Available online at www.elixirpublishers.com (Elixir International Journal)

Applied Biology

Elixir Appl. Biology 57A (2013) 14479-14493

ISSN: 2229-712X

Compensatory reticulocytosis as a surrogate marker of recent PF malaria infection in a holoendemic region of western Kenya

Okoth Patrick, Ofulla A.V, Onyango D and M, Odada Sumba

Masinde Muliro University of Science and Technology, P.O Box 190 50100 Kakamega, Kenya.

ARTICLE INFO

Article history:

Received: 9 November 2012;

Received in revised form:

18 April 2013;

Accepted: 24 April 2013;

Keywords

Compensatory,

Region,

Infection.

ABSTRACT

Reticulocytosis is an important previously unappreciated surrogate marker of recent *Plasmodium falciparum* reticulocyte parasitation. Evidence from studies in murine and primate models show increased prevalence of *Plasmodium falciparum* parasitisation of reticulocytes. The increase in reticulocyte infection has been attributed to compensatory reticulocytosis and increased parasite densities. However, the prevalence of *P. falciparum* parasitisation of reticulocytes in human populations in holoendemic areas of malaria is unknown. Therefore, the current study examined the prevalence of *P. falciparum* parasitation of reticulocytes and its association with clinical, parasitological and haematological outcomes in children (n=164) and adults (n=41) with acute *P. falciparum* malaria and healthy malaria-negative controls (children, n=132 and adults, n=60) from a holoendemic area of malaria of western Kenya. Malaria diagnosis and species identification was performed using Giemsa-stained thick and thin blood smears while reticulocyte parasitation was determined using New Methylene Blue- Giemsa counter-stained thin blood films. Results of this study showed that children with malaria presented with increased numbers of infected reticulocytes compared to adults with malaria (p=0.006; Mann-Whitney U test). In addition, children with malaria presented with higher reticulocyte counts compared to those without malaria (p<0.0001; Mann-Whitney U test).

Introduction

Background of the Study

An insufficient number of circulating reticulocytes has been observed in humans with P. falciparum malaria (Philips et al., 1986; Abdalla et al., 1990; Kurtzhals et al., 1997) as well as in murine models of malaria (Chang et al., 2004). This has been attributed to suppression of erythropoiesis during malaria infection and is considered as a host protective mechanism that limits the number of RBCs available for parasitisation (Chang et al., 2004). Insufficient RBC production has been attributed to bone marrow suppression, decreased erythropoietin production and/or impairment of the maturation of erythroid precursors into erythrocytes (el Hassan, 1997; Abdalla, 1990; Villeval et al., 1990). These factors would lead to a reduction in the number of circulating reticulocytes. Suppression of reticulocyte production has previously been studied in rodent models during Plasmodium chabaudi infection (Chang et al., 2004). Erythropoietic suppression has also been observed in acute Plasmodium berghei infection, as evidenced by decreased transcription of erythroid specific genes in the spleen and bone marrow (Sexton et al., 2004).

The number of reticulocytes produced and destroyed in the late-stage of P. berghei infection has been associated with compensatory erythropoiesis in response to haemolytic anaemia and high parasite burdens (Sexton et al., 2004). This has, however, not been done for P. falciparum in humans in holoendemic regions. The observed level of reticulocytes in circulation is the net result of the production of reticulocytes in the bone marrow and spleen and their subsequent maturation or destruction in the circulation. Studies in P. berghei-infected animals indicate insufficient observed numbers of circulating reticulocytes which has been attributed to increased parasitisation and destruction and not just a reduction in their production. Preferential infection of reticulocytes over erythrocytes has also been observed in P. vivax (Kitchen 1938; Garnham, 1966), and P. berghei (Singer 1953).

Plasmodium falciparum is a major cause of economic loss and underdevelopment in the developing countries. In order to control malaria and accelerate economic development, novel integrated measures are urgently required for controlling and preventing malaria infections. Recent studies highlight new insights into parasite biology and host mechanisms of protective immunity (Hill et al, 2006). The prospects of a paediatric vaccine capable of alleviating the burden of malaria in the developing world or even more dauntingly, a vaccine capable of completely preventing malaria remain a distant prospect. Novel strategies have been undertaken to generate a whole organism vaccine candidate but the fact that malaria parasite progresses through a succession of stages in the human host with stage specific expression of proteins and utilization of a myriad mechanisms to evade host immune response poses a great challenge. Understanding the biology of P. falciparum invasion of erythrocytes may lead to novel approaches of parasite manipulation hence contributing to integrated management and prevention of malaria.

Malaria is an important etiologic factor for severe anaemia among children and pregnant women in malaria-endemic areas of sub-Saharan Africa (Dreyfuss et al., 2000; Steketee et al., 2001). The mortality rate of malaria-related anaemia is between

Tele:

E-mail addresses: cdc.govk@yahoo.co.uk

5.6% and 16% for children, and 6% for pregnant women, largely in primigravidae (Chang et al., 2004). Increased destruction of infected and uninfected red blood cells (RBCs), and decreased replenishment of the RBCs due to bone marrow dysfunction have been implicated in the pathogenesis of severe malarial anaemia and, the underlying mechanisms found to be largely multifactorial (Chang et al., 2004). Furthermore, previous studies have demonstrated that blood-stage P. chabaudi infection in mice suppresses reticulocytosis in proportion to the level of parasitaemia (Dormer et al., 1983). Development of late erythroid precursors is severely suppressed during blood-stage malaria, which may ultimately lead to the decreased production of reticulocytes observed in malaria-infected experimental animals and patients (Wickramasingh et al., 1982; Dormer et al., 1983; Chang et al., 2004). In humans, P. vivax has been demonstrated to show strong preference for invasion of reticulocytes (Kitchen et al., 1938), while P. falciparum can efficiently invade RBC of all ages (Bruce–Schwart et al, 1948). P. falciparum propensity for reticulocytes has been observed in in vitro studies in experimental animal models (Clough et al., 1998), and in vivo (Hegner, 1938; Pasvol et al., 1980). Decreased circulating levels of reticulocytes has often been relied on as evidence for erythropoietin suppression in humans with P. falciparum infection, but this does not account for the preferential invasion and destruction of reticulocytes by P. falciparum (Srichaikul et al.,1967).

This study aimed at delineating prevalence of reticulocyte infection by P. falciparum and their cumulative effect on the erythropoietic processes, their attendant effect on clinical parasitological and haematological outcomes in children exposed to holoendemic malaria. The results of this study will be importantin in understanding reticulocytosis as an important surrogate marker of malaria pathogenesis which will help in designing novel tools for malaria management and control strategies.

Statement of the problem

Reticulocytes are erythroid cells in a descrete penultimate phase of maturation. The observed level of reticulocytes in circulation is the net result of the production of reticulocytes in the bone marrow and spleen and their subsequent maturation or destruction in the circulation. Studies in P. berghei-infected animals indicate insufficient observed numbers of circulating reticulocytes which has been attributed to increased parasitisation and destruction and not just a reduction in their production. Prevalence of reticulocyte parasitisation in children and adults has received relatively little attention but may offer unique insights into the processes that govern reticulocyte parasitation. Studies in areas with seasonal malaria have reported association between reticulocyte parasitation with clinical, parasitological, anthropometric and haematological factors but the case of children and adults reporting to hospitals with acute malaria is poorly understood. Prevalence of reticulocyte infection levels are yet to be defined. Compensatory reticulocytosis has been described in animal models but it remains to be known whether reticulocyte parasitation has an effect on erythrocytic indice in holoendemic regions of malaria. The number of reticulocytes produced and destroyed in the late-stage of P. berghei infection has been associated with compensatory erythropoiesis in response to haemolytic anaemia and high parasite burdens. But the case of P. falciparum in humans in holoendemic regions remains unclear. Preferential infection of reticulocytes over erythrocytes has also been observed in P. vivax (Kitchen 1938; Garnham, 1966), and P. berghei (Singer 1953).

Rationale of the Study

Several experimental studies in human and animal models have demonstrated Plasmodial species predilection for circulating reticulocytes, but the extent of Plasmodium falciparum cellular tropisms and prevalence of reticulocyte parasitisation in populations exposed to holoendemic malaria is unknown. The prevalence of reticulocyte parasitisation in children and adults with asymptomatic acute malaria in holoendemic areas have received relatively little attention but may offer unique insights into the processes that control reticulocyte parasitation in malaria. Several studies in areas with seasonal malaria have reported that increased reticulocyte infection is associated with clinical, parasitological, anthropometric and haematological factors but the case of children and adults reporting to hospitals with acute malaria is poorly understood. Prevalence of reticulocyte infection levels are yet to be defined in holoendemic regions of malaria. Compensatory reticulocytosis has been described in animal models but it remains to be known whether increased reticulocyte parasitation has an effect on erythrocytic parameters in holoendemic regions of malaria. Since levels of reticulocyte parasitation are unknown, this study sought to delineate and document the prevalence of reticulocyte parasitisation in children and adults with uncomplicated malaria in western Kenya. The results of this study documented the levels of reticulocyte parasitation in children with asymptomatic paediatric malaria and its association with clinical, parasitological and haematological outcomes in a holoendemic region of malaria of western Kenya. The results of this study shades further light on reticulocytosis and offer unique insights into the reticulocyte parasitisation and its cumulative effect on reticulocytosis as an important surrogate marker in malaria infection which may help in designing novel integrated measures in malaria management and control strategies.

Research Questions

(a) What is the prevalence of P. falciparum reticulocyte parasitation in children and adults with uncomplicated malaria?

(b) Which of the clinical, parasitological and haematological factors are associated with P. falciparum reticulocyte parasitation in children and adults with uncomplicated malaria?

General Objective

To determine the prevalence of P. falciparum parasitation of reticulocytes and its association with clinical, parasitological and haematological outcomes in children and adults with uncomplicated malaria in a holoendemic region of western Kenya.

Specific objectives

a) To delineate the prevalence of P. falciparum reticulocyte parasitation in children and adults with uncomplicated malaria.

b) To determine the association of reticulocyte parasitisation with clinical, parasitological and haematological outcomes in children and adults with uncomplicated malaria.

Limitations / Confounding Factors of the Study

HIV screening was done to the study subjects but other Co-morbid conditions such as sickle Cell trait, bacteraemia, hemoglobinopathies and nutritional status were not investigated and therefore their confounding effect on erythropoietic process were not determined.]

Materials And Methods

Study Area

This was a cross-sectional study performed as part of larger study investigating the development of immunity in children naturally exposed to malaria. The population of this area is

approximately 0.6m people out of which 15% are children <5 years of age.

The study was performed at Chulaimbo Rural Health Training Centre (CRHTC) and Kanyawegi area, Kisumu District, Nyanza Province, western Kenya (See Appendix I). Populations in western Kenya experience holoendemic P. falciparum transmission with annual entomologic inoculation rates of 150-450 per person with peak transmissions occurring during and following the long rains in April to August and November to January (Bloland et al., 1999).

Study Population

The study was conducted at Chulaimbo Rural Health Training Centre (CRHTC) in Kisumu District, Nyanza Province, western Kenya (See Appendix I). The population of this area is approximately 600,000 people with 15% children <5 years. The Chulaimbo Provincial Rural Health Centre is located about 25 Km North West of Kisumu City, Kisumu County, Nyanza Province (Latitude: 0 ^{o} -2 ^{o} 0 N; Longitude: 34 ^{o} 37' 60 E). The area is within the lowland region at an elevation of about 1133m above sea level adjacent to the shows of Lake Victoria.

Populations in this area experience holoendemic P. falciparum transmission with annual entomologic inoculation rates of 150-450 per person with peak transmissions occurring during and following the long rains in April to August and November to January (Bloland et al., 1999). P. falciparum is the principal species causing malaria accounting for 97% of malaria cases in infants (Bloland et al., 1999). This region has an equatorial altitude of 1140 -1400 m above sea level and rainfall of 800 -2000mm per year. The main mosquito vector is A. Gambie (Beire et al., 1994).

Sample Size

The study consisted of 164 parasitaemic children and 41 parasitaemic adults with asymptomatic acute uncomplicated malaria. 132 age-matched control children and 60 adults were also enrolled. Since haematological measures of children with malaria vary with endemicity and age (Bloland et al., 1999), healthy controls were used for evaluating haematological changes during reticulocyte parasitation in malaria. Sample size was determined using Power and Sample Size Calculation Programme (version 2.1.31, USA) based on Dupont and Plummer (1990) for studies analysed using Chi-square and Fisher's exact tests. Sample sizes for children and adults were calculated separately based on studies by Awander et al., 2006, investigating the genetic study at Siaya District Hospital (SDH) showing associations between high density parasitaemia and MIF -173 polymorphisms. The probability of exposure of controls was obtained from previous studies in an adjacent study area in western Kenya showing that children aged 1-4 years have a P. falciparum malaria prevalence of 88.3% (Bloland et al., 1999). The probability of malaria infection cases was set at >99.5%. Therefore using the alpha value of 0.05 and a power of 80%, the sample size for acute cases versus controls was determined using Power and Sample Size calculation for children and adults as shown in sections (i) and (ii) below.

i) Sample size calculation for children.

The study involved independent cases and controls with 0.8 control(s) per case. Prior data indicated that the probability of exposure among controls was 0.6. If the true odds ratio for disease in exposed subjects relative to unexposed subjects is 0.5, we will need to study 162 case patients and 130 control patients to be able to reject the null hypothesis that this odds ratio equals 1 with probability power of 0.8. The type I error probability associated with this test of null hypothesis is 0.05. Fisher's exact test was used to evaluate this null hypothesis. Therefore, 164 children with malaria and 132 controls were enrolled into the study.

ii) Sample size calculation for adults.

The study involved independent cases and controls with 1.5 control(s) per case. Prior data indicated that the probability of exposure among controls was 0.6. If the true odds ratio for disease in exposed subjects relative to unexposed subjects is 0.30, we will need to study 41 case patients and 61.5 controls to be able to reject the null hypothesis that this odds ratio equals 1 with probability power of 0.8. The type I error probability associated with this test of null hypothesis is 0.05. Fisher's exact test was used to evaluate this null hypothesis. Therefore, 41 adults with malaria and 60 controls were enrolled into the study.

Inclusion criteria

Children less than five years of age and permanent residents of Kisumu District, living within a radius of 50 Km of Chulaimbo Rural Health Training Hospital (CRHTH). Ethical clearance to draw blood from human volunteers was obtained. The child had to have an auxiliary temperature of $\geq 37.5^{0}$C and parasitaemia greater than 5,000 P. falciparum parasites /µL of blood for acute cases but healthy controls did not have to conform to this in order to reflect a natural population. Malaria diagnosis and species identification achieved by microscopic examination of thick blood smears stained with Giemsa, which is the routine standard examination according to (WHO) recommendations. Slides were independently examined by two experienced microscopists and the third one as a tie breaker. Parasitemia was reported as the number of asexual parasites/µL after counting the number of asexual parasites/200 leukocytes in high-magnification fields.

Exclusion criteria

Children with Hb levels <5.0g/dL and/or other indicators of complicated P. falciparum malaria (Marsh et al., 1995; WHO, 2000) or evidence for other aetiologies of fever such as lower respiratory tract infection, patients who presented chronic diseases likely to create anemia and pregnant women were excluded from the study for ethical reasons.

Ethical Considerations

Informed parental/guardian consent was sought for children meeting inclusion criteria. The consent form was available in Dholuo, Kiswahili and English. Approval for this study was obtained from the Ethical Review Committee at the Kenya Medical Research Institute (KEMRI) and Human Investigations Institutional Review Board at the University Hospitals of Cleveland and Case Western Reserve University (CWRU), Cleveland, Ohio, USA. The geographic, clinical, and haematological indices of children with varying severities of malaria were tabulated. Phlebotomists carried out the process of venipuncture in a sterile manner to minimize risk of infection and discomfort to children. All clinical cases were treated according to Ministry of Health guidelines.

Experimental Procedures/Methods

Collection and processing of blood samples

Sterile techniques were used to obtain finger prick blood into ethylene diamine tetracetic acid (EDTA) tubes. Approximately, 0.5 mL of blood was drawn from children and adults by finger prick. It was then transported at room temperature to CWRU/KEMRI laboratories located at Centre for Global Health Research (CGHR), Kisumu where complete blood count (CBC) was done (See Appendix II showing blood collection form).

Thick and thin blood films were prepared and stained with 5% Giemsa stain. The parasites and leucocytes were counted in

the same fields until 200 leucocytes were counted. Peripheral blood parasite densities were calculated per µL of blood using the total WBC count for each individual. Slides were considered negative upon confirmation of absence of asexual parasites in 200 high-power ocular fields of thick smear. Species diagnosis was determined from thin smears.

Haematological evaluations

Complete Blood Counts were determined using an 8-parameter [white blood cells (WBC, $\times 10^3/\mu L$), lymphocyte (%), monocyte (%), granulocyte (%), RBC ($\times 10^6/\mu L$), Hb (g/dL), haematocrit (Hct, %), MCV (fL), MCHC (g/dL), platelets $\times 10^3/\mu L$) and MPV] automated haematology analyzer (Beckman Coulter Inc., Fullerton, CA, USA). Blood samples were obtained before administration of treatment. Haemoglobin concentrations were determined using a portable B-haemoglobin photometer (Hemocue AB, Angel Holm, Sweden).

Whole blood was collected in EDTA tubes and s aspirated into the coulter counter and the CBC recorded. The WBC and RBC measure the total number of cells per volume of blood. Hb and Hct report signs of anaemia. MCV measures average size of RBC and may indicate microcytosis or macrocytosis or iron deficiency anaemia or thalassemias. The MCHC measures the concentration of Hb inside the RBC. The platelet count is the number of platelets in a given volume of blood. Both increases and decreases in platelets can point to abnormal conditions of excess bleeding or clotting.

Whole blood in the EDTA anticoagulant blood tubes was mixed thoroughly before aliquoting 2.0 µL on the Coulter (TM) machine followed by dilution using isotone lysing solution to denature RBC to allow for haemoglobin (Hb) determination and white blood cell count. To determine haematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) and platelet (Plt) count, a fraction of blood was channelled to the RBC chamber.

Parasitological examination for P. falciparum

Thick and thin blood films were stained with 5 % Giemsa and examined for presence of the parasites and leucocytes which were counted in the same field until 200 leucocytes had been enumerated. Slides were considered negative if no asexual parasites were found in 200 high power ocular fields of thick smear while thin films were used for the diagnosis of different species. Giemsa-stained thick and thin blood smears were examined at ×1000 under oil immersion for the presence and parasitaemia levels of P. falciparum. Malaria parasitaemia was determined as the number of parasites/µL of blood by counting asexual parasites per 200 leucocytes, assuming 8000 leucocytes/µL of blood.

Fresh blood was used to make thin smears, which were stained with new methylene-blue followed by Giemsa, and observed under light microscopy for differential counts of mature versus young red cells, infected with Plasmodium parasites. A minimum of 1,000 RBCs were counted to enhance enumeration precision, on evenly stained blood smears, with a uniform cellular distribution. Ruled ocular devices that standardized the area to be counted [i.e.; the Miller ocular devise] were used to lower counting variability. A comparative analysis of the number of reticulocytes and normocytes infected with P. falciparum and presence of multiple infections was also examined. All children testing positive for blood stage malaria parasites were treated free of charge following the Kenya national guidelines for the treatment of uncomplicated malaria infection.

Reticulocyte counting

Supravital staining technique for reticulocyte counting was used (Brecher, 1949). An isotonic solution of new methylene blue (supra-vital) stain was incubated with blood. The live RBCs were stained so as to detect reticulocytes. Reticulocytes were counted microscopica1ly on a thin blood film preparation and were recognized by the presence of violet-blue stained granules of ribosomal RNA (reticulin) in the cytoplasm (See Appendix IV for optimized protocol on reticulocyte staining and enumeration). A few drops of the supravital dye solution [1.0% w/v of new methylene blue or brilliant cresyl blue] was mixed with an equal volume of EDTA anti-coagulated peripheral whole blood and incubated for several minutes. A thin smear of the stained blood preparation was then made, counterstained with Wright's stain, and the slide examined by light microscopy. An adequate number of red cells totalling 1000 in a well stained area were observed and the proportion of reticulocytes determined. The cells that displayed a blue granular precipitate, and which could vary from one individual to the other with small blue granules to a network of blue reticular material, and had a faint, diffuse basophilic hue (a condition described as hypochromasia) were enumerated. Reticulocyte counts were reported as percentages of total RBC examined.

The normal mean percentage reticulocyte count by NMB light microscopy is 1% to 1.5%, with 3% being the upper limit of normal (Deiss et al., 1970). Since the relative reticulocyte count is misleading when RBC count is abnormal and/or erythropoietic stimulation to the bone marrow is taking place, like in severe anaemia, a mathematical correction was applied to the relative count in cases of high parasitaemia (Koepke et al., 1986). Reticulocytes were differentiated on the basis that immature reticulocyte contain the largest amount of precipitated ribosomal RNA while the least immature contain only a few dots or strands of ribosomal RNA material.

Reticulocyte enumerations were determined using 1% new methylene blue staining of thin blood films. Reticulocyte count (%), absolute reticulocyte numbers (ARN, $\times 10^9/L$) and Reticulocyte Production Index (RPI) was calculated as follows: RPI= reticulocyte index (RI)/maturation factor (MF), where RI = [reticulocyte count (%) × Hct/0.36] and MF = b+ (m) (x), where b = 1, m = 0.05 and x = (avg. normal population Hct of patient).

Data Management and Statistical Analysis

Data entry and cleaning was performed using Excel software. Statistical analysis was performed using SPSS software package version 15.0 (SPSS Inc., Chicago, USA). Fisher's exact test was used to compare differences in the proportions of categorical data across groups. Differences in medians for continuous data between P. falciparum [+] and P. falciparum [-] individuals in the children and adults were examined using the Mann-Whitney U tests. Associations between the number of infected reticulocytes and haematological indices were compared using the Spearman rank correlation coefficient. Logistic multivariate regression models were used to determine predictors of reticulocyte infection in children and adults with P. falciparum malaria. In these models the confounding effect of age and gender was controlled for. All tests were two-tailed and an alpha value of 5% was used for statistical inferences.

Results

4.1: Gender, Age, and Temperature of the Study Participants

The gender, age, temperature, clinical and laboratory characteristics of the study participants are summarized in Table 1 and 2. Gender distribution did not differ significantly between

the aparasitaemic and parasitaemic individuals in the adults (p=0.736; Fisher's exact test) and children (p=0.140; Fisher's exact test) groups. Although age was similar in the aparasitaemic and parasitaemic adults (p=0.471; Mann-Whitney U test), children with P. falciparum infection were younger (p=0.001; Mann-Whitney U test). Although axillary temperature was similar in aparasitaemic and parasitaemic adults (p=0.787; Mann-Whitney U test), it was elevated in parasitaemic children compared to aparasitaemic children (p<0.0001; Mann-Whitney U test).

Parasitological analyses (Table 2) revealed that children with malaria presented with higher median parasitaemia (p<0.0001; Mann-Whitney U test) and geometric mean (p=0.007; t test) parasite densities compared to adults with malaria. Consistent with higher parasite densities, the prevalence of high density parasitaemia (HDP, ≥10,000 parasites/µL of blood) was higher in parasitaemic children relative to parasitaemic adults (47.0% vs. 2.4%; p<0.0001; Fisher's exact test). Infection with multiple species (P. falciparum and P. malariae) was presented among 4 (9.8%) and 6 (3.9%) parasitaemic adults and children, respectively (p=0.222; Fisher's exact test).

The prevalence of infected reticulocytes was higher among parasitemic children compared to parasitaemic adults [43 (27.7%) vs. 3 (7.3%); p=0.006; Fisher's exact test). In addition, the prevalence of multiple infected (two or more parasites per RBC) RBC [23 (14.0%) vs. 0 (0.0%); p=0.009; Fisher's exact test] and reticulocytes [1 (0.6%) vs. 0 (0.0%)] was more common in parasitaemic children compared to parasitaemic adults.

Data are presented as medians (Q1-Q3). Median parasitaemia/µL; HDP, high density parasitaemia (≥10,000 parasites/µL); Retics, reticulocytes; [a]Mann Whitney test; [b]Fisher's exact test; [d]versus adults; [e]Only P. malariae and P. falciparum co-infections were observed. [f,g]Ring and trophozoite stages.

4.2: Leucocytic and Thrombocytic Indices of the Study Participants

The leucocytic and thrombocytic indices of the study participants are shown in Table 3 and **Figure 1 and 2.** These haematological analyses did not show significant alterations in the total WBC counts, lymphocytes, monocytes, granulocytes, platelets, prevalence of thrombocytopaenia and MPV levels in parasitaemic versus aparasitaemic individuals among the children and adult groups (p>0.05 for all; Mann-Whitney U test). 4.3: Erythrocytic Indices of the Study Participants

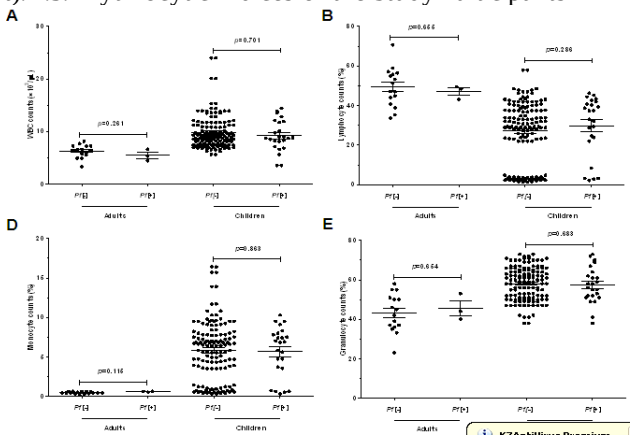

Figure 1: Leukocytes in the study participants categorized according to *P. falciparum* (*Pf*) infection status. (A) total white blood cell counts (WBC); (B) lymphocytes; (C) monocytes; and (D) granulocytes in *Pf* [-] and *Pf* [+] adults (n=16 and n=3) and children (n=132 and n=33), respectively. Statistical significance was determined by the Mann-Whitney U test.

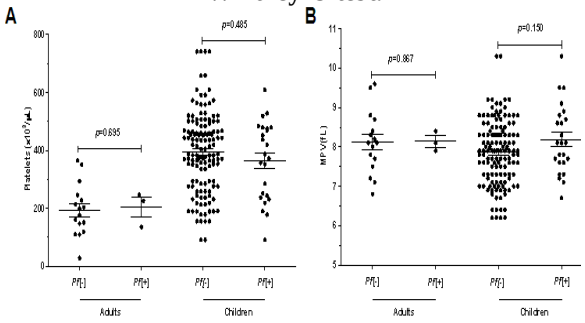

Figure 2*:* Leukocytes in the study participants categorized according to *P. falciparum* infection status. (A) Platelet count (×10^3/µL) (B) MPV (fL) in *Pf* [-] and *Pf* [+] adults (n=16 and n=3) and children (n=132 and n=33), respectively. Statistical significance was determined by the Mann-Whitney U test.

The relationship between erythrocytic indices was examined among the study participants (Table 4 and **Figure 3 and 4**). There were no significant differences in the erythrocytic indices in parasitaemic individuals compared to aparasitaemic individuals in the adult group. Although the RBC (p=0.917; Mann-Whitney U test) and ARN (p=0.828; Mann-Whitney U test) counts did not differ significantly in the parasitaemic compared to aparasitaemic children, reticulocytes were elevated in the parasitaemic children (p<0.0001; Mann-Whitney U test). The RPI was higher in the parasitaemic children (p=0.318; Mann-Whitney U test) while nearly all the children with malaria had increaseds erythropoiesis, however, the difference was non-significant (p=0.012; Mann-Whitney U test). Although Hb levels were higher in children with malaria (p<0.0001; Mann-Whitney U test), the prevalence of SMA (p=0.078; Fisher's exact test) and haematocrit (p<0.0001; Mann-Whitney U test) were lower in the children with malaria. The MCV (p=0.961; Mann-Whitney U test) and MCHC (p=0.098; Mann-Whitney U test) levels were not significantly different in the infected versus uninfected children.

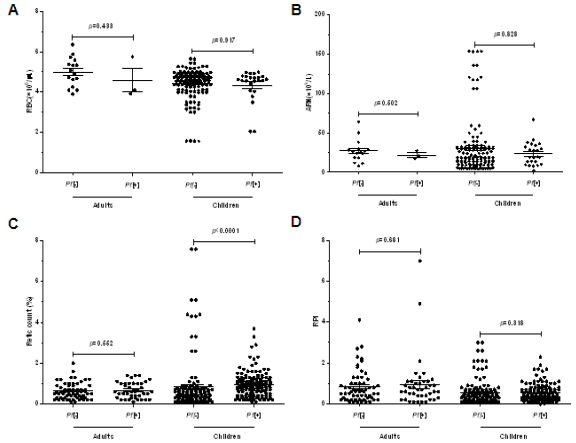

Figure 3: RBC indices in the study participants categorized according to (*Pf*) infection status. (A) RBC (×10^6/µL); (B) absolute reticulocyte numbers, ARN (×10^{9}L); (C) reticulocyte count (%); and (D) reticulocyte production index (RPI) in *Pf* [-] and *Pf* [+] individuals was as follows: A and B. Adults (n=16 and n=3) and children (n=132 and n=33); C and D. Adults (n=60 and n=41) and children (n=164 and n=132) in *Pf* [-] and *Pf* [+], respectively. Statistical significance was determined by the Mann-Whitney U test.

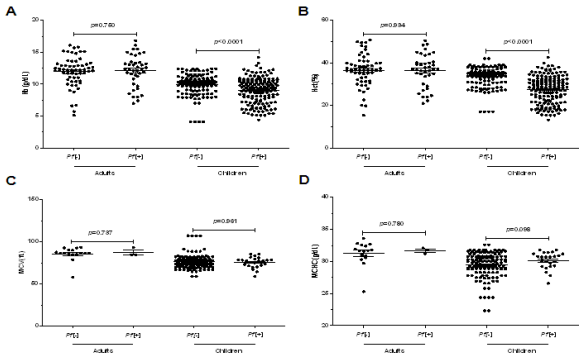

Figure 4: RBC indices in the study participants categorized according to P. falciparum infection status. (A) Hb (g/dL); (B) Hct(%) , (C)MCV(fL); and (D) MCHC (g/dL) Pf [-] and Pf [+] adults (n=16 and n=3) and children (n=132 and n=33), respectively. Statistical significance was determined by the Mann-Whitney U test.

Data are presented as medians (Q1-Q3) or as indicated. RBC, red blood cells; ARN, absolute reticulocyte numbers ($\times 10^9$/L); RPI, reticulocyte production index; SMA, severe malarial anaemia (Hb<6.0 g/dL); MCHC, mean cell haemoglobin concentration. [a]Mann-Whitney U test; [b]Fisher's exact test.

4.4: Association between Infected Reticulocytes and Clinical, Parasitological, and Haematological Measures.

To determine whether infected reticulocytes were associated with alterations in the parasitological, clinical or haematological measures, median levels of these variables were compared between infected reticulocyte and uninfected reticulocytes in children with P. falciparum malaria (Table 5). Haematological measures were not available to perform these analyses in adults with P. falciparum malaria. The median and geometric parasite densities were higher in the individuals presenting with infected reticulocytes in both the adult (p=0.010 and p<0.0001; Mann-Whitney U test) and children (p<0.0001 and p<0.0001; Mann-Whitney U test) groups, respectively.

Consistent with elevated parasitaemia, the prevalence of HDP was higher in the individuals presenting with infected reticulocytes in both the adults (p=0.073; Fisher's exact test) and children (p<0.0001; Fishers exact test) with malaria. However, the axillary temperature was only elevated in individuals with infected reticulocytes (p=0.013; Mann-Whitney U test) among children with malaria. Haematological analyses (Table 6) did not show significant perturbations in the total WBC (p=0.112; Mann-Whitney U test), monocyte (p=0.145; Mann-Whitney U test), granulocyte (p=0.965; Mann-Whitney U test), platelet (p=0.217; Mann-Whitney U test), thrombocytopaenia (p=0.999; Fisher's exact test), and MPV (p=0.536; Mann-Whitney U test) levels among children presenting with infected reticulocytes relative to those presenting without infected reticulocytes among children with malaria. However, lymphocyte counts were elevated in individuals with infected reticulocytes compared to those without infected reticulocytes among children with malaria (p=0.031; Mann-Whitney U test).

Erythrocytic analyses (Table 7) showed non-significant elevations in the RBC (p=0.094; Mann-Whitney U test) and ARN (p=0.186; Mann-Whitney U test) counts, and significant elevations in the reticulocyte counts (p<0.0001; Mann-Whitney U test) and RPI (p<0.0001; Mann-Whitney U test) in children presenting with infected reticulocytes compared to those presenting without infected reticulocytes during P. falciparum malaria. Other erythrocytic indices including inappropriate erythropoiesis (RPI<2.0; p=0.238; Mann-Whitney U test), Hb (p=0.295; Mann-Whitney U test), SMA (p=0.552; Mann-Whitney U test), haematocrit (p=0.369; Mann-Whitney U test), MCV (p=0.234; Mann- Whitney U test) and MCHC (p=0.947; Mann-Whitney U test) did not differ significantly between individuals with infected reticulocytes versus those without infected reticulocytes among children with malaria. 4.5: Predictors of Reticulocyte Infection in Adults and Children with Malaria

In order to determine the association of infected reticulocytes with parasitological, clinical and haematological parameters, Spearman rank correlation was performed. These analyses showed that proportion of parasitised reticulocytes were correlated with parasitaemia (rho=0.481, p<0.0001) and reticulocyte count (rho=0.218, p=0.006) in children with malaria. Among adults with malaria, infected reticulocytes were associated only with parasitaemia (rho=0.408, p=0.008).

Multivariate logistic modelling was performed to identify predictors of infected reticulocytes only for children with malaria, since there were too few individuals among adults with malaria following dichotomisation into various groups to perform these analyses. These analyses demonstrated that increased infection of reticulocytes in children with malaria was independently associated with HDP (OR, 6.845; 95% CI, 2.887-16.231; p<0.0001) and axillary temperature >37.5^{o}C (OR, 2.485; 95% CI, 1.082-5.708; p=0.032).

Discussion

5.1: Prevalence of Plasmodium falciparum Reticulocyte Parasitation in Children and Adults Exposed to Holoendemic Malaria

Reticulocytosis is an important surrogate marker of a recent malaria infection in children and adults. Age-structured red blood cell (RBC) susceptibility to P. falciparum is an important, previously unappreciated determinant of reticulocyte parasitisation. Insights into RBC reticulocyte parasitation are fundamental in understanding malaria pathogenesis. The relationship between red cell age and susceptibility to invasion by Plasmodium falciparum has been examined by several methods including short-term cultures of parasitized human blood (Chang et al., 2004). The results of this study indicated that the prevalence of Plasmodium falciparum reticulocyte parasitation was higher in children compared to adults with malaria (Table 2, 4 and 7). This observation has important theoretical, clinical and practical implications but its mechanism and extent of reticulocyte parasitisation in holoendemic regions of malaria remains as yet unclear.The total reticulocyte count in parasitaemic children was 1496 out of which 39 were infected which translated to 2.6% reticulocyte parasitation. However, within the infected category, 162,935 normocytes were counted and out of which 1061 normocytes were infected which translated to 0.65% parasitized normocytes among children. This indicates that either way, there was high prevalence of reticulocytes parasitation than normocytes in children exposed to holoendemic malaria. Similarly, the adult category presented 273 reticulocyte counts with only 3 reticulocytes parasitized translating to 1.09% reticulocyte parasitation against 39724 normocytes counted and only 99 of them parasitized translating to 0.2% normocyte parasitation. Taken together, there was increased prevalence of reticulocyte parasitation in children and adults that normocytes in both children and adults. The relationship between erythrocytic indices was examined among the study participants (Table 4 and Figure 3 and 4). There were no significant differences in the erythrocytic indices in parasitaemic individuals compared to aparasitaemic individuals in the adult group. Although the RBC and ARN counts did not differ significantly in the parasitaemic compared to

aparasitaemic children, reticulocytes were elevated in the parasitaemic children (Table 7). The RPI was higher in the parasitaemic children while nearly all the children with malaria had increased erythropoiesis, however, the difference was non-significant. Hb levels were higher in children with malaria and haematocrit levels were lower in the children with malaria. Results of this study indicate that increased reticulocyte infection was associated with compensatory reticulocytosis and increased parasite densities (Table 7).

Evidence from murine and human malaria has demonstrated that plasmodial species have a predilection for circulating reticulocytes (Abdalla et al., 1980). However, the impact of P. falciparum on reticulocytes in human populations residing in holoendemic areas is unknown. This study showed that increased reticulocyte infection in children with uncomplicated P. falciparum malaria was associated with increased reticulocytosis; higher parasite densities and febrile illness (axillary temperature >37.5 (Table 5).

In order to investigate the prevalence and impact of reticulocyte parasitation during uncomplicated P. falciparum malaria, children and adults with malaria and their respective controls were studied. Although there were no significant differences in presented demographic characteristics between adults with and without malaria, children with malaria were relatively younger and presented with elevated axillary temperature (Table 1). These results are similar to previous studies indicating that younger children are more susceptible to malaria infection and that febrile illness is a common manifestation of acute malaria (Verhoeff et al., 2002). It can also be argued that since adults have higher levels of acquired immunity, they are unlikely to manifest with common syndromes of malaria unless the infection is exacerbated by the presence of immune suppression such as with HIV co-infection (Kurtzhals et al., 199 Of importance, were findings presented here showing that children with malaria presented with increased levels of parasite densities and higher prevalence of HDP (Table 5). In addition, the infected children had higher levels of infected reticulocytes, mixed malaria infections and multiple stages of the P. falciparum parasites (Table 2). These results are consistent with previous studies showing that children with uncomplicated malaria often had higher parasite densities and multiple Plasmodium spp and stage infections (Orago et al., 2001). Previous studies showing that children with uncomplicated malaria had higher reticulocyte counts (or RDW an indicator of reticulocyte production) also support results of this study (Kurzhals et al., 1997). Thus, since antiparasite immunity develops later after clinical immunity, it appears children are more susceptible to parasitological manifestations of malaria. Consistent with a higher prevalence of infected reticulocytes, children with malaria had higher levels of erythrocytes, reticulocyte count and haemoglobin counts (Table 4).

However, the lower hematocrit levels in the presence of higher erythrocyte and reticulocyte count suggest presence of multi-sized erythrocytes. Thus, children with malaria presenting with increased reticulocyte counts appear to have increased/compensatory reticulocytosis (Table 4).

Additional haematological analyses revealed that children with infected reticulocytes had higher levels of lymphocyte counts. Although these observation are dependent on the coulter (TM) count, higher erythrocyte counts suggested increased inflammatory response (Table 6). These results are similar to previous studies showing that children with uncomplicated malaria presented with lymphocytosis since increased lymphocyte counts may also suggest alterations in lymphocyte subsets (Casuals-Pascual et al., 2006). The increase in lymphocyte count in children with infected reticulocytes may be related to the higher parasite densities in these individuals, which previously has been associated with higher plasma IL-10 levels (Casuals-Pascual et al., 2006).

In order to determine unique predictors of infected reticulocytes, additional correlation and regression analyses were performed. These analyses revealed a positive correlation between parasitized reticulocytes and parasitaemia, and reticulocyte count in children with malaria, while only parasitaemia was correlated with infected reticulocytes in adults with malaria. However, multivariate logistic modelling showed that increased infection of reticulocytes was independently associated with HDP and axillary temperature only in children with malaria. Thus, these analyses confirm that children are more likely to present with infected reticulocytes that can be predicted by presence of high parasite densities and febrile illness.

Increased reticulocyte infection has been attributed to compensatory reticulocytosis and increased parasite densities (Chang et al., 2004). Evidence from murine and human malaria has demonstrated that plasmodial species have a predilection for circulating reticulocytes (Abdalla et al., 1980). However, the impact of P. falciparum on reticulocytes in human populations residing in holoendemic areas of malaria was unknown. This study showed that increased reticulocyte infection in children with uncomplicated P. falciparum malaria was associated with increased reticulocytosis and higher parasite densities. Progression of P. falciparum infection through basophilic, polychromatic, orthochromatic and reticulocyte stages to mature erythrocytes is poorly understood.

Susceptibility to invasion of reticulocytes and the potential for complexity of host interactions may be vastly greater than hitherto known. During low parasite densities, more normocytes are infected (Chang et al., 2004). The presence of parasites in reticulocytes is pronounced during periods of severe immune pressure and peak parasitaemia implying that unremitting parasitaemia is associated with infection of normocytes (Chang et al., 2004). In marked contrast, however, the results from this study showed that chronically infected subjects apparently displayed a pronounced predilection for reticulocyte infection.

While decreased levels of circulating reticulocytes have been used as indicators of erythropoietic suppression in murine malaria (Collins et al., 2003), this study reports that reticulocytosis is an important surrogate marker of a recent malaria infection in children exposed to sholoendemic malaria (Table 4). Reticulocyte parasitation does contribute, in varying degrees, to the reduced levels of circulating reticulocytes in human P. falciparum malalaria in children. This is similar with previous in vitro and in vivo studies showing preferential invasion of reticulocytes by P. falciparum (Hegner, 1938; Pasvol et al., 1980; Clough et al., 1998). In the presence of preferential parasitisation of reticulocytes, increased erythropoiesis did not translate into a comparable increase in observed reticulocyte numbers and RBC levels since newly created reticulocytes served as targets for parasitisation, and do not therefore contribute to the RBC count (Clough et al., 1998). Also, other studies have shown that decreased reticulocyte production is a result of polycythemia and bone marrow irradiation which leads to reduced patency and peak parasitaemia, and increased reticulocytosis (Singer, 1954; Zukerman, 1957).

Table 1: Gender, age and temperature of the study participants

Characteristic		Adults, n=101			Children, n=296		
		P. falciparum[-] n=60	P. falciparum[+] n=41	p	P. falciparum[-] n=132	P. falciparum[+] n=164	p
Gender	Male, n (%)	20 (33.3)	15 (36.6)	0.736[a]	60 (45.5)	89 (54.2)	0.140[a]
	Female, n (%)	40 (66.7)	26 (63.4)		72 (54.5)	75 (45.8)	
Age, years		37.50 (24.50-50.45)[d]	37.90 (24.30-61.40)	0.471[b]	2.50 (2.40-2.70)	2.30 (1.40-3.10)	0.001[b]
Axillary temperature, ^{o}C		36.50 (36.10-36.90)[e]	36.50 (36.15-36.90)	0.787[b]	36.80 (36.50-37.00)	37.70 (36.98-38.55)	<0.0001[b]

Data are presented as medians (Q1-Q3). [a]Fisher's exact test; [b]Mann Whitney test;

Table 2: Clinical characteristics of children and adults with *P. falciparum* infection

Characteristic	Adults n=41	Children n=164	*p*
Median parasitaemia/µL	200 (80-580)	9,760 (1,120-42,680)	<0.0001[a,d]
HDP, n (%)	1 (2.4)	77 (47.0)	<0.0001[b,d]
[e]Mixed infections, n (%)	4 (9.8)	6 (3.9)	0.222[b,d]
Infected Retics, n (%)	3 (7.3)	43 (27.7)	0.006[b,d]
[f]Multiple infected RBCs, n (%)	0 (0.0)	23 (14.0)	0.009[b,d]
[g]Multiple infected Retics, n (%)	0 (0.0)	1 (0.6)	-

Data are presented as medians (Q1-Q3). Median parasitaemia/µL; HDP, high density parasitaemia (≥10,000 parasites/µL); Retics, reticulocytes; [a]Mann Whitney test; [b]Fisher's exact test; [d]versus adults; [e]Only *P. malariae* and *P. falciparum* co-infections were observed. [f,g]Ring and trophozoite stages.

Table 3: Leucocytic and thrombocytic indices of the study participants

Characteristic	Adults, n=101			Children, n=296		
	P. falciparum[-] n=60	P. falciparum[+] n=41	p	P. falciparum[-] n=132	P. falciparum[+] n=164	p
WBC ($\times10^3$/µL)	6.40 (5.60-7.20)	5.40 (4.90-6.00)	0.261[a]	8.90 (7.60-11.20)	8.75 (7.35-11.85)	0.701[a]
Lymphocytes (%)	49.15 (41.53-56.30)	48.40 (43.10-48.95)	0.655[a]	29.40 (22.00-38.70)	35.25 (22.48-41.70)	0.286[a]
Monocytes (%)	0.40 (0.33-0.60)	0.60 (0.50-0.60)	0.115[a]	6.40 (3.50-8.00)	6.85 (3.55-7.95)	0.863[a]
Granulocytes (%)	43.25 (36.58-50.78)	44.10 (40.20-48.65)	0.654[a]	59.00 (50.63-64.90)	56.80 (51.15-63.28)	0.683[a]
Platelets ($\times10^3$/µL)	187 (126-241)	226 (135-237)	0.695[a]	405 (285-484)	380 (239-478)	0.485[a]
Thrombocytopaenia, n (%)	5 (31.3)	1 (33.3)	0.943[b]	2 (1.5)	1 (4.2)	0.396 [b]
MPV, fL	8.10 (7.55-8.63)	8.10 (7.90-8.25)	0.867[a]	7.90 (7.30-8.40)	8.10 (7.60-8.78)	0.150[a]

Data are presented as medians (Q1-Q3) or as indicated. WBC, white blood cells; thrombocytopaenia (platelets<150×10^3/µL); MPV, mean platelet volume. [a]Mann-Whitney U test; [b]Fisher's exact test.

Table 4: Erythrocytic indices of the study subjects

Characteristic	Adults, n=101		p	Children, n=296		P
	P. falciparum[-] n=60	P. falciparum[+] n=41		P. falciparum[-] n=132	P. falciparum[+] n=164	
RBC ($\times10^6$/µL)	4.98 (4.35-5.54)	4.10 (3.93-4.93)	0.433[a]	4.57 (4.26-4.82)	4.61 (4.16-4.78)	0.917[a]
ARN ($\times10^9$/L)	26.03 (18.54-29.54)	20.50 (17.28-24.01)	0.502[a]	18.84 (10.37-31.85)	19.68 (13.70-35.42)	0.828[a]
Reticulocyte count (%)	0.50 (0.40-0.68)	0.50 (0.30-0.60)	0.552[a]	0.39 (0.30-0.70)	0.40 (0.30-0.80)	<0.0001[a]
RPI	0.95 (0.59-2.16)	0.74 (0.45-0.93)	0.681[a]	0.39 (0.21-0.64)	0.42 (0.21-0.61)	0.318[a]
RPI<2.0, n (%)	54 (90.0)	38 (92.7)	0.642[b]	124 (93.9)	163 (99.4)	0.012[b]
Haemoglobin (g/dL)	14.40 (12.10-15.18)	11.80 (10.80-13.65)	0.750[a]	10.20 (9.10-10.80)	10.40 (8.93-10.88)	<0.0001[a]
SMA, n (%)	2 (3.3)	0 (0.0)	0.513[b]	4 (3.0)	13 (8.4)	0.078[b]
Haematocrit (%)	43.95 (38.45-49.15)	36.70 (34.70-42.70)	0.934[a]	34.70 (31.00-35.90)	34.15 (30.63-35.78)	<0.0001[a]
Mean cell volume (fL)	86.30 (85.05-90.08)	84.50 (84.50-88.95)	0.737[a]	76.70 (71.70-79.70)	77.15 (71.60-79.18)	0.961[a]
MCHC (g/dL)	31.70 (30.68-32.55)	31.80 (31.20-31.95)	0.780[a]	29.70 (28.80-30.80)	30.70 (29.23-31.20)	0.098[a]

Data are presented as medians (Q1-Q3) or as indicated. RBC, red blood cells; ARN, absolute reticulocyte numbers ($\times10^9$/L); RPI, reticulocyte production index; SMA, severe malarial anaemia (Hb<6.0 g/dL); MCHC, mean cell haemoglobin concentration. [a]Mann-Whitney U test; [b]Fisher's exact test.

Table 4: Clinical parameters of the study participants and reticulocyte infectivity

Characteristic	Adults, n=41		p	Children, n=164		p
	Non-infected Retics n=38	Infected Retics n=3		Non-infected Retics n=125	Infected Retics n=39	
Median parasitaemia/µL	160 (80-480)	6,600 (3,600-20,960)	0.010[a]	3,120 (680-19,600)	60,120 (41,320-129,600)	<0.0001[a]
HDP, n (%)	0 (0.0)	1 (33.3)	0.073[b]	43 (34.4)	33 (84.6)	<0.0001[b]
Axillary temperature, °C	36.5 (36.5-36.5)	36.4 (35.8-36.9)	0.666[a]	37.30 (36.80-38.10)	38.0 (37.23-38.90)	0.013[a]

Data are presented as medians (Q1-Q3) or proportions. Median parasitaemia/µL, HDP, high density parasitaemia (≥10,000 parasites/µL); Retics, reticulocytes. [a]Mann Whitney U test; [b]Fisher's exact test.

Table 5: Leukocytic and thrombocytic indices of the study participants and association with reticulocyte infection

Characteristic	Children, n=164		P
	Non-infected Retics n=125	Infected Retics n=39	
WBC ($\times10^3$/µL)	8.75 (7.85-12.10)	8.20 (5.60-8.50)	0.112[a]
Lymphocytes (%)	54.10 (50.53-61.20)	67.30 (54.80-72.30)	0.031[a]
Monocytes (%)	5.15 (0.60-0.74)	7.80 (0.70-8.30)	0.145[a]
Granulocytes (%)	37.40 (7.03-42.15)	24.70 (3.20-46.30)	0.965[a]
Platelets ($\times10^3$/µL)	403 (243-479)	285 (218-475)	0.217[a]
Thrombocytopaenia, n (%)	1 (0.8)	0 (0.0)	0.999[b]
MPV, fL	8.10 (7.30-8.90)	7.80 (7.70-8.80)	0.536[a]

Data are presented as medians (Q1-Q3) or as indicated. WBC, white blood cells; thrombocytopaenia (platelets<150×10^3/µL); MPV, mean platelet volume; fL,femtolitres. [a]Mann-Whitney U test; [b]Fisher's exact test.

Table 6: Association between reticulocyte infectivity and erythrocytic indices.

Characteristic	Children, n=164		p
	Non-infected Retics n=125	Infected Retics [+] n=39	
RBC ($\times10^6$/µL)	4.57 (4.07-4.71)	4.80 (4.04-4.92)	0.094[a]
ARN ($\times10^9$/L)	18.88 (9.83-36.38)	32.32 (19.68-34.51)	0.186[a]
Reticulocyte count (%)	0.40 (0.28- 0.80)	0.70 (0.40-0.80)	<0.0001[a]
RPI	0.39 (0.20-0.59)	0.63 (0.49-0.67)	<0.0001[a]
RPI<2.0, n (%)	125 (100.0)	38 (97.4)	0.238[b]
Hb, g/dL	10.10 (8.9-10.9)	10.70 (10.40-10.70)	0.295[a]
SMA, n (%)	12 (9.6)	2 (5.1)	0.552[b]
Haematocrit (%)	33.80 (30.40-35.8)	35.10 (30.38-35.60)	0.369[a]
MCV, fL	77.15 (72.00-79.05)	78.50 (73.20-83.40)	0.234[a]
MCHC, g/dL	30.05(29.20-31.20)	30.8(27.80-30.90)	0.947[a]

Data are presented as medians (Q1-Q3) or as indicated. RBC, red blood cells; ARN, absolute reticulocyte numbers ($\times10^9$/L); RPI, reticulocyte production index; SMA, severe malarial anaemia (Hb<6.0 g/dL); MCV, mean cell volume; fL, femtolitres; MCHC, haemoglobin concentration. [a]Mann Whitney U test; [b]Fisher's exact test.-

Earlier studies have attributed insufficient RBC production to bone marrow suppression, decreased erythropoietin production and /or impairment of the maturation of erythroid precursors into erythrocytes which collectively would lead to reduced peripheral reticulocyte levels (Abdalla et al., 1990). However, in view of the results presented in this study, there is a higher likelihood that the low levels of circulating reticulocytes observed in the parasitemic group could have variously been caused by increased parasitisation and destruction by P. falciparum. This inference is also similar to studies done in rodent models during P. chabaudi infection indicating increased parasitisation and destruction of reticulocytes (Chang et al., 2004).

While this study aimed at investigating the prevalence of P. falciparum reticulocyte parasitation and reticulocytosis as a surrogate marker of a recent malaria pathogenesis, previous data suggests that haematological indices and anthropometric parameters are also important indicators of disease severity and are influenced by host- and parasite derived factors including nutritional and environmental factors (Chang et al., 2004). The results of this study showed that increased reticulocytosis in children with uncomplicated P. falciparum malaria was associated with increased reticulocyte infection. In light of this observation, preferential invasion of reticulocytes could have been responsible for the reduced levels of circulating reticulocytes during uncomplicated P. falciparum malaria.

Plasmodium falciparum species propensity and infection of circulating reticulocytes contributes in varying degrees to the lower levels of reticulocytes in children with uncomplicated malaria. However, the infection kinetics of RBCs and reticulocytes needs to be investigated to shed light on how this influences erythropoiesis in uncomplicated P. falciparum malaria. The findings in this study are similar to studies showing increased erythropoietic activity in Kenyan children with asymptomatic malaria (Verhoeff et al., 2002).

5.2: Reticulocyte Parasitisation and its Association with Clinical, Parasitological and Haematological Outcomes

To determine whether infected reticulocytes were associated with alterations in the parasitological, clinical or haematological measures, median levels of these variables were compared between infected and uninfected reticulocytes in children with P. falciparum malaria (Table 5). The median and geometric parasite densities were higher in the individuals presenting with infected reticulocytes in both children and adult groups. Consistent with elevated parasitaemia, the prevalence of HDP was higher in the individuals presenting with infected reticulocytes in both the adults and children with malaria (Table 5). However, the axillary temperature was only elevated in individuals with infected reticulocytes among children with malaria.

Haematological analyses (Table 6) did not show significant perturbations in the total WBC, granulocyte, thrombocytopaenia, and MPV levels among children presenting with infected reticulocytes relative to those presenting without infected reticulocytes among children with malaria. However, lymphocyte counts were elevated in individuals with infected reticulocytes compared to those without infected reticulocytes among children with malaria

Erythrocytic analyses (Table 7) showed non-significant elevations in the RBC and ARN counts, and significant elevations in the reticulocyte counts in children presenting with infected reticulocytes compared to those presenting without infected reticulocytes during P. falciparum malaria. Other erythrocytic indices including inappropriate erythropoiesis (RPI<2.0), Hb, SMA, haematocrit, MCV and MCHC did not differ significantly between individuals with infected reticulocytes versus those without infected reticulocytes among children with malaria

Age, sex, ethnic background and sociodemographic factors influence hematological indices (Evans et al., 1999). Differential leukocyte count was performed microscopically to derive cardinal WBC values. The results of this study reported low lymphocyte count during acute uncomplicated clinical P falciparum malaria indicating lymphocytopenia which documented to be attendant with paediatric malaria. In contrast, neutrophil levels increased significantly in peripheral circulation during acute clinical malaria compared. Eosinophils levels reflected high prevalence of helminth infection given their close proximity to Lake Victoria. Lymphocytes, monocytes and WBC showed positive correlated positively with parasitemia. This probably was because lymphocytes are important during inflammation and immunity to P. falciparum malaria through secretion of proinflammatory cytokines including tumor necrosis factor-alpha (TNF-ά), interferon-γ and other cytokines leading to a cascade of inflammatory responses (Sowunmi et al., 1995). Presence of these cytokines contributes to disease severity in humans (Day et al., 1999; Biemba et al., 2000).

Monocyte levels which were low during reticulocyte parasitation may be as a result of the immunosuppressive nature of Plasmodium falciparuma. Monocytes exert their effect through a plethora of mechanisms including antibody-dependent cellular inhibition of parasite growth (Tebo et al., 2001), phagocytosis of parasite-infected RBCs, and cytokine and reactive oxygen species secretion (Nielson and Theander, 1989).

The leucocytic and thrombocytic indices of the study participants are shown in Table 3 and Figure 1 and 2. These haematological analyses did not show significant alterations in the total WBC counts, lymphocytes, monocytes, granulocytes, platelets, prevalence of thrombocytopaenia and MPV levels in parasitaemic versus aparasitaemic individuals among the children and adult groups

Although anaemia in developing countries such as Kenya is defined by Hb<11.0 g/dL, the degree of anaemia in this malaria region may not be proportional to Hb levels since most normal children have lower levels of Hb (Were et al., 2006). In addition, none of the children in the current study presented with intraleukocytic hemozoin which has previously been associated with malaria anaemia severity (Casuals-Pascual et al., 2006; Were et al., 2006).

Anaemia development during malaria infection is multi-factorial and often accompanied by alterations in the erythropoietic process (Verhoeff et al., 2002). Murine and human malaria studies have demonstrated associations between compensatory increases in the erythropoietic response and the degree of anaemia (Verhoeff et al., 2002; Chang et al., 2004). Other studies however, show associations between reduced circulating reticulocytes and reduced RBC production. Both compensatory reticulocytosis and low numbers of circulating reticulocytes may result from preferential infection and destruction of parasitized reticulocytes. This premise is supported by results presented in this study illustrating adaptive physiological compensatory response following increased destruction of reticulocytes during malaria infection (Abdalla et al., 1980). In addition, increased preference of reticulocytes invasion leading to eventual destruction may lead to higher levels of reticulocyte production in the infected group (Kurtzhals et al., 1997).

The lower MCV and MCHC levels observed in the children relative to adults in this study suggests a mild microcytic hypochromic anaemia, a finding characteristic of iron deficiency, which may be attributed to sequestered iron (Hoffbrand et al., 2007). These observations are consistent with the fact that anaemia due to iron and folate deficiencies is a common phenomenon among children from western Kenya (Hoffbrand et al., 2007). The lower MCV and MCHC in the children relative to adults was also consistent with lower RBC, reticulocyte, ARN and RPI suggesting that other mechanisms including presence of co-infections such as HIV and hookworms may contribute to low Hb levels and iron status in children and adults from western Kenya. However, it remains to be determined if folate and iron deficiency are important contributors to suppression of erythropoiesis in young children from western Kenya. Anaemia or inflammation (possibly resulting from other underlying infections) may be a plausible reason for the results presented in this study; iron sequestration in the context of chronic inflammation would therefore have the combined effect of denying the erythropoietic process of crucial iron.

Different erythropoietic indices have been used in studying reticulocytosis during malaria (Kurtzhals et al., 1997). In the current study, erythropoietic indices (RBC, ARN, and reticulocyte count, RPI, Hb, Hct, MCV and MCHC) were higher in acute cases relative to normal controls. Other studies indicate that reduced RPI, ARN and reticulocyte counts are associated with reduced erythropoietic response in children with malarial anaemia (Were et al., 2006). While the transferrin receptor compensates for a shift in the erythron iron stores, the RPI accounts for the degree of anaemia, and the ARN accounts for the circulating RBC count (Were et al., 2006), it is important to perform concurrent investigations on these erythropoietic markers and related growth-factors to fully understand the erythropoietic activity in children with malaria.

Although haemolysis is normally preceded by increased MCV and reticulocyte counts (Hoffbrand et al., 2007), in this study, the higher MCV in the acute cases could reflect increased in reticulocytosis (i.e., reticulocyte counts and ARN) possibly due to variable cell size (anisocytosis) commonly observed in malaria (McElroy et al., 1999). A higher MCV during haemolysis is also normally associated with a low MCHC (Bloland, 1999), but the higher MCHC in this study parallels the higher Hb and Hct % levels in the acute cases. Although a higher RDW was associated with increased erythropoietic activity in children with SMA (Kurzhals et al., 1997), a higher RDW could also result from anisocytosis due to iron deficiency or mixed iron and vitamin B12 deficiencies which are common phenomena in malaria endemic areas (Kurtzhals et al., 1997).

In this study, leukocytic characteristics of children presenting with acute malaria showed lower total WBC and lymphocyte counts, and higher monocyte and granulocyte (>98% neutrophils) counts. These results indicate that lymphopaenia, neutrophilia and monocytosis are frequently associated with acute paediatric P. falciparum malaria (Bloland, 1999). The findings of lower platelet counts and higher frequency of thrombocytopaenia in children with malaria are consistent with studies by Labda et al., (1966) illustrating that thrombocytopenia is a common phenomenon in paediatric malaria. Although the previous study showed that thrombocytopaenia occurs in >50% of children with severe malaria (Labda et al., 1996), thrombocytopaenia was only present in ~4% of children in the current study. The reasons for this difference may be related to the fact that such studies were performed in children with severe malaria while this study focused on children with mild malaria. Nevertheless, thrombocytopenia is an important indicator of paediatric malaria severity and prognosis that may result from bone marrow suppression, a characteristic phenomenon in clinical malaria (Ong'echa et al., 2006)).

Results in the acute uncomplicated cases in this study showing associations between circulating reticulocyte counts, RPI and ARN among individuals with infection in their reticulocytes illustrate associations between parasitaemia and reticulocytosis in children with malaria (Orago et al., 2001). Further results showing associations between reticulocyte counts, RPI and ARN recapitulate increased reticulocytosis during acute malaria. However, additional analysis showed a lack of association between the degree of parasitaemia and the number of infected reticulocytes is consistent with the fact that the levels of peripheral parasitaemia and indices of anaemia are independently associated in children from western Kenya (Ong'echa et al., 2006), and suggest that the degree of parasitaemia may not be the sole feature of reticulocyte and erythrocyte infection in children exposed to holoendemic malaria.

The observed significant differences in haematological indices between adults and children are probably due to the fact that physiologically, children have higher total WBC counts than adults (Ongecha et al., 2006). This is consistent with studies among blood donors in Kisumu region showing that healthy adult blood donors have higher leucocyte, granulocyte, monocyte and lymphocyte counts. The higher lymphocyte counts observed in children compared to adults is partly, similar to studies in western Kenya (Orago et al., 2001), showing that lymphocyte counts are higher in children than in adults. This finding may be related to the active development of immunity in children following exposure to malaria. The lack of significant difference of lymphocytes between control and acute cases may be due to other infections such as bacteraemia that are known to alter haematological indices (Orago et al., 2001).

In order to determine the association of infected reticulocytes with parasitological, clinical and haematological parameters, these analyses showed that proportion of parasitised reticulocytes were correlated with parasitaemia and reticulocyte count in children with malaria. Among adults with malaria, infected reticulocytes were associated only with parasitaemia. Multivariate logistic modelling was performed to identify predictors of infected reticulocytes only for children with malaria, since there were too few individuals among adults with malaria following dichotomisation into various groups to perform these analyses. These analyses demonstrated that increased infection of reticulocytes in children with malaria was independently associated with HDP and axillary temperature $>37.5^{o}$C.

The results of this study demonstrate that Plasmodium falciparum parasitation of reticulocytes is associated with compensatory reticulocytosis which cumulatively is an important surrogate marker of erythropoietic process during malaria infection.

Conclusions And Recommendations

Conclusions

1. The prevalence of Plasmodium falciparum reticulocyte parasitation was higher in children compared to adults with uncomplicated malaria in western Kenya.
2. Increased reticulocyte parasitation in children with uncomplicated malaria is associated with compensatory reticulocytosis.

3. High Density Parasitaemia (HDP) is an important predictor of increased reticulocyte parasitation in children with uncomplicated malaria in western Kenya.
4. A febrile response to malaria associated with increased reticulocyte response is related to higher parasite densities in children with malaria.

Recommendations

Reticulocyte enumeration should be adopted as a routine clinical diagnostic tool in hospitals for children presenting with malaria as an important assessment of the integrity of the bone marrow, determine the cause of anaemia and therefore give more informed management of malaria patients.

Suggestions for Future Studies

i). Determine the prevalence of Plasmodium falciparum parasitation of reticulocytes in children with severe malaria and its association with clinical outcomes.

ii). Investigate the effect of co-infections such as HIV-1 and bacteraemia on increased reticulocyte parasitsation in children with severe malaria.

References

Abdalla DJ, Weatherall SN, Wickramasinghe, Hughes M (1980): The anaemia of P. falciparum malaria, Br. J. Haematol. 46: 171–183.

Abshire TC (2001): The anaemia of inflammation. Pediatric Clin North Am. 43 623-637.

Abdalla SH (1990): Haematopoiesis in human malaria. Blood Cells 16:401-416.

Aikawa M (1988): Morphological changes in erythrocyte induced by malarial parasites. Biol cell 64,173-181.

Aikawa M, Iseki M, Barnwell J, Taylor DO and Howard P (1990): The pathology of human cerebral malaria.AM J Trop Med Hyg. 43, 30-37.

Allred DR, Gruenberg JE and Sherman IW (1986): Dynamic rearrangement of erythrocyte membrane internal architecture induced by infection by Plasmodium falciparum. J Cell Sci 81, 1-16.

Awander GA, Ouma Y, Ouma C, Were T, Otieno R, Keller CC, Davenport GC, Hittner JB, Vulule J, Ferrel R, Ongecha JM and Perkins DJ (2007): The role of monocyte acquired hemozoin in suppression of macrophage migration inhibitory factor in children with severe malarial anaemia. Infect Immun, 7, 5201-210

Bloland PB, Lackritz PN, Kazembe JB, Were R, Steketee CC and Campbell (1993): Beyond chloroquine: implications of drug resistance for evaluating malaria therapy efficacy and treatment policy in Africa. J Infect Dis, 167: 932-937.

Bloland PB, Boriga DA, Ruebush TK, McCormick JB, Roberts JM, Oloo AJ, Hawley A, Lal A, Nahlen B and Campbell CC (1999): Longitudinal cohort study of the epidemiology of malaria infections in an area of intense malaria transmission II. Descriptive epidemiology of malaria infection and disease among children. Am J Trop Med Hyg 60: 641- 648.

Biemba G, Gordeu VR Thuma KP, Weiss G (2000): Markers of inflammation in children with severe malarial anemia. Tropical Medicine and International Health, 5: 256-262.

Bessman JD, Kai H, Chang MT and Mary MS (2004): Inappropriately low reticulocytosis in severe malarial anemia correlates with suppression in the development of late erythroid J Infect Dis. 189:723-729.

Beier JC, Oster CN, Onyango FK., Bales JD, Sherwood JA,Perkins PV, Chumo DK, Koech DV, Whitmir RE and RobertsCR (1994): Plasmodium falciparum incidence relative to entomologic inoculation rates at a site proposed for testing malaria vaccines in Western Kenya, Am.J. Trop.Med Hyg., 50:529-536.

Davis BH and Bigelow NC (1994): Reticulocyte analysis and reticulocyte maturity index. Methods in Cell Biology 42:263–74.

Bock A and Herkner KR (1994): Reticulocyte maturity pattern analysis as a predictive marker of erythropoiesis in paediatrics. Evaluation of age dependence values. Clin Lab Haematol 16; 247-251.

Brecher G (1970): New methylene blue as reticulocyte stain. Clin Pathol, 53: 895-896.

Breman JG (2001): The ears of hippopotamus: manifestations, determinants, and estimates of the malaria burden. Am J Trop Med Hyg, 64(1-2 Supp): 1-11.

Bruce-Chwatt LJ (1948): Infection of reticulocytes by Plasmodium falciparum and Plasmodium malariae in hyperendemic indigenous malaria. Ann. Trop. Med. Parasitol. 42, 101-112.

Cavill I (1995): The rejected reticulocyte. Br Haematol. 84: 563-565

Casuals-Pascual C, Kai O, Cheung JO, Williams S, Lowe B, Nyanoti M, Williams TN, Maitland K, Molenyux M, Newton CR, Peshu N,Watt SM and Roberts DJ (2006): Suppression of erythropoiesis in malarial anaemia is associated with hemozoin in vitro and in vivo. Blood, 108: 2569-2577.

Chang KH, Tam M and Stevenson MM (2006): Modulation of the course and outcome of blood-stage malaria by erythropoietin-induced reticulocytosis. J Infect Dis. 189, 735-743.

Chang KH, Tam M, Stevenson MM (2004): Inappropriately low reticulocytosis in severe malarial anaemia correlates with suppression in the development of late erythroid precursors. Blood. 103: 3727-3735.

Checchi F, Cox M, Balkan J, Tamrat S, Priotto A, Alberti G and Guthmann JP (2006): Malaria epidemics and interventions, Kenya, Burundi, southern Sudan, and Ethiopia, Emerg Infect Dis, 12, 1477-1485.

Clough B, Atilola FA and Pasvol G (1998): The role of ressetting in the multiplication of Plasmodium falciparum: rosette formation neither enhances nor targets parasite invasion into uninfected red cells. Brit. J. Haematol. 100: 99-104.

Coetzee M, Craig M, and Le Sueur D (2002): Distribution of African malaria mosquitoes belonging to the Anopheles gambiae complex. Parasitol Today, 16, 74-77. Cohen A (1996): Hematologic emergencies. Lippincott Williams and Wilkins;859273.

Collins WE, Jeffery GM, Roberts JM (2003): A retrospective examination of anemia during infection of humans with Plasmodium vivax. Am. J. Trop. Med. Hyg. 68, 410–412.

Coulombel L, Tchernia G and Mohandas N (1979): Human reticulocyte maturation and its relevance to erythropoietic stress. J Lab Clin Med; 94:467-474.

Crosby S (1981): A quick and easy method for the staining of reticulocytes. Am. J. Clin.

Pathol. 72:1011-1013.

Crowmer D Evans KJ, Schofield Land Davenport MP (2006): Preferential invasion of reticulocytes during late stage plasmodium berghei infection accounts for reduced circulating reticulocyte levels. Int.J Parasitol, 36, 1389-1397.

Davenport PM, Cromer D, Krystal JE and Schofield L (2006): Preferential invasion of reticulocytes during late-stage Plasmodium berghei infection accounts for reduced circulating reticulocytes levels. Int. J. Parasitol. 36: 1389-1397.

Day NP, Hien TT, Schollaardt T, Loc P, Chuong V, Chau TT, Mai T, Phu NH, Singh NJ, White MH (1999): The prognostic

and pathophysiologic role of pro- anti-inflammatory cytokines in severe malaria. Journal of Infectious Diseases, 180: 1288-1297

Deiss A and Kurth D (1979): Circulating reticulocytes in normal adults as determined by the new methylene blue method: A M J Clin Pathol; 53:481-484.

Diggs LW, Sturm D and Bell A (1985): The morphology of human blood cells in wright stained smears of peripheral blood and bone marrow. Abott laboratories, North Chicago,IL

Dormer P, Dietrich M, Kern P, Horstmann RD (1983): Ineffective erythropoiesis in acute human P. falciparum. Blut. 46: 279-288.

Dreyfuss ML, Stoltzfus R J, Shrestha J B (2000): Hookworms, malaria and vitamin A deficiency contribute to anaemia and iron deficiency among pregnant women in the plains of Nepal. J. Nutr.130: 2527-2536.

Dupont WD and Plummewr WD (1990): Power and sample size calculation. A review and computer programme.Control Clin Trials, 11,116-128

Dzeing-Ella A, Nze Obiang PC, Tchoua R, Planche T, Mboza B, Mbounja M, Muller-Roemer U, Jarvis J, Kendjo E, Ngou-Milama E, Kremsner PG, Krishn S, and Kombila M (2005): Severe falciparum malaria in Gabonese children: clinical and laboratory features. Malaia.r J, 4, 1.

Ekvall H (2003): Malaria and anemia. Miller, L. Curr Opin Hematol,10,108-114

Egan AF, Fabucci ME, Saul A, Kaslow DC (2002): Aotus New World monkeys: models for studying malaria-induced anemia. Blood. 99, 3863- 3866.

English M (2000): Life threatening severe malarial anaemia.Trans R Soc Trop Med Hyg. 94, 585-588.

Evans DM, Fraze IH, Martin NG (1999): Genetic and environmental causes of variation in basal levels of blood cells. Twin Res 2: 250-7.

Evans DM, Zhu G, Duffy DL, Montgomery GW, Frazer IH, and Martin NG (2004): Multivariate QTL linkage analysis suggests a QTL for platelet count on Chromosome 19 q. Eur J Hum Genet, 12, 835-842.

el Hassan AM, Saeed J, Fandrey M and Jelkmann W (1997): Decreased erythropoietin response in Plasmodium falciparum malaria-associated anaemia, Eur. J. Haematol. 59, 299–304.

Fisher JW (1997): Erythropoietin: Physiology and pharmacological aspects. Proc.Soc.Exp. Biol. Med 216: 358-369

Freeman RR and Trejdosiewicz AJ (1980): Protective monoclonal antibodies recognizing stage-specific merozoitic antigens of rodent malaria parasite. Nature, 284: 366-368

Galinksi MR, Corredor-Medina C, Ingravello P, and Barnwell JW (2000): A reticulocyte-binding protein complex of Plasmodium vivax merosoites. Cell.69:1213-1226.

Garnham PC (1966): Malaria parasites and other Haemosporidia. Blackwell Scientific Publication, Oxford.

Githeko AK, Lindsay SW, Confalonieri UE and Patz JA (2000): Climate change and vector-borne diseases: a regional analysis. Bull World Health Organ, 78, 1136-1147.

Goris H, Bungart B, Loeffler M, Schimitz S and Nijihof W (1990): Migration of stem cells and progenitors between bone marrow and spleen following thiamphenical treatment of mice. Exp. Hematol. 18. 400- 407.

Greenwood BM, Marsh K, Snow RW (1991): Why do some children develop severe malaria? Parasitol Today, 7:277-81.

Greenwood BM (1989): Tumour necrosis factor production in falciparum malaria and its association with schizont rupture. Clin Exp Immunol, 77: 361-366.

Greenwood BM, Bradley AK, Greenwood AM, Byasss P, Jammeh K, Marsh K, Tulloch S, Oldfield FSJ and Hayes R (1987): Mortality and morbidity among children in a rural area of the Gambia, West Africa. Trans. R. Soc. Trop. Med. Hyg, 81:478-486.

Hegner R (1938): Relative frequency of ring stage Plasmodia in reticulocytes and mature erythrocytes in man and monkey, Am. J. Hyg. 27: 690–718.

Hara H, Ogawa M (1984): Erythropoietic precursors in mice under erythropoietic stimulation and suppression. Exp.Hematol. 5: 141-148.

Hay SI, Simba M, Busolo M, Noor AM, Guyatt HL, Ochola SA and Snow RW (2002): Defining and detecting malaria epidemics in the highlands of western Kenya. Emerg Infect Dis, 8, 555-562.

Hermiston and Mentzer (2002): Differential diagnosis of hereditary hyporegenerative anaemia. J. Parasitol 54: 345-352.

Hertenstein B, Kurrle E, Redenbercher M, Arnold R, Heimpel H (1993): Pseudoreticulocytosis in a patient with myelodysplasia. Ann Hematol, 67:127-128

Hill AV (2006): Pre-erythrocytic malaria vaccines: Towards greater efficacy. Nat Rev Immuno 6 (1):21-32.

Hviid L (1998): Clinical disease, immunity and protection against Plasmodium falciparum malaria in populations living in endemic areas. Exp. Rev. Mol. 100,172-206

Hoffbrand AV, Moss PAH and Pettit JE (2007): Essential haematology Pg 58-71.

Irwin JJ and Kirchner JT (2001): Anaemia in children. Am Fam Physician 64: 1379-1385.

Kitchen SF (1938): The infection of reticulocytes by Plasmodium vivax. Am J. Trop. Med. 18, 347-359.

Koepke JF, Koepke JA (1986): Reticulocytes. Clin. Lab. Haematol. 8:169-179.

Kurtzhals JA, Addae MM, Akanmori BD, Dunyo S, Koram KA, Appawu MA, Nkrumah FK and Hviid L (1999): Anaemia caused by asymptomatic Plasmodium falciparum infection in semi-immune African schoolchildren. Trans R Soc Trop Med Hyg, 93:623-627.

Kurtzhals JA, Rodrigues O, Abdae M, Commey JO, Nkrumah FK, Hviid L (1997): Reversible suppression of bone marrow response to erythropoietin in P. falciparum malaria. Br Haematol. 97:169-174.

Kwiatkowski D, Cannon JD, Manogue KR, Cerami A, Dinarello CA and Ladda R and Lalli F (1989): The course of Plasmodium berghei infection in the polycythemic mouse, J. Parasitol. 52: 383–385.

Greenwood M (2002): Tumour necrosis factor production in falciparum malaria and its association with schizont rupture. Clin Exp Immunol, 77: 361-366.

Lesperance L, Bernstein H (2002): Screening for Iron Deficiency. Peds in Review; 23: 171-178.S

Marsh K, Forster D, Waruiru C, Mwangi I, Winstanley M, Marsh V, Newton C, Winstanely P, Warn P, Peshu N (1995): Indicators of life-threatening malaria in African children. N Engl J Med, 332: 1399-1404.

McNally J, O'Donovan SM and Dalton JP (1992): Plasmodium berghei and Plasmodium chabaudi chabaudi: development of simple in vitro erythrocyte invasion assays, Parasitology 105; 355–362.

McElroy PD, Lal AA, Hawley WA, Bloland PB, ter Kuile FO, Oloo AJ, Harlow SD, Lin X and Nahlen BL (1999): Analysis of repeated haemoglobin measures in full-term, normal birth weight children between birth and 4 years of age III. The Asembo Bay Cohort Project. Am J Trop Med Hyg 61: 932–940.

Mentzer WC (1973): Differentiation of iron deficiency from thallasemia. Lancet 1(7808):882.
Miller LH, Baruch DI, Marsh K and Doumbo OK (2002): The pathogenic basis of malaria. Nature, 415: 673-679.
Mitchell GH, Hadley TSJ, Mc Ginniss MH, Klotz FW, and Miller LH (1986): Invasion of erythrocytes by P. falciparum malaria parasites: evidence for receptor heterogeneity and two receptors. Blood, 67:1519-1521.
Mohan K and Stevenson MM (1998): Dyserythropoeisis and severe anaemia associated with malaria correlate with deficient interleukin-12 productions. Br J Haematol, 103, 942-949.
Murphy PT, Hutchinson RM (1994): Identification and treatment of anaemia in older patients. Drugs Aging; 4: 113-127.
Murphy SC and Breman JG (2001): Gaps in the childhood malaria burden in Africa: cerebral malaria, neurological sequelae, anaemia, respiratory distress, hypoglycemia, and complications of pregnancy, Am. J. Trop. Med. Hyg. 64: 57–67.
Munga S, Minakawa N, Zhou G, Mushinzimana E, Barrack OO, Githeko AK and Yan G (2006): Association between land cover and habitat productivity of malaria vectors in western Kenyan highlands. Am J Trop Med Hyg, 74: 69 -75.
Nakazawa S, Looareesuwan S, Fujioka H, Pongponratn E, Luc KD, Rabbege J and Aikawa MA (1995): A correlation between sequestered parasitized erythrocytes in subcutaneous tissue and cerebral malaria. Am J Trop Med Hyg . 53, 544-546.
Newton CR (1997): Severe anaemia in children living in a malaria endemic area of Kenya. Trop Med Int Health. 2:165-178.
Nijihof M (1993): The splenic microenvironment in rodents appears well suited for expansion of erythroid precursors compared to the bone marrows. Br J Haematol, 103: 813-828.
Nielson H and Theander TJ (1989): Blood monocyte oxidative burst activity in acute Plasmodium falciparum malaria. Acta Pathologica Microbiologica et Immunologica Scandinavica, 97: 469-471.
Njoroge J (2002): Malaria takes its constant toll: a postcard from Kenya. Bull World Health Org, 80: 919.
Ogawa M and Leary AG (1984): Erythroid progenitors in Hematopoiesis: Churchill Livinstone, New York, NY, pp.123-132.
Ogun SA and AA, Holder (1996): A high molecular mass P. yoelii rhoptry protein binds to erythocytes. Molec.Bioch.Parasitol:76:321324s
Ongecha J, Keller CC, Were T, Ouma C, Otieno RO, Landis-Lewis Z, Ochiel D, Shigluff JL, Mogere S, Ogonji JA, Orago AS, Vulule JM, Kaplan SS, Day RD, and Perkins DJ (2006): Parasitaemia, anaemia and malarial anaemia in infants and young children in a rural holoendemic Plasmodium falciparum transmission area. Am J Trop Med Hyg 74: 376 - 385.
Orago AS, Ouma C, Keller CC, Opondo DA, Were T, Otieno RO,Otieno MF,Ongecha JM, Vulule JM,Ferrel RE and Perkins DJ(2001): Polymorphisms with malarial anaemia and high density parasitaemia in infants and young children. AM Troph Med Hyg, 74: 573-577.
Pasvol G, Weatherall DJ and Wilson RJ (1980): The increased susceptibility of young red cells to invasion by the malaria parasite Plasmodium falciparum. Br. J. Haematol. 45: 285-295.
Phillips RE, Looareesuwan S, Warrell DA, Lee SH and Karbwang J (1986): The importance of anaemia in cerebral and uncomplicated falciparum malaria: role of complications, dyserythropoiesis and iron sequestration, Q. J. Med. 58: 305 – 323.
Plowe CV, Roper C, Barnwell JW, Happi CT, Joshi HH, Mbacham W, Meshnick SR, Mugittu K, Naidoo I, Price RN, Shafer RW, Sibley CH, Sutherland CJ, Zimmerman PA and Rosenthal PJ (2007): World Antimalarial Resistance Network (WARN) III: molecular markers for drug resistant malaria. Malar. J, 6, 121.
Rayner JC, Galinksi MR, Ingravello P and Barnwell JW (2000): Two P. sfalciparum genes express merozoitic proteins that are related to P. vivax and P. yoelii adhesive proteins involved in host cell selection and invasion. Proc Natl.Acad.Sa,USA 97: 9648-9653.
Rainey JJ, Omenah D, Sumba PO, Moormann A, Rochford R and Wilson ML (2007): Spatial clustering of endemic Buckitts lymphoma in high risk regions of Kenya. Intl.J. Cancer, 120: 121-127.
Riley SR, Ben-Ezra MJ and Tidwell A (2001): Reticulocytes and reticulocyte enumeration. J. Clin. Lab An. 15:267-294.
Sexton AC, Good RT, Hansen DS, D'ombrain MC, Buckingham L, Simpson K, Schofield L (2004): Malaria is an important cause of anaemia in primigravidae: evidence from a district hospital in coastal Kenya. Trans R Soc Trop Med Hyg. 90: 535-539.
Sexton RT, Good RT, Hansen DS, D'Ombrain MC, Buckingham L, Simpson K and Schofield L (2000): Transcriptional profiling reveals suppressed erythropoiesis, up-regulated glycolysis, and interferon-associated responses in murine malaria, J. Infect. Dis. 189. 1245 –1256.
Sexton AC (1998): Extramedullary erythropoiesis observed in humans with chronic hematological disorders, such as thalassemia J. Infect. Dis. 125; 272–331.
Singer R (1953): The effect of X irradiation on infections with Plasmodium berghei in the white mouse, J. Infect. Dis. 9: 97–104.
Singer (1954): The course of infection with Plasmodium berghei in inbred CF 1 mice, J. Infect. Dis. 94, pp. 237–240.
Snow RW, Guerra CA, Noor AM, Myint HY and Hay SI (2005): The global distribution of clinical episodes of Plasmodium falciparum malaria, Nature 434: 214–217.
Snow, R.W., J.A. Omumbo, B. Lowe, C.S. Molyneux, J.O. Obiero, A. Palmer, M.W. Weber, M. Pinder, B. Nahlen, C. Buoyed, C. Newbold, S. Gupta, K. Marsh (1997): Relation between severe malaria morbidity and level of Plasmodium falciparum transmission in Africa. Lancet, 349:1650-4.
Snow RW, Omumbo JA, Molyneux CS, Lowe B, Obiero JO, Palmer A, Weber MW, Pinder M, Nahlen B, Buoyed C, Newbold C, Gupta S and Marsh K (1999): Relation between severe malaria morbidity and lesvel of Plasmodium falciparum transmission in Africa. Lancet, 349: 1650-4.
Snow RW, Gouws E, Omumbo J, Rapuoda B, Craig MH, Tanser FC, Sueur D and Ouma J (1998): Models to predict the intensity of Plasmodium falciparum transmission: applications to the burden of disease in Kenya.Trans R Soc Trop Med Hyg, 92: 601-606.
Snow RW, Craig M, Deichmann U and Marsh K (1999): Estimating mortality, morbidity and disability due to malaria among Africa's non-pregnant population. Bull World Health Org, 77:624-40.
Soubes SC, Reid ME, Kaneko O and Miller LH (1999): Search for the sialic acid-independent receptor on red blood cells for invasion by P. falciparum. Vox Song. 76: 107-114.
Sowumni A, Akindele JA, and Balogun MA (1995): Leucocyte counts in falciparum malaria in African children from an endemic area.Afr J Med Sci, 24: 145-149

Srichaikul T, Panikbutr N and Jeumtrakul P (1967): Bone marrow changes in human malaria. Ann Trop.Med.Parasitol.61:40-51.

Srichaikul T, Wasanasomsithi M, Poshyachinda V. Panikbutr N and Rabieb T, (1969): Ferrokinetic studies and erythropoiesis in malaria, Arch. Intern. Med. 124: 623–628.

Steketee R W., Nahlen B. L., Parise M. E and Menendez C (2001): The burden of malaria in pregnancy in malaria-endemic areas. Am J Trop Med Hyg. 64: 28-35.

Tebo AE, Kremsner PG, Luty AJ (2001): Plasmodium falciparum: a major role for IgG3 in antibody-dependent monocyte-mediated cellular inhibition of parasite growth in vitro. Exp Parasitol, 98: 20-28.

Van Eijk AM, Ayisi JG, ter Kuile FO , Misore AO, Otieno JK, Rosen DH, Kager PA, Steketee RW and Nahlen BL (2002): Risk factors for malaria in pregnancy in urban and pre-urban population in western Kenya. Trans R Soc Trop Med Hyg, 96: 586 – 592.

Verthof H, West CE, Kraaijenhagen R, Nzyuko SM, King R, Mbandi MM, van Laatum S, Hogervost R, Schep C and Kok FJ (2002): Malaria anaemia leads to adequately increased erythropoietin in asymptomatic Kenyan children. Blood. 100: 3489-3494.

Vigario AM, Belnoue E, Cumano A, Marussig M, Miltgen F, Landau I, Mazier D, Gresser I and Renia L (2001): Alpha through the inhibition of the production of its target cell, the reticulocyte, Blood. 97: 3966 –3971.

Villeval KM (1990): Changes in hemopoietic and regulator levels in mice during fatal or non -fatal malarial infections. I. Erythropoietic populations, Exp. Parasitol. 71: 364–374.

Were T, Hitner JB, Ouma C, Otieno RO, Orago AS, Ongecha JM, Vulule JM, Keller CC and Perkins DJ (2006): Suppression of rantes in children with Plasmodium falciparum malaria. Haematologica, 91: 1396-1399.

White NJ, Breman JG, Braunwald E, Fauci AS, Isselbacher KJ, Kasper DL, Hauser SL, Longo DL and Jameson JL (2001): Principles of Internal Medicine, eds. (McGraw–Hill, New York) 1203–1213.

W.H.O. (2002): WHO report: reducing risks promoting healthy life, World Health Organization, Geneva.

WHO and UNICEF (2005): World Malaria Report 2005. In: Roll Back Malaria.

WHO (2000): Severe P. falciparum malaria.World Health Organization, Communicable

Disease Cluster.Trans R Soc Trop Med Hyg, 94 Suppl 1, S1-90

W.H.O. (1996): Malaria Fact sheet No.94, World Health Organization, Geneva.

Werheimer SP, and JW, Barnwell K (1989): Plasmodium vivax interactions of parasite receptor-like proteins. EXP Parasitol: 69:340-350.

Widnes JA, Madan A, Grindaenu LA, Zimmerman MB,Stevenson DK (2005): Reduction in red blood cell, Pediatrics 115: 1299-1306.

Wickramasinghe SN (2000): Blood and bone marrow changes in malaria, Baillieres Best Pract. Res. Clin. Haematol. 13; 277–299.

Wickramasinghe SN, Abdalla S and Weatherall DJ (1982): Cell cycle distribution of erythroblasts in P. falciparum malaria. Scand J Haematol. 29: 83-88.

Wickramasinghe SN., Philips, RE, Looareesuwan S, Warell DA and Hughes M (1987): The bone marrow in human cerebral malaria: parasite sequestration within sinusoids. Br.J. Haematol: 66: 295-306.

Yawson AE, McCall PJ, Wilson MD and Donnelly MJ (2004): Species abundance and insecticide resistance of Anopheles gambiae in selected areas of Ghana and Burkina Faso. Med Vet Entomol, 18: 372-377.S

Zuckerman A and Yoeli M (1954): Age and sex as factors influencing Plasmodium berghei infections in intact and splenectomized rats, J. Infect. Dis. 94; 225–236. Zukerman A (1957): Blood loss and replacement in plasmodial infections. Plasmodium berghei in untreated rats of varying age and in adult rats with erythropoietic mechanisms manipulated before inoculation, J. Infect. Dis. 100: 172–206.