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Hematological Indices in Malian Children Change Significantly during a Malaria Season and with Increasing Age: Implications for Malaria Epidemiological Studies

Mahamadou Diakite,[1]* Kazutoyo Miura,[2] Ababacar Diouf,[2] Drissa Konate,[1] Abdoul S. Keita,[1] Saibou Doumbia,[1] Seidina Diakite,[1] Karim Traore,[1] Mory Doumbouya,[1] Jennifer M. Anderson,[2] Rick M. Fairhurst,[2]† and Carole A. Long[2]†

[1]*Faculty of Medicine, Pharmacy, and Odontostomatology (FMPOS), Malaria Research and Training Center (MRTC), University of Sciences, Techniques, and Technologies of Bamako (USTTB), Bamako, Mali;* [2]*Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland*

Abstract. Standard hematological indices are commonly used in malaria epidemiological studies to measure anemia prevalence and calculate blood parasite densities. In Africa, few studies have investigated how these indices change during a malaria transmission season and with increasing age. To address these knowledge gaps, we collected blood from 169 healthy Malian children aged 3–12 years before (May 2010) and after (January 2011) a transmission season. Red blood cell (RBC) count, hemoglobin (Hb) level, hematocrit (Ht), white blood cell (WBC) count, and WBC subsets were measured in paired blood samples, and the data were stratified by month (May, January) and age group (3–5, 6–8, and 9–12 years). From May to January, RBC count (4.53–4.70 × 10^6/μL; $P < 0.0001$), Hb level (11.5–11.9 g/dL; $P < 0.0001$), and Ht (37.1–39.2%; $P < 0.0001$) increased, and WBC count (6.46–5.96 × 10^3/μL; $P = 0.0006$) decreased. From May to January, the prevalence of WBC subsets also changed: 35–43% neutrophils, 6.5–7.6% monocytes, and 53–45% lymphocytes ($P < 0.001$). These seasonal changes were not associated with the number of malaria episodes experienced in the interim or the presence of RBC polymorphisms. In May, Hb (11.2, 11.4, and 11.8 g/dL; $P = 0.0013$) and Ht (36.5%, 36.7%, and 38.1%; $P = 0.0154$) increased and WBC count (8.04, 6.43, and 5.76 × 10^3/μL; $P < 0.0001$) decreased with age group; similar differences were observed in January. These data suggest that season- and age-based reference values for hematological indices are needed to better estimate anemia prevalence and parasite density in malaria epidemiological studies.

INTRODUCTION

About 3.2 billion people remain at risk of malaria, and an estimated 214 million new cases of malaria and 438,000 deaths occurred in 2015.[1–3] *Plasmodium falciparum* malaria remains a major cause of morbidity and mortality among African children, who experience multiple parasite infections and repeated febrile illnesses until they acquire clinical immunity to malaria.[4] During this time, alterations in hematological indices are frequently encountered during acute malaria episodes as red blood cells (RBCs) are turned over and white blood cells (WBCs) expand and activate to produce inflammatory mediators.[5,6] The pathogenesis of malarial anemia is complex, involving the destruction of both infected and uninfected RBCs in the spleen as well as dyserythropoiesis.[7–10]

Although not specific to malaria, anemia may be used to support the diagnosis or grade the severity of this disease. Changes in WBC counts and subsets, however, are not generally useful in malaria diagnosis or prognosis. Hematological indices, on the other hand, have been very useful in epidemiological studies of malaria. Different grades of anemia are commonly defined by hemoglobin (Hb) level cutoff values,[11] and parasite densities are often calculated using WBC counts in Giemsa-stained thick blood films. For example, assuming an average WBC count of 7,500/μL, parasite densities can be estimated by counting the number of parasites until 250 WBCs have also been counted. This count, when multiplied by 30, gives an estimate of parasites/μL of whole blood.

Although hematological indices are not uncommonly reported for malaria episodes, few studies have investigated how hematological indices change with seasonal malaria transmission or with increasing age. Herein, we compare five hematological indices in a cohort of healthy Malian children aged 3–12 years before and after a 7-month malaria transmission season.

METHODS

Study site and participants. This study was conducted in three villages (Kenieroba, Fourda, and Bozokin) located approximately 75 km southwest of Bamako, where malaria transmission is highest during and after the long rainy season (June–January). The populations of these villages are primarily of Malinke ethnicity, and practice fishing and subsistence farming of maize, cassava, and banana. In May 2008, we initiated a 4-year longitudinal cohort study of malaria incidence in children aged 0.5–17 years who were permanent residents of Kenieroba, Fourda, and Bozokin. Details of the entire cohort study have been described previously.[12] These children were genotyped for HbS and HbC, glucose-6-phosphate dehydrogenase (*G6PD**A−) deficiency, α-thalassemia, and ABO/Rh blood group antigens as described.[13,14] Using these data, we established a subcohort of 249 children (details of the subcohort have been described elsewhere[15]), which included all HbAS and HbAC children aged 3–12 years and HbAA children matched for age and the aforementioned RBC polymorphisms.

Study design and sample collection. During the 2010 transmission season, we passively followed the subcohort of children for episodes of malaria, defined by axillary temperature

*Address correspondence to Mahamadou Diakite, Faculty of Medicine, Pharmacy, and Odontostomatology (FMPOS), Malaria Research and Training Center (MRTC), University of Sciences, Techniques, and Technologies of Bamako (USTTB), Route du Point-G, Bamako 1805, Mali. E-mails: mdiakite@icermali.org or mdiakite65@icermali.org
†These authors contributed equally to this work.

> 37.5°C or history of fever in the past 2 days (with or without symptoms such as headache and malaise), any parasite density observed by microscopic examination of a Giemsa-stained thick blood film, and no other apparent etiology of febrile illness. From a subset of 169 children, we were able to collect blood samples both before (May 2010) and after (January 2011) the transmission season. These paired samples were used to determine RBC count, Hb level, hematocrit (Ht), WBC count, and WBC subsets using an automated cell counter (Beckman Coulter LH 750; Beckman Coulter, Inc., Brea, CA).

Ethical clearance. This study was approved by the Ethics Committee of the Faculty of Medicine, Pharmacy, and Odontostomatology, University of Bamako, and the Institutional Review Board of the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Written informed consent was obtained from the parents or guardians of children. The study is registered with Clinicaltrials.gov, number NCT00669084.

Statistical analysis. Hematological indices in May or January were compared using Mann–Whitney test and among three groups differing in age or Hb type using Kruskal–Wallis test (if significant, followed by Dunn's multiple comparison test). Hematological indices between May and January were compared using Wilcoxon signed rank test. Age-related difference in malaria incidence was investigated using Kruskal–Wallis test, and in parasitemia prevalence with χ^2 test. Correlations between delta values (changes from May to January in individual children) for hematological indices and number of malaria episodes were evaluated using Spearman rank test. GraphPad Prism 6 (GraphPad Software, La Jolla, CA) and STATA version 9.2 (Stata Corp., College Station, TX) were used for statistical analysis. Two-sided P values < 0.05 were deemed significant.

RESULTS

We measured hematological indices in 169 healthy Malian children aged 3–12 years before (May 2010) and after (January 2011) the malaria transmission season. In May, median values for these parameters were: RBC count, $4.53 \times 10^6/\mu L$; Hb, 11.5 g/dL; Ht, 37.1%; and WBC count, $6.46 \times 10^3/\mu L$ (Table 1). From May to January, median RBC count, Hb, and Ht increased significantly ($P < 0.0001$), and WBC count decreased significantly ($P = 0.0006$) (Table 1). Nearly all of these changes remained significant when data were stratified into age groups 3–5, 6–8, and 9–12 years (Table 1). These data suggest that seasonal changes in hematological indices may have resulted from a 7-month increase in age, the effects of *P. falciparum* infection, or both.

To further investigate whether changes in hematological indices during the transmission season were associated with increasing age, we stratified children into three age groups (3–5, 6–8, and 9–12 years), and tested for age group–related differences in hematological indices. At the end of the dry season in May, when children had not experienced malaria for several months, RBC count ($P = 0.0538$), Hb ($P = 0.0013$), and Ht ($P = 0.0154$) increased, and WBC count ($P < 0.0001$) decreased with age group (Table 1, Figure 1). These differences (except for RBC count) were also seen in January (Table 1). In various comparisons between two age groups in either May or January, we also identified significant

TABLE 1
Hematological indices in 169 healthy Malian children, stratified by age group and month

Age group	RBC ($10^6/\mu L$)			Hb (g/dL)			Ht (%)			WBC ($10^3/\mu L$)				
	May	January	P*	May	January	P*	May	January	P*	May	January	P*	IR	% Pf+
All ($N = 169$)	4.53 (4.21–4.84)	4.70 (4.39–5.07)	< 0.0001	11.5 (10.7–12.0)	11.9 (11.0–12.5)	< 0.0001	37.1 (34.6–39.2)	39.2 (37.2–42.2)	< 0.0001	6.46 (5.61–7.95)	5.96 (5.01–7.37)	0.0006	1.1 (0.9–1.3)	20
3–5 years ($N = 40$)	4.52 (4.21–4.72)	4.68 (4.39–5.04)	0.0116	11.2 (10.2–11.7)	11.3 (10.8–12.1)	0.0202	36.5 (34.2–38.9)	38.2 (36.4–41.9)	0.0011	8.04 (6.52–9.75)	7.43 (5.51–8.97)	0.0149	1.7 (1.3–2.0)	15
6–8 years ($N = 66$)	4.45 (4.18–4.72)	4.75 (4.35–5.09)	< 0.0001	11.4 (10.4–11.9)	11.8 (11.0–12.4)	0.0003	36.7 (34.2–38.7)	38.9 (36.9–41.3)	< 0.0001	6.43 (5.66–7.88)	5.92 (5.17–7.16)	0.0581	1.3 (1.0–1.5)	17
9–12 years ($N = 63$)	4.65 (4.24–4.99)	4.70 (4.39–5.11)	0.0082	11.8 (11.1–12.5)	12.1 (11.5–12.8)	0.0194	38.1 (35.9–40.4)	40.1 (37.9–43.3)	< 0.0001	5.76 (4.92–6.91)	5.53 (4.52–6.65)	0.0867	0.6 (0.4–0.9)	27
P**	0.0538	0.7842		0.0013	0.0035		0.0154	0.0521		< 0.0001	0.0001			
P***													< 0.0001	0.2243

Hb = hemoglobin level; Ht = hematocrit; IR = incidence rate (mean [95% confidence interval] number of malaria episodes in the 2010 transmission season); % Pf+ = proportion of healthy children with *Plasmodium falciparum* parasitemia on thick blood smear in January 2011; RBC = red blood cell; WBC = white blood cell.
Median (interquartile range, IQR) values are shown unless otherwise specified.
* Data for May and January were compared using Wilcoxon signed rank test.
** Data for three age groups were compared using Kruskal–Wallis test.
*** Data for three age groups were compared using Kruskal–Wallis test (IR) or χ^2 test (% Pf+).

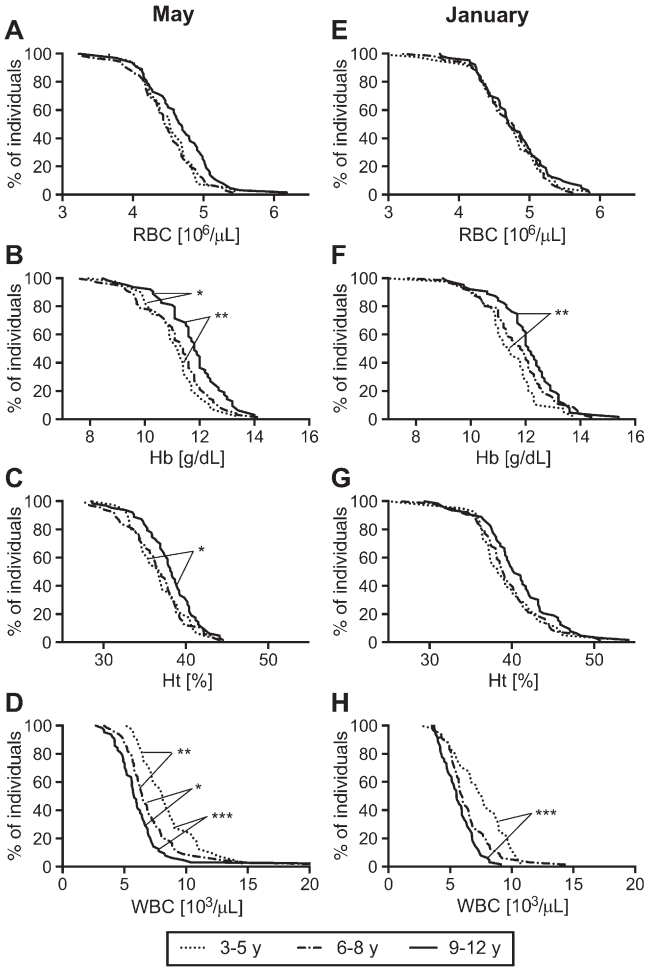

FIGURE 1. Hematological indices in 169 healthy Malian children, stratified by month. Reverse cumulative distribution plots for three age groups (3–5, 6–8, and 9–12 years) are shown in each panel. (**A**, **E**) Red blood cell (RBC) count, (**B**, **F**) hemoglobin (Hb), (**C**, **G**) hematocrit (Ht), and (**D**, **H**) white blood cell (WBC) count were measured before ([**A**–**D**] May 2010) and after ([**E**–**H**] January 2011) the malaria transmission season. Data among the three age groups were compared using Kruskal–Wallis test followed by Dunn's multiple comparison test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

differences in several hematological indices (Figure 1). For example, in both May and January, Hb was higher in children aged 9–12 years than in those aged 3–5 years ($P < 0.01$).

We then explored whether changes in hematological indices were associated with the number of malaria episodes experienced in the 2010 transmission season. When we stratified children into four groups (0, 1, 2, and 3–5 episodes), we found no malaria incidence–related differences in these parameters ($P > 0.05$, Figure 2). We also found no relationship between changes in these parameters and the presence of HbAS, HbAC, G6PD-deficiency, or α-thalassemia (Supplemental Tables 1–3). Finally, we investigated whether season-associated decreases in WBC count were accompanied by changes in the prevalence of WBC subsets (Figure 3). Significant decreases in the proportions of lymphocytes (53–45%; $P < 0.001$) and eosinophils (2.6–2.5%; $P = 0.012$) were associated with concomitant increases in the proportions of

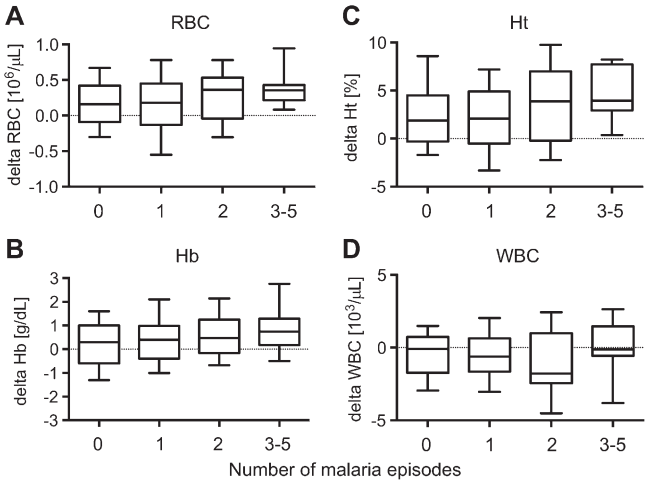

FIGURE 2. Changes in hematological indices in 169 healthy Malian children, stratified by malaria incidence rates. The delta values (change from May to January) of four hematological indices were calculated for each child and then grouped by the number of malaria episodes experienced during the 2010 transmission season. Whisker plots show the median, interquartile range, and 10th/90th percentiles. Delta values for all four indices did not significantly associate with malaria incidence rate: (**A**) red blood cell (RBC), $P = 0.126$; (**B**) hemoglobin (Hb), $P = 0.405$; (**C**) hematocrit (Ht), $P = 0.070$; and (**D**) white blood cell (WBC), $P = 0.163$; Spearman rank test. There were 59, 59, 33, and 18 children with 0, 1, 2, and 3–5 malaria episodes, respectively.

monocytes (6.5–7.6%; $P < 0.001$) and neutrophils (35–43%; $P < 0.001$).

DISCUSSION

This study investigated associations between hematological indices, age, and season in a cohort of 169 healthy children aged 3–12 years living in a malaria-endemic area of Mali.

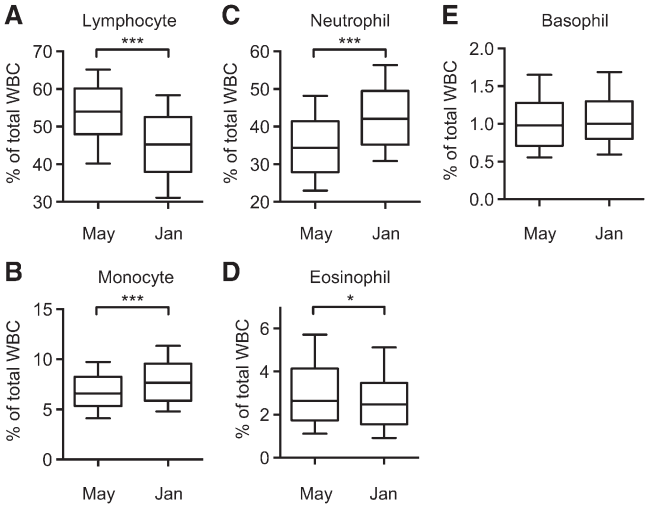

FIGURE 3. Differential white blood cell (WBC) counts in 169 healthy Malian children, stratified by month. The proportions of (**A**) lymphocytes, (**B**) monocytes, (**C**) neutrophils, (**D**) eosinophils, and (**E**) basophils in total WBCs were measured before (May 2010) and after (January 2011) the malaria transmission season. Whisker plots show the median, interquartile range, and 10th/90th percentiles. WBC subset counts between months were compared using Wilcoxon signed rank test (*$P < 0.05$, ***$P < 0.001$).

As variously reported for healthy Ugandan,[16] Tanzanian,[17] Kenyan,[18] and Gambian[19] children, we also found that RBC indices (RBC count, Hb, and Ht) increased and WBC count decreased with age in healthy Malian children. Children in all three age groups (3–5, 6–8, and 9–12 years) also showed increased RBC indices and decreased WBC counts as they aged through a single 7-month transmission season, irrespective of whether they experienced one or more episodes of malaria or carried malaria-protective RBC polymorphisms. Even when all factors were analyzed together using a multivariate regression model, none of the RBC polymorphisms significantly impacted the hematological indices in this study population (data not shown). As shown in Supplemental Tables 1–3, only a minority of children carried RBC polymorphisms, suggesting that additional studies are required to determine the impact of these RBC polymorphisms on hematological indices. We also observed changes in WBC subsets, with the proportion of lymphocytes decreasing and the proportions of monocytes and neutrophils increasing through the transmission season. Together, these data suggest that Malian children experience significant changes in hematological parameters as they age through multiple transmission seasons.

There are several potential explanations for these observations. From age 3 to 12 years, Malian children actively acquire immunity to malaria, which suppresses both parasite density and malaria incidence.[12,13,20] By mitigating the anemia-producing effects of high parasite densities and acute malaria episodes, this immunity may help to increase RBC count, Hb, and Ht with increasing age by reducing the destruction of infected and uninfected RBCs in the spleen, and increasing the production of new RBCs in the bone marrow. Our provision of directly observed, effective antimalarial therapy (artesunate–amodiaquine), which minimizes the duration and morbidity of malaria episodes, may also have contributed to increases in RBC indices during the transmission season. Age-associated changes in alimentation (such as increased consumption of meat) or control of other anemia-causing infections (such as schistosomiasis) may also be contributory. It is less clear how WBC counts and the relative proportion of lymphocytes decrease through a single transmission season and with increasing age. One possibility is that, each year, WBCs become progressively tolerant to repeated infections with *P. falciparum* or other seasonal pathogens.

Our findings have several important implications for epidemiological studies of malaria. First, the use of "average" WBC counts (e.g., 7,500/μL) to calculate parasite densities in whole blood may underestimate parasite densities in younger children and overestimate those in older children. Our data suggest that it may be more accurate to establish "average" WBC counts that are more age- and season-appropriate for a given study population. A similar recommendation was recently made for malaria patients in the Brazilian Amazon, where an assumed WBC count of 5,500/μL (instead of 8,000/μL) was shown to more accurately estimate parasite density.[21] Second, it may be difficult to establish a single reference interval for hematological indices in healthy African children. For example, in children aged 6–12 years, the median Ht that we observed in Mali (36.7–40.1%) was higher or similar to that reported in Uganda (34.4%)[16] or Tanzania (38.4%),[17] respectively. Likewise, the median WBC count in Mali (5.53–6.43 × 10^3/μL) was lower than that observed in Uganda (7.1 × 10^3/μL). Multiple environmental and genetic factors probably account for such differences between populations.

Finally, our study has several strengths and limitations. We analyzed paired data from before (when no child was found to be parasitized) and after (when most children had developed asymptomatic parasitemia or malaria) a well-defined transmission season. Importantly, we accounted for the number of malaria episodes experienced in the interim and the presence of malaria-protective RBC polymorphisms,[13,14,22]—both of which may impact RBC indices.[23] In addition, our inclusion of 169 children met the minimal 120 sample size recommended by the Clinical and Laboratory Standards Institute for establishing reference ranges.[24] One limitation of our study is that we studied only one transmission season; therefore, we are unable to generalize our findings to others. However, the 2010 transmission season was typical of those in 2008, 2009, and 2011 in terms of its duration and total number of malaria episodes. Also, significant associations between changes in hematological indices and age group suggest that the seasonal changes that we observed occurred in multiple previous years. Moreover, we did not collect anthropometric data, which may have identified stunting due to chronic malnutrition, or iron and micronutrient deficiencies that may affect hematological indices in African children.[19,25] Furthermore, we did not screen for hookworm infections or schistosomiasis, which can influence hematological indices and are prevalent in some regions of southern Mali.[26,27]

In summary, our study highlights the complexity of establishing reference intervals for hematological indices in African children, and using these indices to estimate anemia prevalence and parasite density. In our cohort of Malian children aged 3–12 years, RBC and WBC indices changed significantly not only between age groups, but also within each age group during a 7-month transmission season. Establishing reference intervals for these indices may improve the quality of malaria epidemiological studies, especially where spatiotemporal changes in indices may occur due to intensified malaria control or emerging drug resistance.

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Authors' addresses: Mahamadou Diakite, Drissa Konate, Abdoul S. Keita, Saibou Doumbia, Seidina Diakite, Karim Traore, and Mory Doumbouya, Faculty of Medicine, Pharmacy, and Odontostomatology (FMPOS), Malaria Research and Training Center (MRTC), University of Sciences, Techniques, and Technologies of Bamako (USTTB), Bamako, Mali, E-mails: mdiakite@icermali.org, dkonate@icermali.org, akeita@icermali.org, saibou@icermali.org, sdiakite@icermali.org, ktraore@icermali.org, and moryd@icermali.org. Kazutoyo Miura, Ababacar Diouf, Jennifer M. Anderson, Rick M. Fairhurst, and Carole A. Long, Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, E-mails: kmiura@niaid.nih.gov, dioufa@niaid.nih.gov, jenanderson@niaid.nih.gov, rfairhurst@niaid.nih.gov, and clong@niaid.nih.gov.

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