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

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ABSTRACT
Reticulocytosis is an important previously unappreciated surrogate marker of recent Plasmodium falciparum reticulocyte parasitisation. 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 parasitisation 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 parasitisation 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/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
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 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 important 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 discrete 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. bergheri-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 parasitisation. Studies in areas with seasonal malaria have reported association between reticulocyte parasitisation 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 parasitisation has an effect on erythrocytic parameters in holoendemic regions of malaria. Since levels of reticulocyte parasitisation 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 parasitisation 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 parasitisation in children and adults with uncomplicated malaria?

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

**General Objective**

To determine the prevalence of P. falciparum parasitisation 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 parasitisation 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
of 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 of age. The Chulaimbo Provincial Rural Health Centre is located about 25 Km North West of Kisumu City, Kisumu County, Nyanza Province (Latitude: 0° -2.0° N; Longitude: 34° 37° 60 E). The area is within the lowland region at an elevation of about 1133m above sea level adjacent to the shores 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 -2000 mm per year. The main mosquito vector is A. Gambe (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 parasitisation 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 ≥37.5°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 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 \( \times 1000 \) 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 were used to lower counting variability. A comparative analysis of the number of reticulocytes and normocyttes 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 microscopically 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 (Koecke 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 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 parasiticemic children compared to parasiticemic 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; aMann Whitney test; bFisher’s exact test; cversus adults; Only P. malariae and P. falciparum co-infections were observed. dRing 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 thrombocytopenia 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 (×10³/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 increased 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=0.001; 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 (×10⁹/L); (B) absolute reticulocyte numbers, ARN (×10¹/L); (C) reticulocyte count (%); and (D) reticulocyte production index (RPI) in Pf [-] and Pf [+] adults. These infection status. (A) Platelet count (×10³/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.
Data are presented as medians (Q1-Q3) or as indicated. RBC, red blood cells; ARN, absolute reticulocyte numbers (×10^6/L); RPI, reticulocyte production index; SMA, severe malarial anaemia (Hb<60 g/dL); MCHC, mean cell haemoglobin concentration. 1Mann-Whitney U test; 2Fisher’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; Fisher’s 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), thrombocytopenia (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.

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°C (OR, 2.485; 95% CI, 1.082-5.708; p=0.032).

5.1: Prevalence of Plasmodium falciparum Reticulocyte Parasitisation 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 parasitisation 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 parasitisation 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 parasitisation. 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 parasitisation 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 parasitisation against 39724 normocytes counted and only 99 of them parasitized translating to 0.2% normocyte parasitisation. Taken together, there was increased prevalence of reticulocyte parasitisation 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°C) (Table 5).

In order to investigate the prevalence and impact of reticulocyte parasitisation 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 (Kurzhals et al., 1999). 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 (Orogo 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 holoendemic malaria (Table 4). Reticulocyte parasitisation does contribute, in varying degrees, to the reduced levels of circulating reticulocytes in human P. falciparum malaria 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 potency and peak parasitaemia, and increased reticulocytosis (Singer, 1954; Zukerman, 1957).
Data are presented as medians (Q1-Q3). Median parasitaemia/µL; HDP, high density parasitaemia (≥10,000 parasites/µL); Retics, reticulocytes; *Mann Whitney test; †Fisher’s exact test; ‡versus adults; *Only *P. malariae* and *P. falciparum* co-infections were observed.  

### Table 3: Medical characteristics of children and adults with *P. falciparum* infection

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Adults, n=101</th>
<th>Children, n=296</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P. falciparum-[+]</td>
<td>P. falciparum[+]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=60</td>
<td>n=41</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P. falciparum-[+]</td>
<td>P. falciparum[+]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=132</td>
<td>n=164</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>Male, n (%)</td>
<td>Female, n (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 (33.3)</td>
<td>40 (66.7)</td>
<td>0.736b</td>
</tr>
<tr>
<td></td>
<td>15 (36.6)</td>
<td>26 (63.4)</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>23.90 (24.50-30.45)</td>
<td>23.90 (24.30-30.64)</td>
<td>0.471b</td>
</tr>
<tr>
<td></td>
<td>36.50 (36.10-36.90)</td>
<td>36.50 (36.15-36.90)</td>
<td>0.787b</td>
</tr>
<tr>
<td></td>
<td>36.80 (36.50-37.00)</td>
<td>37.70 (36.98-38.55)</td>
<td>&lt;0.001b</td>
</tr>
</tbody>
</table>

Data are presented as medians (Q1-Q3). Median parasitaemia/µL; HDP, high density parasitaemia (≥10,000 parasites/µL); Retics, reticulocytes; *Mann Whitney test; †Fisher’s exact test; ‡versus adults; *Only *P. malariae* and *P. falciparum* co-infections were observed.  

### Table 3: Leucocyte and thrombocyte indices of the study participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Adults, n=101</th>
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<th>p</th>
</tr>
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<tbody>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>n=60</td>
<td>n=41</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P. falciparum-[+]</td>
<td>P. falciparum[+]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=132</td>
<td>n=164</td>
<td></td>
</tr>
<tr>
<td>WBC (×10^9)/µL</td>
<td>6.40 (5.60-7.20)</td>
<td>5.40 (4.90-6.00)</td>
<td>0.261a</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>49.15 (41.53-56.30)</td>
<td>48.40 (43.10-48.95)</td>
<td>0.655a</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>0.40 (0.33-0.60)</td>
<td>0.60 (0.50-0.60)</td>
<td>0.115b</td>
</tr>
<tr>
<td>Granulocytes (%)</td>
<td>43.25 (36.58-50.78)</td>
<td>44.10 (40.20-48.65)</td>
<td>0.654b</td>
</tr>
<tr>
<td>Platelets (×10^3)/µL</td>
<td>187 (126-241)</td>
<td>226 (135-237)</td>
<td>0.695b</td>
</tr>
<tr>
<td>Thrombocytopenia, n (%)</td>
<td>5 (31.3)</td>
<td>1 (33.3)</td>
<td>0.943b</td>
</tr>
<tr>
<td>MPV, fl</td>
<td>8.10 (7.55-8.63)</td>
<td>8.10 (7.90-8.25)</td>
<td>0.867b</td>
</tr>
</tbody>
</table>

Data are presented as medians (Q1-Q3) or as indicated. WBC, white blood cells; thrombocytopenia (platelets<150×10^3/µL); MPV, mean platelet volume. *Mann-Whitney U test; †Fisher’s exact test.

### Table 4: Erythrocyte indices of the study subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Adults, n=101</th>
<th>Children, n=296</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P. falciparum-[+]</td>
<td>P. falciparum[+]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=60</td>
<td>n=41</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P. falciparum-[+]</td>
<td>P. falciparum[+]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=132</td>
<td>n=164</td>
<td></td>
</tr>
<tr>
<td>RBC (×10^6)/µL</td>
<td>4.98 (4.35-5.54)</td>
<td>4.10 (3.93-4.93)</td>
<td>0.433a</td>
</tr>
<tr>
<td>ARN (×10^9)/L</td>
<td>26.03 (18.54-29.54)</td>
<td>20.50 (17.28-24.01)</td>
<td>0.502a</td>
</tr>
<tr>
<td>Reticulocyte count (%)</td>
<td>0.50 (0.40-0.68)</td>
<td>0.50 (0.30-0.60)</td>
<td>0.552a</td>
</tr>
<tr>
<td>RPI</td>
<td>0.95 (0.59-2.16)</td>
<td>0.74 (0.45-0.93)</td>
<td>0.681a</td>
</tr>
<tr>
<td>RPI&lt;2.0, n (%)</td>
<td>54 (90.0)</td>
<td>38 (92.7)</td>
<td>0.642b</td>
</tr>
<tr>
<td>Haemoglobin (g/dL)</td>
<td>14.40 (12.10-15.18)</td>
<td>11.80 (10.80-13.65)</td>
<td>0.750a</td>
</tr>
<tr>
<td>SMA, n (%)</td>
<td>2 (3.3)</td>
<td>0 (0.0)</td>
<td>0.513b</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>43.95 (38.45-49.15)</td>
<td>36.70 (34.70-42.70)</td>
<td>0.934a</td>
</tr>
<tr>
<td>Mean cell volume (fl)</td>
<td>86.30 (85.05-90.08)</td>
<td>84.50 (84.50-88.95)</td>
<td>0.737a</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>31.70 (30.68-32.55)</td>
<td>31.80 (31.20-31.95)</td>
<td>0.780a</td>
</tr>
</tbody>
</table>

Data are presented as medians (Q1-Q3) or as indicated. RBC, red blood cells; ARN, absolute reticulocyte numbers (×10^9/L); RPI, reticulocyte production index; SMA, severe malarial anaemia (Hb<6.0 g/dL); MCHC, mean cell haemoglobin concentration. *Mann-Whitney U test; †Fisher’s exact test.
Table 6: Clinical parameters of the study participants and reticulocyte infectivity

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

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

Table 6: Leukocytic and thrombocytic indices of the study participants and association with reticulocyte infection

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Non-infected Retics n=125</th>
<th>Infected Retics n=39</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (×10³/µL)</td>
<td>8.75 (7.85-12.10)</td>
<td>8.20 (5.60-8.50)</td>
<td>0.112&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>54.10 (50.53-61.20)</td>
<td>67.30 (54.80-72.30)</td>
<td>0.031&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>5.15 (0.60-0.74)</td>
<td>7.80 (0.70-8.30)</td>
<td>0.145&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Granulocytes (%)</td>
<td>37.40 (7.03-42.15)</td>
<td>24.70 (3.20-46.30)</td>
<td>0.965&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Platelets (×10³/µL)</td>
<td>403 (243-479)</td>
<td>285 (218-475)</td>
<td>0.217&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Thrombocytopaenia, n (%)</td>
<td>1 (0.8)</td>
<td>0 (0.0)</td>
<td>0.999&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MPV, fL</td>
<td>8.10 (7.30-8.90)</td>
<td>7.80 (7.70-8.80)</td>
<td>0.536&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as medians (Q1-Q3) or as indicated. WBC, white blood cells; thrombocytopaenia (platelets<150×10³/µL); MPV, mean platelet volume; fL, femtolitres. <sup>a</sup>Mann Whitney U test; <sup>b</sup>Fisher’s exact test.

Table 6: Association between reticulocyte infectivity and erythrocytic indices.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Non-infected Retics n=125</th>
<th>Infected Retics [+], n=39</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (×10⁶/µL)</td>
<td>4.57 (4.07-4.71)</td>
<td>4.80 (4.04-4.92)</td>
<td>0.094&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ARN (×10⁹/L)</td>
<td>18.88 (9.83-36.38)</td>
<td>32.32 (19.68-34.51)</td>
<td>0.186&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Reticulocyte count (%)</td>
<td>0.40 (0.28-0.80)</td>
<td>0.70 (0.40-0.80)</td>
<td>&lt;0.0001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>RPI&lt;2.0, n (%)</td>
<td>0.39 (0.20-0.59)</td>
<td>0.63 (0.49-0.67)</td>
<td>&lt;0.0001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hb, g/dL</td>
<td>10.10 (8.9-10.9)</td>
<td>10.70 (10.40-10.70)</td>
<td>0.295&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SMA, n (%)</td>
<td>12 (9.6)</td>
<td>2 (5.1)</td>
<td>0.552&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>33.80 (30.40-35.8)</td>
<td>35.10 (30.38-35.60)</td>
<td>0.369&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCV, fL</td>
<td>77.15 (72.00-79.05)</td>
<td>78.50 (73.20-83.40)</td>
<td>0.234&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCHC, g/dL</td>
<td>30.05 (29.20-31.20)</td>
<td>30.8 (27.80-30.90)</td>
<td>0.947&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as medians (Q1-Q3) or as indicated. RBC, red blood cells; ARN, absolute reticulocyte numbers (×10⁹/L); RPI, reticulocyte production index; SMA, severe malarial anaemia (Hb<6.0 g/dL); MCV, mean cell volume; fL, femtolitres; MCHC, haemoglobin concentration. <sup>a</sup>Mann Whitney U test; <sup>b</sup>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 parasiticemic 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 parasitisation 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 haematological indices (Evans et al., 1999). Differential leucocyte 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 pro-inflammatory cytokines including tumor necrosis factor-alpha (TNF-α), 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 parasitisation may be as a result of the immunosuppressive nature of Plasmodium falciparum. 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 (Nielsen 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 (Casuas-Pascual et al., 2006; Were et al., 2006).

Anaemia development during malaria infection is multifactorial 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 (Kurtzhal 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 lymphopenia, 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 thrombocytopena 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°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 parasitisation 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 bacteremia on increased reticulocyte parasitisation in children with severe malaria.

**References**


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