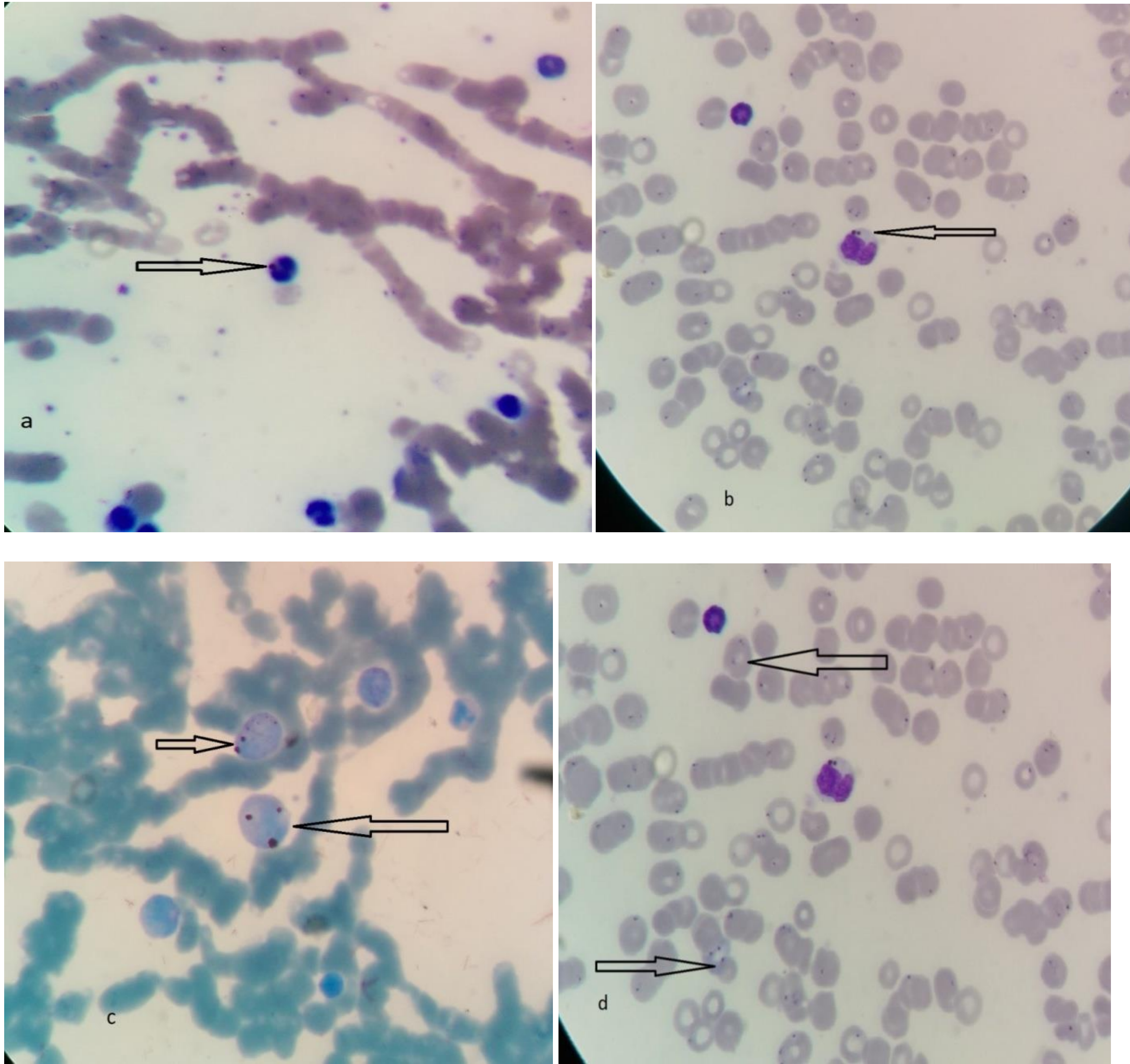

Figure 1.Hemozoin containing leukocytes of children infected with malaria, *a) Hemozoin containing neutrophil, b) Hemozoin containing monocyte c) Hemozoin containing Neutrophil (above) and Hemozoin containing monocyte (down), d) P. falciparum infected RBCs* at Pawe General Hospital, July 30-December 31, 2014, Pawe Northwest Ethiopia.

5.4. Association of hematological parameters and hemozoin-containing leukocytes with parasite density

The parasite density (/µl) of the study subjects was ranged from 102 to 721,430, with a mean of 36,000 (95%CI=28,028 – 45,149). Among the total study subjects, 6.1% (n=23), 46.2% (n=174), 37.7% (n=143) and 10% (n=37) had parasitemia (/µl) <1,000, 1,000−9,999, 10,000−100,000 and >100,000, respectively. Parasitaemia(/µl) was both associated and correlated negatively with age (r= -0.131, p=0.011), Hb (r= -0.117, p=0.023) and Hct (r= -0.119, p=0.021) and positively with axillary body temperature (r=0.106, p=0.04), WBC count (r=0.213, p<0.001), mean HCM percentage (r=0.343, p<0.001) and mean HCNs percentage (r=0.414, p<0.001). However, parasitemia was neither associated nor correlated with gender (p=0.797) and PLT count (r= -0.050; p=0.335).

5.5. Factors associated with hematological abnormalities

Anemia was significantly associated with younger age (p=0.001), acute malnutrition (p<0.001), splenomegaly (p<0.001), *P. falciparum* infection (p=0.024), and hyperparasitemia (p=0.002). However, it was not associated with sex (p=0.58) and fever (p=0.942) (Table 5).

Table 5. The prevalence of anemia in relation to age, sex, nutrition, fever, splenomegaly, parasite species, and parasite density among malaria infected children attending Pawe General Hospital from July 30 to December 31, 2014

Variables	Yes N (%)	No N (%)	Total	X^2-value	P-value
Age (months)					
12-59	67 (52.8)	60 (47.2)	127	11.54	0.001
>59	85 (34)	165 (64)	250		

Sex					
Male	91 (41.7)	127 (58.3)	218	0.31	0.58
Female	61 (38.4)	98 (61.6)	159		
Nutritional status					
Acute malnutrition	109 (47.8)	119 (52.2)	228	12.67	<0.001
Normal	43 (28.9)	106 (71.1)	149		
Splenomegaly					
Yes	33 (63.5)	19 (39.5)	52	12.33	<0.001
No	119 (36.6)	206 (63.4)	325		
Fever					
Yes	98 (40.7)	143 (59.3)	241	0.005	0.942
No	54 (39.7)	82 (60.3)	136		
Parasite species					
P. falciparum	125 (44.2)	158 (55.8)	283	7.487	0.024
P. vivax	18 (31.6)	39 (68.4)	57		
Mixed	9 (24.3)	28 (75.7)	37		
Parasitemia>100,000/µl					
Yes	24 (64.9)	13 (35.1)	37	9.161	0.002
No	128 (34.7)	212 (65.3)	340		

Key: N (%)- number (percentage), X^2- chi-square value

Leukocytosis was significantly associated with younger age [26% (33/127) vs. 10% (25/250) in 12-59 months and >59 months, respectively; x^2=16.529, p<0.001] and hyperparasitemia [29.7% (11/37) vs. 13.8% (47/340) in those with and without hyperparasitemia, respectively; x^2=5.972, p=0.015]. However, it was not associated with sex (p=0.109), fever (p=0.131), malnutrition (p=0.061), splenomegaly (p=0.408) and parasite species (p=0.120).

Leukopenia was significantly associated with older age [(5.5% (7/127) vs. 12.8% (32/250) in children aged 12-59 and >59 months, respectively; x^2=4.823, p=0.028], absence of hyperparasitemia [(0% (0/37) vs. 11.5% (39/340) in those with and without hyperparasitemia, respectively; x^2=4.876, p=0.027] and *P. falciparum* infection [12.4% (35/283) vs. 5.3% (3/57) vs. 2.7% (1/37) in *P. falciparum*, *P. vivax* and mixed infection, respectively; x^2=6.288, p=0.043].However, it was not associated with sex (p=0.878), fever (p=0.302), malnutrition (p=0.215) and splenomegaly (p=0.499). Thrombocytopenia was not associated with any of the factors (p>0.05).

The presence of HCMs was significantly associated with younger age (p=0.001) as 89.8% (114/127) of children aged 12-59 months had HCMs compared to 75.2% (188/250) of children aged above 59 months; x^2=11.209, p=0.001. It was also significantly associated with *P. falciparum* infection as 83.4% (236/283) of children with *P. falciparum* infection had HCMs compared to 70.2% (40/57) and 70.3% (26/37) of children with *P. vivax* and mixed infection; x^2=7.691, p=0.021. In addition, HCM was significantly associated with hyperparasitemia as 100% (37/37) of children with hyperparasitemia had HCMs compared to 77.6% (264/340) of the children without hyperparasitemia; x^2=17.888, p<0.001. However, it was not associated with sex (p=0.141), fever (p=0.581), malnutrition (p=0.157) and splenomegaly (p=0.615).

The presence of HCNs was significantly associated with hyperparasitemia as 89.2% (33/37) of children with hyperparasitemia had HCNs compared to 55.6% (189/340) of the children without hyperparasitemia; x^2=13.642, p<0.001. In addition, HCN was significantly associated with acute malnutrition as 63.2% (144/228) of malnourished children had HCN compared to 52.3% (78/149) of children with normal nutrition; x^2=4.349, p=0.037. However, it was not associated with age (p=0.169), sex (p=0.577), fever (p=0.985), splenomegaly (p=0.088) and parasite species (p=0.400).

5.6. Hematological parameters and hemozoin-containing leukocytes of children with uncomplicated and severe malaria

Children with SM had significantly lower mean Hb value ($p<0.001$), Hct ($p<0.001$), RBC count ($p=0.002$), age ($p<0.001$) and higher WBC count ($p<0.001$), mean HCMs percentage ($p<0.001$), mean HCNs percentage ($p<0.001$) and parasite density (/µl) ($p<0.001$) than those with UM. However, there was no significant difference in the mean PLT count ($p=0.464$) and axillary body temperature ($p=0.172$) between the two groups (Table 6).

Table 6. Comparison of mean values of hematological parameters and hemozoin-containing leukocytes of children with uncomplicated and severe malaria using Student T-test at Pawe General Hospital from July 30 to December 31, 2014

Parameters	UM	SM	T-value	P-value
Hb (g/dl)	12.1 (1.93)	10.6 (2.44)	6.31	<0.001
Hct (%)	34.02 (4.75)	30.05 (6.63)	5.538	<0.001
RBC ($x10^{12}$/L)	4.51 (0.55)	4.18 (0.95)	3.237	0.002
WBC ($x10^{9}$/L)	7.09 (3.26)	9.31 (4.32)	-5.436	<0.001
PLT ($x10^{9}$/L)	151 (80)	160 (116)	-0.735	0.464
HCMs (%)	7.3 (7.2)	16.2 (10.9)	-7.618	<0.001
HCNs (%)	1.25 (1.6)	2.8 (3.1)	-4.685	<0.001
HCMs (/µl)	48.8 (56.1)	166.3 (220.1)	-5.327	<0.001
HCNs (/µl)	56.2 (79.8)	165.6 (209.5)	-5.140	<0.001
Parasitemia ($x10^{3}$/µl)	14.7 (16.868)	95.7 (144.899)	-5.632	<0.001
Age (years)	9.4 (4.7)	6.0 (4.5)	6.287	<0.001
Temperature (^{o}c)	37.66 (0.94)	37.82 (1.13)	-1.401	0.162

Key: Hb- hemoglobin, Hct- hematocrit, HCM- hemozoin-containing monocytes, HCN- hemozoin-containing neutrophils, PLT- platelet, RBC- red blood cell, SM- severe malaria, UM- uncomplicated malaria, WBC- white blood cell

5.7. Association between hematological abnormalities and malaria severity

Anemia (x^2=17.8, p<0.001), leukocytosis (x^2=11.0, p=0.001), HCM (x^2=19.72, p<0.001) and HCN (x^2=7.91, p=0.005) were significantly associated with severity of malaria. However, thrombocytopenia (x^2=0.2, p=0.623), severe thrombocytopenia (x^2=0.035, p=0.851) and leukopenia (x^2=3.0, p=0.09) were not associated with severity of malaria (Table 7).

Table 7. Comparison of hematological abnormalities of children with uncomplicated and severe malaria using Chi square (X^2) test at Pawe General Hospital from July 30 to December 31, 2014

Hematological abnormalities		**UM** N (%)	**SM** N (%)	**X^2-value**	**P-value**	**Total**
Anemia	Yes	93 (33.8)	59 (57.8)	17.847	<0.001	
	No	182 (66.2)	43 (42.2)			
Leukocytosis	Yes	32 (11.6)	26 (25.5)	10.970	0.001	
	No	243 (88.4)	76 (74.5)			
Leukopenia	Yes	33 (12)	6 (5.9)	3.002	0.083	
	No	242 (88)	96 (94.1)			
Thrombocytopenia						
	Yes	154 (56)	60 (58.8)	0.242	0.623	
	No	121 (44)	42 (41.2)			
Severe thrombocytopenia						
	Yes	20 (7.3)	8 (7.8)	0.035	0.851	
	No	255 (92.7)	94 (92.2)			

HCMs	Yes	205 (74.5)	97 (95.1)	19.721	<0.001	
	No	70 (25.5)	5 (4.9)			
HCNs	Yes	150 (54.5)	72 (70.6)	7.909	0.005	
	No	125 (45.5)	30 (29.4)			
Total		**275**	**102**			**377**

Key: HCM- hemozoin containing monocyte, HCN- hemozoin containing neutrophil, N- number, SM- severe malaria, UM- uncomplicated malaria

5.8. Association between hemozoin-containing leukocytes and severity of anemia

To determine the association between mean percentage of HCMs and HCNs with anemia severity, children were categorized into those with mild anemia (Hb: 8.0–10.9) (n=133), moderate anemia (Hb: 5.0–7.9) (n=14) and those with severe anemia (Hb<5.0) (n=5).The mean percentage of HCMs increased significantly as the severity of malarial anemia increases (p<0.001). However, this association was not significant for HCNs (p=0.893) (Table 8).

Table 8. The mean percentage of hemozoin-containing leukocytes in malaria infected children with varying severities of anemia attending Pawe General Hospital from July 30 to December 31, 2014

Hemozoin-containing leukocytes	Mild anemia	Moderate anemia	Severe anemia	F-value	P-value
HCMs	12.5	21.4	34.6	19.7	<0.001
HCNs	2.0	1.8	2.4	0.113	0.893

Key: F- fisher's value, HCMs- hemozoin containing monocytes, HCNs- hemozoin containing neutrophils

CHAPTER SIX: DISSCUSION

In this facility-based cross sectional study which was conducted at Pawe General Hospital, 377 malaria infected children were involved. Anemia, thrombocytopenia, hemozoin-containing monocytes and hemozoin-containing neutrophils were the most common hematological abnormalities in children infected with malaria. Anemia, leukocytosis, the presence and mean percentage of monocytes- and nerutrophils- containing hemozoin were significantly associated with severe malaria.

In this study, anemia was found in 40.3% (n=152) of malaria infected children, out of which 87.5% (n=133), 9.2% (n=14) and 3.3% (n=5) had mild, moderate and severe anemia, respectively. This finding is relatively similar with studies done in Nigeria and Kersa and Halaba Kulito districts in southern Ethiopia which showed that the prevalence of anemia was 43.8% (2) and 43.2% (38), respectively. But this result is much lower than studies done in Venezuela and western Kenya which showed 94.9% (58) and 60% (9), respectively. This difference may be explained by differences in the age of the study subjects such that children in the latter studies were younger than the children in our study. Beside this, the difference may also be attributed to difference in inclusion and exclusion criteria of the studies as children co-infected with intestinal helminthes and/or HIV were excluded in our study, however, these children in latter studies were not excluded. Moreover, about 42% of anemic children in western Kenya had some forms of hemoglobinopathies.

Our study revealed that anemia was significantly more common among children aged 1−5 years than those aged above 5 years (p=0.001) and this was in agreement with studies done in Nigeria (2) and Mount Cameroon Region (63). This might be due to the fact that in malaria endemic areas as a result of repeated infections children aged above 5 years had well developed malarial immunity that protects them from severe illness including the development of anemia. Furthermore, our study showed that splenomegaly was more common among under-five years children than those aged above five years [19.7% (25/127) vs. 10.8% (27/250); x^2=5.591, p=0.018) and this may further contribute to the anemia. Gender had no effect on the Hb of children enrolled in this study. This finding is in line with a study done in Nigeria (2) which showed no significant difference in Hb value between boys and girls. The disparity in Hb with

gender commonly observed in adults (69) may therefore be hormonal since only children who were pre-pubertal were enrolled in our study.

The finding of this study showed that Hb value had a negative correlation with peripheral parasite density (r= -0.117, p = 0.023). This association implied that peripheral destruction of parasitized RBCs was one of the key mechanisms responsible for malarial anemia. This study was in agreement with studies done in Kisumu western Kenya (9) and Mount Cameroon Region (63) which showed significant association between Hb and parasite density. However, this finding was in contrary to studies done in Nigeria (2) and Siaya district hospital western Kenya (17) which showed the lack of association between Hb and parasitemia. These differences may be due to the fact that the etiology of anemia in malaria is multifactorial involving the increased destruction of both infected and non-infected RBCs and decreased erythropoiesis which could vary among children in different clinical settings (11, 12). In addition to the density of parasitemia, the findings of our study showed that splenomegaly and malnutrition were also thought to contribute to anemia as they were significantly associated with malarial anemia ($p<0.001$). This finding was in agreement with a study conducted in Mount Cameroon Region which showed that anemia was significantly higher in children with enlarged spleen and with malnutrition than those with normal spleen size and normal nutrition (63).

This study showed that thrombocytopenia was found in 56.8% (n=214) of malaria infected children; out of which 41.6% (n=89), 45.3% (n=97), and 13.1% (n=28) had mild, moderate and severe thrombocytopenia, respectively. This finding is relatively similar with studies done in Venezuela and at Kilifi district hospital in Kenya which found thrombocytopenia in 59% (58) and 56.7% (45) of malaria infected children, respectively. But this result is higher than studies done in Paris, Kisumu district of western Kenya and Nigeria which found thrombocytopenia in 45.6% (59), 49% (9) and 12.8% (43) of children with malaria, respectively; and lower than studies done in Pakistan and India which found thrombocytopenia in 91% (61) and 71% (60) of malaria infected children, respectively. These differences may be explained by the fact that the prevalence of thrombocytopenia in malaria varies with levels of malarial endemicity and immunity, age, malarial species and malarial severity (43).

The finding of our study revealed that PLT count was neither associated nor correlated with parasite density (r=-0.050; p=0.335) and this was in agreement with a study conducted in Nigeria which showed that PLT count was not associated with parasite density (2). However, our finding was discordant with studies done in Dakar Senegal (27) and Uganda (28) which showed that PLT count was negatively associated with parasite density. This difference may be explained by the fact that there might be widespread sequestration of infected erythrocyte clumps in latter studies than in our studies that might be indicated by a large number of CM cases in latter studies as opposed to a single case of CM in our study. Moreover, during malaria infection PLTs can bind to infected RBCs through CD36 and P-selectin, a phenomenon termed as PLT-mediated clumping of infected erythrocytes, which facilitate the adhesion of PRBCs to endothelial cells. As the parasite load increases a large number of PLT-mediated clumps of infected RBCs are formed which result in subsequent activation and consumption of large number of PLTs which is directly linked to the parasite burden of the patient (70, 71).

Our study revealed that the mean PLT count of children with SM was not significantly different from those with UM (p=0.46) and this was in agreement with studies done in Gabon (48) and Mozambique (52) which showed relatively similar PLT count in children with SM and in those with UM. However, our finding was discordant with studies done in Houston Texas (57), Dakar Senegal (27) and Uganda (28) which showed that children with SM had significantly lower PLT count than children with UM. This difference may be explained by the fact that PLTs play a role in the pathogenesis of malaria particularly CM (42) and the number of cerebral malaria cases in the latter studies is by far higher than in our study in which there was only one cerebral malaria case.

Concerning, WBC count and malaria, the finding of this study showed that leukocytosis and leukopenia were found in 15.4% (n=58) and 10.3% (n=39) of the study subjects, respectively. This finding is similar with a study done in Burkina Faso which found leukocytosis in 15.4% of malaria infected children (46). But this result is higher than a study done in Kenya which found leukocytosis in 8% of malaria infected children (9) and a study done in Nigeria which found leukocytosis and leukopenia in 9.5% and 3%, respectively (2); and lower than studies done in

Uganda and Kenya which found leukocytosis in 41.7% (28) and 20.1% (45), respectively. This difference may be explained by the fact that leukocytic changes in malaria are in general variable and depend on many factors such as acuteness of infection, parasitemia, disease severity, state of the host immunity to malaria, and concurrent infections (30).

The result of this study showed that leukocyte count was associated and correlated positively with parasite density ($r=0.213$, $p<0.001$). This finding is in line with a study conducted at Kilifi district hospital, in Kenya which showed that leukocytosis was significantly associated with hyperparasitemia (45). However, our finding was discordant with studies done in Kenya (9) and Nigeria (2) which showed the lack of association between WBC count and parasite density. This difference may be explained by the fact that our study includes children with any degree of malaria severity whereas the latter studies include only children with uncomplicated malaria. Moreover, our study showed that children with SM had significantly higher parasite density ($p<0.001$) than children with UM and this may further account for the association between WBC count and parasite density.

Our study revealed that leukocytosis was significantly associated with severe malaria ($p=0.001$). However, leukopenia was not associated with the severity of malaria ($p=0.09$). This finding is in line with studies done in Kenya (45), Uganda (28) and Burkina Faso (46) which showed that leukocytosis was significantly associated with SM. The possible explanation for this could be leukocytes particularly monocytes and/or macrophages, and to a lesser extent neutrophils play a crucial role in host defense against malaria parasites and during the early phase of the infection the number of phagocytes increases and their activity intensifies and as the severity of the disease progress there is over stimulation of leukocytes which result in imbalanced and excessive production of inflammatory cytokines which may further contributes to the severity of the disease (72, 73).

Our study showed that HCMs and HCNs were found in thin blood films of 80.1% (n=302) and 58.9% (n=222) of malaria infected children, respectively. This finding is higher than studies done in Severe Malaria in African Children (SMAC) centre, Kenya and Uganda which reported

HCMs and HCNs in 63% and 37% (47), 47% and 12% (17), and 52.3% and 8.7% (19) of children with malaria, respectively. The reason for this difference may be because children in our study were older and, therefore, likely more immune to malaria. Beside this, the difference may be due to difference in methods of detecting and enumerating Hz-containing leukocytes. In our study, they were detected and enumerated in thin films and a total of 500 leukocytes were counted before declaring as the patient had no Hz-containing leukocytes. However in studies done in SMAC centre and Kenya, they were detected in thick films and only 100 leukocytes were counted.

Moreover, the predominance of HCMs over HCNs in our study might be explained by the fact that there is a significant level of clinical immunity in the study population, which results in a less fulminant course of severe malaria. This was confirmed, to some extent, by the low prevalence of severe anemia (3.3%) and only one cerebral malaria case in the study population, which is located in a region characterized by the highest transmission intensity in Ethiopia (37).

The finding of our study showed that significantly a greater proportion of children with SM had HCMs ($p<0.001$) and HCNs ($p=0.005$) than those with UM. In addition, our study finding showed that children with SM had significantly higher mean HCM ($p<0.001$) and HCN ($p<0.001$) percentage than children with UM. This finding is in line with studies done in Nigeria (18), Uganda (19) and Mali (20) which showed that HCMs and HCNs were significantly associated with the severity of malaria. The possible explanation for this could be malaria infections are characterized by an altered immune status, and the severe forms seem to be caused by activation or an over-activation of the immune system and the accumulation of Hz in the phagocytic cells such as monocytes and neutrophils can cause modulation of host innate and inflammatory responses (64).

Our study revealed that mean percentage of HCM was significantly associated with anemia severity ($p<0.001$). This finding is in agreement with studies done in Kenya (17, 65), Gabon (48), Mali (20) and Uganda (19) which showed that mean percentage of HCM was significantly associated with SMA. The possible explanation for this could be phagocytosis of hemozoin by

monocytes is one of the primary causes of altered production of innate inflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-10, nitric oxide (NO), reactive oxygen species (ROS) and others which cause suppression of erythropoiesis and dyserythropoiesis (12).It has also been shown that *P. falciparum*-Hz modulates the cytokine responses of human peripheral blood mononuclear cells *in vitro* in a dose dependent manner, with a drastic decrease in IL-2, IL-12, and interferon (IFN)-γ levels, whereas IL-10levels increase with the Hz load of monocytes/macrophages in culture which are associated with severity of anemia (74).

Both HCMs ($r=0.343$, $p<0.001$) and HCNs ($r=0.414$, $p<0.001$) in our study were significantly correlated with parasite density which was in agreement with studies done in SMAC centre (47) and Kenya (17, 65) which showed that HCMs and HCNs were significantly associated with parasite density. The possible explanation for this could be the proportion of neutrophils and monocytes containing malaria pigment is affected by total parasite burden, synchronicity of the parasite life cycle and their clearance kinetics. Moreover, since HCNs have been shown to persist in the peripheral circulation for a median of 72 hours (range: 0−168) whereas parasitized RBCs and HCMs for a median of 96 (range: 36−168) and 216 (range: 84−492) hours, respectively, they could serve as surrogate markers for acute or chronic parasite load in the body (21).

Except for anemia, little is known about other hematological parameters in children with malaria and their association with disease severity in Ethiopia. In addition, there is no published study on hemozoin-containing leukocytes in children with malaria and on their association with malaria severity. This study is thus a first study in Ethiopia. This study uses primary data and this is also strength of the study. As a limitation of this study there is no established reference range for hematological parameters in children in the study area. Due to budget constraints we are obliged to use only Hb measurements and parasite density for determining severity of malaria and this is also another limitation of the study. Though bacterial infections can profoundly perturb hematological parameters thereby affecting their association with severity of malaria, the study did not include any microbiological data such as cultures and this is also limitation of the study.

CHAPTER SEVEN: CONCLUSION AND RECOMMENDATION

7.1. Conclusion

Our finding appreciates that infection with malaria parasites induces significant hematological abnormalities in children living in malaria-endemic regions. In particular, anemia, thrombocytopenia, hemozoin-containing monocytes and hemozoin-containing neutrophils were the most common hematological abnormalities in children infected with malaria. Despite its higher prevalence, thrombocytopenia is not associated with severity of malaria. Anemia, leukocytosis, the presence and quantities of HCMs and HCNs were significantly associated with severe malaria. In addition, mean percentage of HCMs $\geq$16.2 and HCNs $\geq$2.7 can be used as indicators of severe malaria. Since HCMs and HCNs are significantly associated with hyperparasitemia, they may be used as a surrogate marker for acute or chronic parasite load. Furthermore, our study also showed that mean percentage of HCMs is significantly associated with the severity of anemia. Beside parasite density; findings of anemia, leukocytosis, hemozoin-containing monocytes, and hemozoin-containing neutrophil were hematological indictors suggestive of severe malaria in children residing in malaria endemic areas.

7.2. Recommendation

Based on the study finding the following recommendations are suggested:

- ⇨ Hematological parameters can be considered, in addition to parasite density, for early identification of patients with severe malaria.
- ⇨ The presence and quantites of hemozoin-containing leukocytes can be considered while identifiying patients with severe malaria.
- ⇨ The presence of hemozoin in leukocytes particularly monocytes and neutrophils, which are better indicators of severe malaria, be reported alongside malaria parasite results.
- ⇨ The mean hemozoin-containing monocyte percentage ≥ 16.2 may be used as indicator of severe malaria.
- ⇨ The mean hemozoin-containing neutrophil percentage ≥ 2.7 may be used as indicator of severe malaria.
- ⇨ The mean percentage of HCMs can be considered for determining the severity of malarial anemia
- ⇨ Since only five severe malarial anemia cases were diagnosed in this study, it did not lent us power for determining the association of hematological parameters with SMA. Therefore, longitudinal studies with large sample size are needed to determine the association of hematological parameters with severe malarial anemia.
- ⇨ Since bacterial infections can significantly affect hematological parameters, further studies that include microbiological data such as cultures are needed to determine the exact role of hematological parameters in determining the severity of malaria.
- ⇨ Researchers who are interested in this area should also consider establishment of reference range for hematological parameters in children in their locality.

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ANNEXES

Annex -I: CELL-DYNE 1800

Principle:

The CELDYN 1800 uses two independent measurement methods; they are:

- Electrical impedance method for determining WBC, RBC, and PLT data
- Modified Methemoglobin Method for determining HGB

During each instrument cycle, the sample is aspirated, diluted, and mixed before each parameter is measured.

Specimen Required

- Collected in EDTA anticoagulant. Follow the manufacturer's guidelines regarding collection and stability.
- Mixed well before processing.
- Fresh whole blood specimens are recommended (process within eight hours after collection).WBC size distribution can shift if specimens are tested within the first 20 minutes following collection or more than eight hours after collection.
- A minimum of 50µL must be collected for micro-collection specimens. This ensures an adequate amount of blood for the 30 µL aspiration.

Procedure

<u>Entering and Running Patient Specimen</u>

Note: prior to running patient specimens, perform daily start-up procedures

When the **ready** message is displayed on the run screen, the instrument is ready to run specimens.

Entering specimen

1. From **run** screen, press **[specimen type**]
2. In the specimen type screen, press **[patient specimen**]

3. The cursor is placed in the **<next id #>** entry field. Use the alphanumeric keys on the pc keyboard to enter a specimen id of up to 16 characters.

Running patient specimen

To run patient specimens, proceed as follows:

1. With the cap tightly secured on the specimen tube, slowly invert the tube 10 to 15 times.
2. Remove the cap from the pre-mixed specimen tube.
3. Place the tube under the aspiration probe and raise tube so that the end of the probe is deeply immersed in the specimen.
4. Press the touch plate to aspirate the run.
5. When the sample has been aspirated from the tube, the probe will move up through the wash block. Remove the specimen tube and replace the cap.
6. After the cycle is completed, run results are displayed on screen and the aspiration probe moves into position to accept a new specimen. The current run data is saved to the Data Log.
7. If Automatic Graphics printout has been specified in the setup menu, a report is printed according to the parameters selected during the setup procedure.
8. If Automatic Graphics printout has not been specified in the setup menu, press **[print report**] to obtain a copy of the results.

Note: if a system has been idle for 15 minutes or more, a normal background should be run immediately prior to running patient specimens.

Quality Control

Quality control checks should be performed daily according to the laboratory's protocol. Commercial controls materials should be properly warmed and mixed according to the manufacturers' recommendations patient controls should be handled according to the laboratory's protocol.

Performing a QC Run

If the system has been idle for fifteen minutes or more, run a background prior to running any control specimens. Be sure to prepare the control product according to directions on the package insert.

To perform a QC run, proceed as follows:

1. From the **MAIN MENU** screen, press **[RUN]**.
2. From the **RUN** menu, press **[SPECIMEN TYPE]** followed by **[QC TYPE]**.
3. Select the desired level of control (**Low, Normal, High or Replicates**).

NOTE: Prepare a permanent record (printed copy) of any files to be deleted or purged, as required, according to your laboratory's protocol. You can also copy the QC Log before you purge it.

4. Using the [↑] and [↓] arrow keys, select the desired control file.
5. Remove the cap from a well-mixed control specimen tube and place the open tube under the Sample Aspiration Probe. Raise the tube so that the end of the probe is deeply immersed in the specimen.
6. Press the Touch Plate to activate the run.
7. When the well-mixed control has been aspirated from the tube, and the probe moves up through the Wash Block, remove the specimen tube and replace the cap.

   NOTE: If a flow error, clog, or other fault message appears on the display screen during **RUN** cycle, press **[CLEAR ORIFICE]** or refer to **Operating Manual: *Troubleshooting and Diagnostics*** and repeat the run.

8. Verify that control results are within your laboratory's acceptable limits.
9. If the control results fall within acceptable limits, review the data for shifts or trends, record the results, and begin to process patient specimens.
10. After analysis is complete and results are displayed, press **[RETURN]** to return to the **RUN** menu.
11. Press **[MAIN]** to return to the **MAIN MENU** screen.
12. Press **[PRINT]** if a report of printed results is desired.

Calibration

Calibration is a procedure that confirms the accuracy of the CELL-DYN 1800 and must conform to guidelines established by the regulatory agencies in your locality.

Annex-II: Blood film preparation and examination

Specimen required

Capillary blood or EDTA anticoagulated whole blood.

Materials for preparing blood films

- Protective quality latex gloves without talcum powder
- Clean and frosted slides
- Slide box or tray
- HB lead pencil

Reagents and materials for staining blood films

- Giemsa stain
- Methanol
- Test tubes of 5 ml capacity
- Distilled or deionized water buffered to pH 7.2
- Pasteur pipette with a rubber teat
- a curved plastic staining tray, plate or rack
- Slide-drying rack
- Drying lamp
- Timer

Materials for blood film examination

- Microscope fitted with paired x10, x40 and x100objectives and a mechanical stage slides
- Immersion oil
- At least two tally counters, one for parasites and one for white blood cells
- Pen.

Method of preparing blood films

1. Write patient unique code number on the frosted end of the slides
2. Using pipette add a single small drop of blood from EDTA anticoagulated blood on the middle of the slide. This is for the thin film.

3. Add other two or three larger drops on the same slide, about 1 cm away from the drop intended for the thin film.
4. Thin film preparation:

⇨ Using another clean slide as a spreader and with the slide with the blood resting on a flat, firm surface, touch the small drop of blood with the edge of the spreader, allowing the blood to run right along the edge.

⇨ Firmly push the spreader along the slide, keeping it at an angle of 45^0. The edge of the spreader must remain in even contact with the surface of the other slide while the blood is being spread.

5. Thick film preparation:

⇨ Handling the slides by the edges or a corner, make the blood film by using the corner of the spreader to join the drops of blood, and spread them to make an even, thick film. Do not stir the blood.

⇨ A circular or rectangular film can be made by three to six quick strokes with the corner of the spreader.

⇨ The circular thick film should be about 1 cm in diameter.

⇨ The thick film should be dried level and be protected from dust, flies, sunlight and extreme heat.

Staining blood films with Giemsa stain

Thick blood films must be completely dry before being stained. They can be dried quickly with warm air from a small hair-drier or by careful warming over a lamp or a light bulb. Avoid overheating slides as they can 'heat fix' and then stain poorly.

Method:

1. Fix the thin film by dabbing it with a pad of cotton wool dampened with methanol or by briefly dipping the film into methanol. Avoid contact between the thick film and methanol, as methanol and its vapors quickly fix the thick film, and it does not stain well.

2. Using a test tube or a small container to hold the prepared stain, make up a 10% solution of Giemsa in the buffered water by mixing three drops of Giemsa from the stock solution, using the Pasteur pipette, with 1 ml of buffered water. Each slide needs approximately 3 ml of stain to cover it.
3. Depending on whether you are using a staining tray, plate or rack, place the slides to be stained face down on the curved staining tray or face upwards on the plate or rack.
4. Pour the stain gently under the staining tray until each slide is covered with stain, or gently pour the stain onto the slides lying face upwards on the plate or rack.
5. Stain the films for 10 min.
6. Gently wash the stain from the slide by adding drops of clean water. Do not pour the stain directly off the slides, or the metallic-green surface scum will stick to the film, spoiling it for microscopy.
7. When the stain has been washed away, place the slides in the drying rack, film side downwards, to drain and dry. Ensure that thick films do not scrape the edge of the rack.

Technique for thick and thin film microscopic examination

Thick film examination method

1. Place the slide to be examined on the stage, and position the thick film in line with the objective lens.
2. Place a drop of immersion oil on the thick film, and allow it to spread.
3. Using paired x10 oculars and x40 objective, scan the film for microfilaria, other large blood parasites and obvious debris. Select the part of the film that is well stained, free of debris and has evenly distributed white blood cells.
4. Raise the revolving nosepiece away from the stage, and swivel the x100 oil immersion objective over the selected portion of the thick film.
5. Raise the mechanical stage until the objective lens gently touches the immersion oil.
6. Using the fine adjustment, focus on the cell elements and confirm that the portion of the film is acceptable for routine examination:

⇨ 15–20 white blood cells per thick film field will give a satisfactory film thickness. Films with fewer white blood cells per field will require more extensive examination.

7. Starting from the selected portion, examine the film carefully, field by field, moving to each contiguous field in horizontal pattern. For efficient examination, continuously focus and refocus using the fine adjustment throughout examination of each field.
8. A minimum of 200 fields are examined before recording the thick film as negative. Examination of 100 oil immersion microscopic fields takes approximately 10 min.
9. If the slide is positive, go to the thin film to identify the species, developmental stages and to detect the presence and quantites of hemozoin-containing leukocytes.

Thin film examination method

1. Place the slide on the stage, sitting the x100 oil immersion objective over the edge of the middle of the thin film.
2. Place a drop of immersion oil on the edge of the middle of the film.
3. Rack the mechanical stage up until the objective lens touches the immersion oil, as above.
4. Examine the blood film following the pattern of movement: moving along the edge of the film, then moving the slide inwards by one field, returning in a lateral movement and so on.
5. Continue examining until the presence and species of malaria parasites have been identified, or up to at least 800 fields before declaring the slide negative.
6. To obtain the same sensitivity of examination as that for thick film at high power fields (with x100 oil immersion objective) for 10 min, you must examine a thin film for at least 30 min.
7. Record the species, stage of development and the presence and quantities of pigment containing leukocytes.
8. Remove the immersion oil from the slide by using soft-lint tissue paper, and store the slide in a covered slide box for later reference.

Annex-III: Detection and enumeration of hemozoin-containing leukocytes

Microscopic detection and enumeration of hemozoin-containing leukocytes is useful for the diagnosis as well as for determining the severity and progression of malaria. The presence of leukocyte pigment is both qualitatively and quantitatively associated with parasite load, and is therefore indicative of a clinically significant infection.

Procedure

Hemozoin containing leukocytes (monocytes and/or neutrophils) are detected and enumerated in 10% Giemsa stained thin blood films. In Giemsa stained thin film, neutrophils are identified by their multi-lobed nucleus and stains a deep purple. Monocytes are the largest of the white blood cells, measuring 12–18 µm in diameter. Their nucleus is large and kidney or bean shaped; the cytoplasm may contain a few granules that stain pinkish or red. Malaria pigment (hemozoin) is amber to black-brown crystal which is found in the cytoplasm of monocytes and/or neutrophis. It does not take up Giemsa stain.

A total of 100 neutrophils and 30 monocytes are counted in Giemsa-stained thin blood films and during which the respective cells (neutrophils, monocytes) containing amber to black solid looking granules of malaria pigment are enumerated. If there is no any monocyte or neutrophils that contain hemozoin detected, the counting will continue until a total of 500 polymorphonuclear leukocytes and 500 mononuclear leukocytes are counted.

Measures of intraleukocytic malaria pigment:

1. **Percent of neutrophils or monocyteswith cytoplasmic pigment=**

(Number of neutrophils or monocytes with cytoplasmic pigment) x 100%

(Number of neutrophils or monocytes counted)

⇨ **<u>Note:</u>** When the percent of monocytes in the differential cell count is less than 1% but pigment is noted on monocyte pigment count, an arbitrary monocyte differential count of 0.5% (n = 9) is assigned.

⇨ **<u>Note:</u>**If the percent of monocytes with pigment is > 0 and < 1% then a value of 0.5% is used.

2. **Absolute number of neutrophils or monocytes with cytoplasmic pigment per microliter=**

(Percent of neutrophils or monocytes with cytoplasmic pigment) × (absolute WBCs count/µl× percent of neutrophils or monocytes in differential count)

Annex-IV: Determination of peripheral parasitemia

Method:

Parasite density is determined from the thick blood film and is reported as the number of parasites per microliter of blood using actual WBC count of each patient obtained from the CELL-DYN 1800 automated hematology analyzer.

Materials:

- Two tally counters (one to count parasites and the other to count leukocytes)
- A simple electronic calculator.

Procedure:

1. Count the number of parasites seen on one tally counter and the number of white blood cells on the other, oil immersion field by field.

2. The number of parasites and WBCs counted depends on how numerous the parasites are. The lower the number of parasites counted, the higher the number of WBCs that should be counted.

- ⇨ If, after 200 WBCs have been counted, 100 or more parasites are found, the results should be recorded on the form in terms of number of parasites per 200 WBCs.
- ⇨ If, after 200WBCs have been counted, the number of parasites is 99 or fewer counting should be continued up to 500 WBCs.

3. Some parasitemias are so heavy that hundreds of parasites are counted per oil immersion field. In this situation, counting up to 100 WBCs or the total number in about five oil-immersion fields (assuming about 15 WBCs per thick-film field) would be appropriate.

4. When counting is completed, the number of parasites relative to the number of leukocytes is calculated and expressed as ‘parasites per microlitre of blood’ from the simple mathematical formula:

Parasites per microlitre=

$$\frac{\text{Number of parasites counted}}{\text{Number of leukocytes counted (200 or 500)}} \times \text{Patients WBC count}/\mu l$$

- In mixed infections (two species or more), all asexual parasites are counted together and expressed as discussed above.

Annex-V: HIV rapid diagnostic testing

Method:

The test was performed by using HIV rapid testing method, based on the National test algorithm. The National rapid test algorithm should use three rapid HIV test kits using the serial method. The algorithm uses three types of tests: the screening test, the confirmatory test, and a tiebreaker. The screening test is the first test in the sequence. The confirmatory test is used to confirm a positive result if the first test is positive. A tiebreaker is the final test which is done when there is a difference between the screening and confirmatory test results. In Ethiopia, we use KHB as a screening test, STAT-PAK as a confirmatory test, and Uni-Gold as a tiebreaker.

KHB test procedure

1. Remove a test cassettes from a foil pouch, and place it on a flat surface
2. Label the test device with patient name or identification number.
3. Use the sample of either serum/plasma or whole blood: add 40µl of sample (precision pipette) to the sample area first, and then slowly instill 1 drop (~40µl) of sample diluent to the sample area. Direct contact of the diluent bottle with the sample area should be avoided.
4. The results can be seen within 2-3 minutes with strong positive samples. Please don't interpret the test result after 30 minutes. Don't record the result on cassettes.

STAT-PAK™ test procedure

1. Remove the Chembio HIV 1/2 STAT-PAK™ test device from its pouch and place it on a flat surface.
2. Label the test device with patient name or identification number.
3. Touch the 5µl sample loop provided to the specimen, allowing the opening of the loop to fill with the liquid.
4. Holding the sample loop vertically, touch it to the sample pad in the center of the sample (S) well of the device to dispense ~5µl of sample (serum, plasma or whole blood) onto the sample pad.

5. Invert the Running Buffer bottle and hold it vertically (not at an angle) over the sample well. Add 3 drops (~ 105µl) of buffer slowly, drop wise, into the sample (S) well.
6. Read the test result between 15 and 20 minutes after the addition of the running buffer. Reactive test results may be observed and read earlier than 15 minutes. To verify a nonreactive test result, wait the entire 15 minutes after starting the test. Do not read results after 20minutes.

Uni-Gold™ Recombigen® HIV-1/2 test procedure

1. Allow the kit (unopened devices and wash solution) to reach room temperature (15−27°C/ 59.0−80.6°F) (at least 20 minutes) if previously stored in the refrigerator. Once at room temperature remove the required number of Uni-Gold™ Recombigen® HIV- 1/2 devices from their pouches. Perform no more than 10 tests at one time.
2. Lay the devices on a clean flat surface.
3. Label each device with the appropriate patient information / ID.
4. Draw up adequate sample to the first gradation on the pipette using one of the disposable pipettes included in the kit. Use only the pipette included in the kit and do not reuse.
5. Holding the pipette vertically over the sample port, add one (1) free falling drop of sample carefully. Do not add the full volume contained within the pipette. Allow the sample to absorb into the paper in the sample port. Ensure air bubbles are not introduced into the sample port. Discard the pipette in a biohazard waste container.
6. Holding the dropper bottle of Wash Solution in a vertical position, add four (4) drops of Wash Solution to the Sample Port.
7. Set the timer for 10 minutes and start timing the test.
8. Read test results after 10 minutes but not more than 12 minutes incubation time.

Annex-VI: Formaldehyde–ether sedimentation technique

Materials and reagents

- Microscope
- Slides
- Cover slips
- Centrifuge
- Test-tubes
- Test-tube rack
- Wooden applicators
- Brass wire filter, 40 mesh (425mm), 7.2 cm diameter
- Small porcelain or stainless steel dish or beaker
- Pasteur pipette
- Formalin
- Ether

Method

1. Using a wooden applicator, remove a small amount (approximately 0.5 g) of feces from both the surface and the inside of the stool specimen.
2. Place the sample in a centrifuge tube containing 7 ml of 10% formalin.
3. Emulsify the feces in the formalin and filter into the dish.
4. Wash the filter (with soapy water) and discard the lumpy residue.
5. Transfer the filtrate to a large test-tube. Add 3ml of ether (or ethyl acetate).
6. Stopper the tube and mix well.
7. Transfer the resulting suspension back to the centrifuge tube and centrifuge at 2000*g* for 1 minute.
8. Loosen the fatty plug with an applicator and pour the supernatant away by quickly inverting the tube.
9. Allow the fluid remaining on the sides of the tube to drain on to the deposit and then mix well. Using the pipette, transfer a drop on to the slide and cover with a cover slip.
10. Use the x 10 and x 40 objectives to examine the whole of the cover slip for ova and cysts.

Annex -VII: Information sheet

Name of the principal investigator: Muluken Birhanu

Name of the organization: Jimma University

Introduction:

This information sheet is prepared by the principal investigator whose main aim is to determine hematological parameters and hemozoin-containing leukocytes in children with malaria and their association with disease severity at Pawe general hospital. It will indicate a true idea about the existence and/or extent of hematological abnormalities and their association with disease severity in children in the study area. The investigators include a senior medical laboratory technologist and senior public health officer.

Purpose:

The purpose of this research is to determine hematological parameters and hemozoin containing leukocytes in children with malaria and their association with disease severity at Pawe general hospital, North West Ethiopia. Malaria is a major public health problem in Ethiopia with varied hematological consequences, particularly in children. Anemia is the most common cause of malaria related morbidity and mortality. Although in the majority of health centers in Ethiopia malaria severity is determined by counting peripheral parasitemia, most studies showed that the level of peripheral parasitemia does not indicate the severity of malarial anemia and parasitemia alone is insufficient indicator of malaria severity. Rather, despite such scanty peripheral parasitemia, numerous pigmented leukocytes are found in the children peripheral blood and are well associated with the severity of malarial anemia and disease severity. Moreover, there is scarcity of information on the occurrences and severity of hematological abnormalities in children with malaria and their association with disease severity in Ethiopia, specifically in this study area. Therefore we have planned to conduct a study in the area to assess hematological parameters and hemozoin containing leukocytes in children with malaria and their association with disease severity, in which the findings of this study may contribute on designing strategies

which help to improve diagnosis of severe malaria and to early identify patients at risk of progressing to severe disease.

Procedure:

You are kindly invited to take part in this project which is aimed at determining the hematological parameters and hemozoin containing leukocytes in children with malaria and their association with disease severity at Pawe general hospital. If you are willing for your child participation in this study, you need to understand and sign the agreement form. You will be then asked to provide some information about your child associated with malaria. For laboratory examinations, your child will provide about 4 ml of blood, and 0.5gm of stool, in which these will be collected following laboratory standard operating procedures. The laboratory examination (including HIV screening) results will be kept confidential using coding systems whereby no one will have access to your laboratory result. If the result of the laboratory examination shows significant results, you will be only communicated to the health professional attending you for further intervention and treatment for malaria, intestinal helminthes infections and any chronic illness.

Risk and discomfort:

There is no discomfort and as such great pain when blood and stool sample is collected.

Benefits:

If your children participate in this research, he/she will get early and appropriate interventions and we will provide drug prescription request for drug seeking individuals. In addition your participation will help us in determining the hematological changes and pigmented leukocytes and their associations with malaria severity which enhances diligent and correct diagnosis of malaria and to stratify patients in the early stages of admission and assessment according to severity markers.

Incentives:

You will not be provided any incentives to take part in this research except getting some treatments for individuals with helminthes infection and establishing medical attention and means of follow-up for any chronic illness.

Confidentiality:

The information we collect from this research project will be kept confidential. Information about you that will be collected from the study will be stored in a file, which will not have your name on it, but a code number assigned on it. It will be kept under lock and key, and it will not be revealed to anyone except the principal investigator and the health professional following you.

Right to refuse or withdraw:

You have full right to refuse from participating in this research if you do not wish to participate.

Whom to contact:

If you have any questions contact any of the following individuals and you may ask at any time you want:

1. Muluken Birhanu(BSc)- Jimma University, College of Public Health and Medical Sciences, Department of Medical Laboratory Sciences and Pathology, Jimma
2. Wondemagegne Adiisu (BSc, MSc)-Jimma University, College of Public Health and Medical Sciences, Department of Medical Laboratory Sciences and Pathology, Jimma
3. Dr.Tilahun Yemane (MD, MSc) - Jimma University, College of Public Health and Medical Sciences, Department of Medical Laboratory Sciences and Pathology, Jimma

Annex- VIII: Consent Form

This informed consent formis prepared for children presenting with symptoms suggestive of malaria and with asexual parasitemia that attend Pawe general hospital for the evaluation of hematological parameters and hemozoin-containing leukocytes and their association with disease severity.

Name of proposal: Hematological parameters and hemozoin-containing leukocytes in children with malaria and association with disease severity at Pawe general hospital, 2014.

Dear Participant

My name is Muluken Birhanu, and I am a postgraduate student at Jimma University in clinical laboratory science specialty hematology and immunohematology. I am going to conduct my thesis on hematological parameters and hemozoin-containing leukocytes in children with malaria and their association with disease severity. Hematological changes are some of the most common complications in malaria and they play a major role in malaria pathology. The purpose of this study is to assess the occurrences and severities of hematological changes and hemozoin-containing leukocytes in children with malaria and their association with disease severity.

I am inviting all children attending Pawe general hospital to take part in this study.

Your participation in this study is entirely voluntary. If you choose not to consent, all the services you receive at this hospital will continue as usual. Even if you agree now but decide to change your mind and withdraw later, the services you receive at the hospital will continue normally.

Your participation will help us to know the presence and extent of hematological changes and hemozoin-containing leukocytes in children infected with malaria in this area and this will benefit the society and future generations.

We will not share the identity of participants in the study with anyone. The information that we collect from this study will be kept strictly confidential. Any information collected about you will have represented in **number instead of your name**. Only the study team members will know what your number is and we will lock that information up. We will share the knowledge

that we get from this study with you before it is made available to the public. Confidential information will not be shared.
This proposal was reviewed and approved by Jimma University ethical committee.

During the data collection time a small amount of blood and stool will be taken. Your child may experience a bit of pain or fear when your vein is punctured. The pain should disappear within a few minutes. The blood will be transferred onto test tubes. The blood sample will be used for the intended purpose. Nothing else will be done with your sample other than detecting malaria parasite/s, enumerating pigmented leukocytes, measuring hematological parameters and screening HIV-1/2 antibodies.
Dear participant, I thank you for your cooperation. I would like to ask you to agree and take part in the study by giving the following declaration.

I have been invited to participate in a study of hematological changes.

I have read the above information, or it has been read to me. I have had the opportunity to ask questions, and any questions that I have asked have been answered to my satisfaction. I consent voluntarily for my child participation in this study.

Code of participant:____________________

Signature___________ Date: ________

Name of the witness ____________________

Signature___________Date: ________

Annex IX: Consent Form (Amharic Version)

ጅማ ዩኒቨርሲቲ

የህብረተሰብ ጤና • ና የህክምና ሳይንስ ኮሌጅ

የህክምና ላቦራቶሪና ፓቶሎጂ ትምህርት ክፍል

የደም ህዋሳትን በተመለከተ ወባ በተÖቁ ህፃናት ላይ የሚደረግ ጥናት

ይህ የምርምር ተሳትፎ ስምምነት መጠየቅያ ቅፅ የተዘጋጀው በû ዳ ሆስú ታል ¨ ባ ለመመርመርለመÖህፃናትነው፡፡

¨ ¬ት ተሳታፊዎች

ስሜ ሙሉቀን ብርሃኑሲሆን በጅማ ዩኒቨርሲቲ በህክምና ላቦራቶሪ ና ፓቶሎጂ ትምህርት ክፍል የክሊኒካል ሄማቶሎጂ የድህረ ምረቃ ተማሪ ስሆን የወባ በሽታ የደም ህዋሳትላÃ ¾ምÁመ× ¨ ¬ ለ¨ ¬ዓ ላÃ ¾ምርምር ስራ በመስራት ላይ እቶኛለሁ፡፡

እንደሚታ¨ ቀ¨ ¬¾ ባ በሽታበሀገራችን ገዳይ ከምባሉበሽታዎች ግምባር ቀÅም መሆኑÃታ¨ ቃል፡፡ በሽታ¨ ¬ም የደም ህዋሳትመጠንናአገልግሎት በማዛባት¨ Å ከፍተኛ ¾ህመም Åረጽ ይቀየራል፡፡ስለሆነም የዚህ ጥናት ዓላማ በወባ በሽታበተÖቁህፃናት ደም ውስጥ Áለ¨ ¬ን ¾Åም ህዋሳትመጠን ለውጥና ከበሽታ¨ ¬ ¾ህመም Åረጽ ፉር Áላችውን ፅንኙነት ለማ¨ ቅ የሚረዳ ነው፡፡

ጥናትና ምርምር ፋላጎትን ያማከለ ሲሆን ማንኛ¨ ¬ም ¾ ባ በሽታምልክቶችየምታÃበትእናወባ በÅሙ ¨ ¬ስዓ ¾ቱቶኘበትህፃን የጥናት መመዘኛውን የሚያሟሉ ህሙማን የምርምሩ አካል እንዲሆኑ ያበረታታል፡፡

ማንኛውም ልጅዎን የተመለከተ መረጃ በሚስጢር እንደሚያዝ ልናረጋግጥልዎት እንወዳለን፡፡

ርስዎም የጥናቱን አስፈላጊነት ለአርስዎም ሆነ ለቀጣዩ ትውልድ ከማሰብ አንፃር እንዲሁም የጥናቱን አስፈላጊነት በመረዳት መልካም ፈቃድዎ ከሆነ ልጅዎ በጥናቱ እንዲሳተፍ ፈቃደኛ ቢሆኑ?

☐ *አ ቃÅኛ ነኝ* ☐ *ፈቃደኛ አይደሉም*

አመሰግናለሁ፡፡

(መልሱ- አ ቃÅኛ ነኝ ከሆነ)

በጥናትና ምርምሩ ልÎ እንድሳተፍ በተጠየኩት መሰረት አላማ በደንብ አንብቤ (ተነቦልኝ) ዕንዲሁም ያልገባኝን ጥይቂ ስለተገነዘብኩ የተሳትፎ ፋቃደኝነቴን በፊርማዬ አረጋግጣሉ፡፡

¾ተሳታኝ¨ ` መለÁ ቁዓ ር -------------------- ኝርማ ------------- ቀን --------------------

¾ተመራማሪ¨ `ስም ---------------------- ኝርማ ------------------ ቀን --------------------

¾ሏማኝ ስም -------------------------------- ኝርማ ------------------ ቀን --------------------

Annex-X: Questionnaire

Jimma University

College of public health and medical sciences

Department of medical laboratory science and pathology

A research questionnaire

AIM: Dear participants, first I would like to thank you for your participation in the study. The aim of this study is to determine hematological parameters and hemozoin-containing leukocytes in children with malaria and their association with disease severity at Pawe general hospital.

Name of the patient_________________________ Card number___________

Code number___________ Address___________ Date___________

PART I: Socio–demographic variables

1. Age in years or months: ☐
2. Sex: 1. Male ☐
   2. Female ☐

3. Place of residence ☐

4. Anthropometric measurements

   4.1. Weight (Kg) ☐

   4.2. If > 5 years old mid-upper arm circumference (MUAC) (mm) ☐

Part II: Clinical data

1. **Clinical data**

1.1. Presenting Symptoms

1. Axillary temperature (^{0}c) [ ]
2. Does the child have fever? 2.1. Yes [ ] 2.2. No [ ]
3. If the answer is yes, how long is the duration of fever?
   - 3.1. 24 hours [ ]
   - 3.2. 48 hours [ ]
   - 3.3. >48 hours [ ]
4. Does the child have vomiting? 4.1. Yes [ ] 4.2. No [ ]
5. Does the child have diarrhea? 5.1. Yes [ ] 5.2. No [ ]
6. If the answer is yes, then specify the duration of diarrhea: ________
7. Does the child have cough? 7.1. Yes [ ] 7.2. No [ ]
8. If the answer is yes, then specify the duration of cough: ______________________________
9. Does the child convulse within the last 24 hours? 9.1. Yes [ ] 9.2. No [ ]
10. If the answer is yes, how many times?
    - 10.1. Once [ ]
    - 10.2. Twice [ ]
    - 10.3. More than two times [ ]
11. Other main complaints: [ ]

1.2. Presenting Signs

12. Is the child pale? 12.1. Yes [ ] 12.2. No [ ]
13. Is the child unable to stand or sit without help? 13.1. Yes [ ] 13.2. No [ ]
14. Does the child have jaundice? 14.1. Yes [ ] 14.2. No [ ]
15. Does the child have respiratory distress? 15.1 Yes [ ] 15.2. No [ ]
16. How much is the child's Blantyre /Glasgow coma scale?

0. [ ] 1. [ ] 2. [ ] 3. [ ] 4. [ ] 5. [ ]

17. Does the child have splenomegaly? 17.1. Yes ☐ 17.2. No ☐

18. Does the child have hepatomegaly? 18.1. Yes ☐ 18.2. No ☐

19. Other presenting signs:

☐

20. Provisional diagnosis?

☐

21. Did the child take antimalarial drugs within the last two weeks?

21.1. Yes ☐ 21.2. No ☐

22. Was the child infected with malaria within the last one year?

22.1. Yes ☐ 27.2. No ☐

Annex-XI: Questionnaire (Amharic version)

ጅማ ዩኒቨርሲቲ

የህብረተሰብ ጤናና ህክምና ሳይንስ ኮሌጅ

የህክምና ላቦራቶሪና ፓቶሎጂ ትምህርት ክፍል

የጥናቱ መጠየቅያ ቅጽ

የጥናቱ አላማ:- ¨Å ተሳታፊዎች፣ በቅድሚያ በጥናቱ ለመሳተፍ ፍቃደኛ ስለሆኑ የልብ ምስጋናችንን ለመፅለêእንወዳለን:: የጥናቱ አላማ የወባ በሽታ በህፃናት የደም ህዋሳት ላይ የሚያመጣውን ለውጥና ከበሽታው የህመም ደረጃ ጋር ያለውን ግንኙነት ለማወቅ የሚረዳ ነው::

¾ታካሚው ስም__________________________ የካርድ ቁጥር____________

¾መለÁ ቁዓር____________አድራሻ____________ቀን____________

¡አል 1.ማህበራዊ እና ግለሰባዊ መረጃዎች

01. እትþ በù መት ☐

02. ኖታ **1.** ወንድ ☐ **2.** ሴት ☐

03. የሚኖርበት አድራሻ ☐

¡አል1.1. ¾ታካሚው መጠን መለክያዎች

04. ክብደት በ ክ.ግ ☐

05. ከ 5 ùመትበላይ ከሆነ የላይኛው ክንድ መሀል ስፋት በ ሚ.ሜ ☐

¡አል 2. ¾በሽታው ስሜቱ እና ምልክቶች

ክፍል 2.1.የበሽታው ስሜቶች

01. የሰውነት ሙቀት ማጠን በ ድ.ሴ ☐

02. ልጅዎ ትኩሳት ነበረው/ት? 2.1. አዎ ☐ 2.2. የለውም ☐

03. በዚህ2 ሳምንትወስጥትኩሳትነበረው 3.1. አዎ ☐ 3.2. የለውም ☐

04. ብርድብርድ ይለው/ላት ነበር 4.1. አዎ ☐ 4.2. አይለውም/ትም ☐

05. የህመም ወይንም የድçም ስሜት ይሰማው ነበር

5.1. አዎ ☐ **5.2.** አይሰማውም ☐

06. ራሱን ያመዋል/ታል 6.1. አዎ [ ] 6.2. አያመውም/ትም [ ]

07. ያስታወክዋል/ታል 7.1.አዎ [ ] 7.2. አያስታውከውም/ትም [ ]

08. ÁስቀምÖª ል/ታል 8.1.አዎ [ ] 8.2.አያስቀምጠውም/ትም [ ]

8.3. መልሰ- አዎ ከሆነ ለምን ያህል ግዜ [ ]

09. Áስለª ል/ታል 9.1. አዎ [ ] 9.2.አያስለውም/ትም [ ]

9.3. መልሰ- አዎ ከሆነ ለምን ያህል ግዜ [ ]

10. በ 24 ሰአት ውስጥ ራሱን/ዋን ሲቶ/ታ ወድቆነበር 10.1. አዎ [ ] 10.2.አልሳተም [ ]

10.3. መልሰዎአዎ ከሆነ ምን ያህል ግዜ [ ]

11. ለላ የምሰማህ/ሽ ስሜት_______________________

¡ አል 2.2. ¾ብሽታው ምልክቶች

12.አይኑ/ዋ፤የእÏ መቴና ¨ Ãም ምላሱ የነጣ ነው 12.1. አዎ [ ] 12.2. አይደለም [ ]

13.ትኩሳት አለው 13.1. አዎ [ ] 13.2. የለውም [ ]

14.አይኑ/ዋ ብጫ ሆኑዋል 14.1. አዎ [ ] 14.2.አልሆነም [ ]

15.የአተነፋፈስ ስነ-ስርአቱ ትክክል ነው 15.1.አዎ [ ] 15.2.አይደለም [ ]

16. ራስ ¾መቆ× ÖC Åረጃ

0. [ ] 1. [ ] 2. [ ] 3. [ ] 4. [ ] 5. [ ]

17.ጣፍያውአብጥዋል 17.1. አዎ [ ] 17.2. አላበጠም [ ]

18.ጉበቱ አብጥዋል 18.1. አዎ [ ] 18.2. አላበጠም [ ]

19.እጥዎቹ አብጥዋል 18.1. አዎ [ ] 18.2.አላበጡም [ ]

20.ሌላ ¾ምታዩ ምልክቶች_______________________

21.ሌላተጨፔር ምርሜዎች_____________________________

22.ከ2 ሳምንት ወድህ የወባ መድሃኒት ወስድዋል 22.1. አዎ [ ] 22.2.አልወሰደም [ ]

23.በዝህአመት ውስጥ ወባ አሞት ያውቃል 23.1. አዎ [ ] 23.2.አያውቅም [ ]

Annex-XII: Laboratory reporting form

1. Parasitological results

1.1.Blood film examination result:

01. Positive ☐ 02. Negative ☐

03. If the result of the blood film examination is positive, then specify and determine:

(i). The *Plasmodium* species

01. P. falciparum ☐ 02. *P. vivax* ☐ 03. *P. ovale* ☐

04. *P. malariae* ☐ 05. Both *P. falciparum* and *P. vivax* ☐

06. Other parasites ☐

(ii). **The developmental stage**

01. The ring stage ☐ 02. Mature trophozoite stage ☐

03. The schizont stage ☐ 04. Gametocyte stage ☐

(iii). **Parassitemia (parasites/µl):** ☐

1.2. **Stool examination result results:** ________________________

2. Serological test results

2.1.Rapid HIV diagnostic test result:

01. KHB a. Reactive ☐ b. Non-reactive ☐ c. Invalid ☐

02. Stat pack a. Reactive ☐ b. Non-reactive ☐ c. Invalid ☐

03. Unigold a. Reactive ☐ b. Non-reactive ☐ c. Invalid ☐

3. Hematological test result

3.1.Hematological parameters test results

Test parameters	Test results	Reference values	Abnormalities (flags)
1. WBC(x 10^9/L)			
2. GRAN(x10^9/L) ⇨ GRAN (%)			
3. LYM (x10^9/L) ⇨ LYM (%)			
4. MID (x10^9/L) ⇨ MID (%)			
5. RBC (x 10^{12}/L)			
6. Hb (g/dl)			
7. Hct (%)			
8. MCV (fl)			
9. MCH (pg)			
10. MCHC (g/dl)			
11. RDW (%)			
12. PLT (x 10^9/L)			
13. MPV (fl)			

3.2.Pigment-containing leukocytes detection and enumeration results

1. **Is/are there any pigment containing leukocyte/s?**

1.1.Yes ☐

1.2. No ☐

1.3. If yes, specify the type of the leukocyte harboring malaria pigment,

a. Neutrophil ☐
b. Monocyte ☐
c. Both neutrophils and monocytes ☐
d. Other leukocytes ☐

2. **Quantities of Pigment-containing leukocytes**

2.1. **Pigment containing leukocytes percentage (%)**

2.1. a. Pigment-containing neutrophils % ☐

2.1. b. Pigment-containing monocytes % ☐

2.2. **Total pigment containing leukocytes per microliter (/µl)**

2.2. a. Total pigment containing neutrophils/µl ☐

2.2. b. Total pigment containing monocytes/µl ☐

Remark

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Annex-XIII: Declaration sheet

We, the undersigned, agree to accept responsibility for the scientific ethics and technical conduct of the biomedical research and for the provision of required progress reports as conditions of your institution in effect of grant provision. Moreover, all investigators will assure to guarantee the safety and proper care of the study participants.

Principal investigator

Muluken Birhanu (BSc, MSc student; Jimma University)

Signature _________ Date___________

Advisors

1. Wondimagegne Adissu (BSc, MSc)

   Signature ________ Date___________

2. Dr. Tilahun Yemane, Assist. Professor, (MD, MSc)

   Signature________ Date____________