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Artigo original

Immunohematological indices among school children and adolescents living in Manhiça District

Eduardo Samo Gudo[1]; António Prista[2] e Ilesh Jani[1]

[1] *Instituto Nacional de Saúde, Ministério da Saúde, Moçambique*
[2] *Facudade de Educação Física e Ciências do Desporto, Universidade Pedagógica Moçambique*

ABSTRACT: Immunohematological reference values reported in text books and obtained in western countries might not be applicable to sub-Saharan Africa. This study was aimed at determining these reference values for immunohematological parameters to be applied to children and adolescents living in highly endemic Malaria rural areas in Mozambique. A cross-sectional study was conducted in a highly endemic Malaria rural area in southern Mozambique, from August to September 2005. Apparently healthy children and adolescents were selected and blood samples were collected from 348 participants, of which 57.6% (190/348) were male. The mean age of the study population was 11.1 years (95%CI, 10.8 – 11.5). *Plasmodium falciparum* was detected in 56.5% (194/343) of the studied subjects. Hemoglobin, hematocrit, mean corpuscular concentration hemoglobin and mean corpuscular volume were lower than those reported in western countries. However, total lymphocyte count, CD4+ T cells and CD8+ T cells absolute counts were higher in our setting. The findings have important implications in the current international guidelines for the management of medical conditions in our settings, particularly for the immunologic assessment of HIV-infected children and adolescents. Immunohematological values obtained in this study were different from those reported for age matched children and adolescents in western countries, with emphasis for CD4+ T cells counts.

Keywords: Immunohematological indices; malaria falciparum, Southern African; children and adolescents

Estudo dos indicadores imuno hematológicos em crianças e adolescentes em idade escolar do Distrito de Manhiça

RESUMO: Embora o peso da malária na Àfrica Sub-Sahariana seja elevado, os valores normais de referência imunohematológica não foram ainda estabelecidos na região. Os valores descritos nos livros de texto, podem não ser aplicáveis ao nosso contexto. Este estudo teve como objectivo determinar se os valores de referência imunohematológica descritos nos livros de texto podem ser generalizados para as crianças e adolescentes vivendo em regiões rurais altamente endémicas para a malária em Moçambique. Para tal, entre Agosto e Setembro de 2005, foi conduzido um estudo transversal em Manhiça, uma região rural altamente endémica para a malária, para o qual crianças e adolescentes aparentemente saudáveis foram selecionados. Para o estudo, foram colhidas amostras de sangue de 348 participantes, dos quais 57.6% (190/348) eram do sexo masculino. A idade média da população foi de 11,1 anos (IC95%, 10.8–11.5). O *Plasmodium falciparum* foi detectado em 56.5% (194/343) dos participantes. Os valores de hemoglobina, hematócrito, concentração da hemoglobina corpuscular média e volume corpuscular médio foram menores quando comparados com aqueles reportados em livros de texto. No entanto, a contagem total de linfócitos, contagem absoluta de células T CD4+ e de células T CD8+ foi mais elevada no nosso estudo. Portanto, Os valores imuno-hematológicos obtidos foram diferentes daqueles reportados para crianças e adolescentes da mesma faixa etária em países desenvolvidos, com ênfase para a contagem de células T CD4. Os nossos achados têm importantes implicações nas directrizes para o manuseio de condições médicas no nosso contexto, particularmente para a avaliação imunológicas de crianças e adolescentes infectadas pelo HIV.

***Palavras-chave*:** Índice Imunohematológica, malária falciparum, África Subsaariana, crianças e adolescentes

Correspondência para: (correspondence to:) esamogudo@yahoo.com.br

INTRODUCTION

Immunohematological measurements serve a wide variety of applications, either in the clinical settings for the management of medical conditions or at epidemiological level for the assessment of health status of a population, guiding public health decisions and interventions (Ichikawa *et al.*, 1989; Hong *et al.*, 1991; Komatsuda, 1991; Sanchez-Ramon *et al.*, 2003; WHO and CDC, 2008). The correct interpretation of these measurements relies on availability of adequate and locally appropriate reference values. Nonetheless, normal ranges of immunohematolgical values have not yet been established in most Sub-Saharan Africa countries, including Mozambique. In the absence of these reference data, internationally derived intervals are commonly used. In Mozambique and other Sub-Saharan countries, reference intervals currently in use are based on data published in text books or peer-reviewed scientific literature which do not reflect the biological context of tropical settings. In fact, scientific evidence has repeatedly demonstrated that immunohematological reference ranges for African populations are markedly different from those for Caucasians (Ogala, 1986; Tatala *et al*., 1987; Tikly *et al*., 1987; Onwukeme and Olomu, 1991; Kassu *et al*., 2001; Bussmann *et al*., 2004; Lugada *et a*l., 2004; Aina *et al*., 2005; Klose *et a*l., 2007;; Ngowi *et al.*, 2009; Buchanan *et a*l., 2010). Several factors such as environmental, geographical, racial, ethnic, nutritional and exposure to pathogens can affect these indices, leading to variability (Williams, 1981; Miyawaki *et al*., 1984; Reichert *et al.*, 1991; Lee *et al*., 1996; Maini *et al*., 1996; Swaminathan *et al*., 2003; Borges *et al*., 2009; Maina *et al*., 2010). Therefore, each region should define their own normal reference values.

Moreover, the Southern Africa region, including Mozambique, which is highly burdened by malaria, parasitic infections, calorie-protein malnutrition, mineral and vitamins deficiencies and where the presence of infection by *Plasmodium falciparum* among apparently healthy individuals, usually denominated asymptomatic *P. falciparum* infection (APFI), has been reported to be high (Njama-Meya *et al*., 2004; Dal-Bianco *et al*., 2007; Baliraine *et al*., 2009). Paradoxically, insufficient data exist regarding the distribution of immunohematological indices in the general population. Scarcity of this information is even more evident for school age children and adolescents, as the few studies conducted in Africa have been restricted either to adults, or pre-school children (Abdurrahman and Adekoje, 1983; Cross and Heyns, 1983; Ogala, 1986; Tsegaye *et al*., 1999; Ngowi *et al*., 2009; Maina *et al.*, 2010).

We conducted this study aiming at firstly, determining if text books reported reference values for immunohematology can be generalized to children and adolescents living in rural areas highly endemic for Malaria in Mozambique; and, secondly, determining the prevalence of APFI in the study group.

METHODS

Study setting

The study was conducted in Calanga, a small village on the eastern coast of southern Mozambique, in the Manhiça district. Housing is precarious with neither canalized water, nor electric power. Calanga has an estimated population of 9.451 inhabitants and is located in an area with high endemicity and transmissibility of malaria. The raining season lasts from November through April. At the time of our study, only one health centre in Segera was available to serve all of the surrounding area.

Study sample

Between August and September 2005 we included consecutively in the study individuals aged less than 20 years old attending 1st (1st - 5th grades) and 2nd (6th - 7th grades) levels of the primary education in Calanga, and who consented to participate in the study through their parents or guardians. Consent to participate in the study was requested and explained in the local language. Clinical evaluation was performed by a physician before inclusion in the study and individuals presenting any sign or symptoms of disease such as fever were excluded from the study. The study was approved by the National Health Bioethics Committee.

Specimen collection

Venous blood (10 ml) was collected in all subjects who consented to participate in the study. Blood was collected from the cubital fossa into a 5ml K_3EDTA tube and a 5ml Serum Separation Tube (both from BD Vacutainer, USA). Specimens were delivered to the laboratory within four hours of collection.

Laboratory assays

Upon delivery of samples at the laboratory, a thick blood smear was mounted from K_3EDTA anticoagulated whole blood. All blood smears were stained using the Giemsa Protocol and screened for *P. falciparum*, *P. malaria*, *P. ovale* and *P. vivax* using light microscopy. Parasite density was estimated by means of a semi-quantitative scale (Payne, 1988).

Hematology parameters, including white blood cell counts and differentials, were determined using an automated five-part differential hematology analyzer (Sysmex SF 3000, Japan). All samples were processed within six hours of collection.

Immunophenotyping was performed on fresh whole blood using a FACSCalibur™ flow cytometer (Becton Dickinson, USA). MultiTEST reagents, TruCOUNT tubes and MultiSet software (all from Becton Dickinson, USA) were employed in a lyse-no wash protocol to determine absolute and percent values for T cell subsets (CD4+ T cells, CD8+ T cells and CD4/CD8 ratio).

Statistical analysis

Data was analyzed using the statistics package STATA 9.0 (College Station, Texas: StataCorp, USA, 2005). The Mann Whitney test was used to compare the study groups regarding numerical variables. Associations between categorical variables were determined using the Pearson Chi-square test.

For the purpose of comparison of immunohematological values, we stratified the participants by age, using the cut-point of 12 years of age. Those with less than 12 years, herein and after will be denoted as children and those with 12 years old or more will be denoted as adolescents.

Baseline characteristics of study subjects

Consent to participate in the study was obtained from 794 subjects, of which, 348 accepted to provide blood samples. Of these, 55% (190/348) were male and the median age of study subjects was 11 years old (Inter-Quartile Range, IQR: 8 - 13). Both sexes were comparable in terms of medians for age (male: 12 years old *versus* female: 11 years old, p=0.08); weight (male: 28.9 Kg *versus* female: 28.4 Kg, p=0.436); height (male: 134.4 cm *versus* female: 133.7 cm, p=0.418); and BMI (male: 16.4 Kg/m^2 *versus* female: 16.1 Kg/m^2, p= 0.309). (Table1).

TABLE 1: General characteristics of study subjects.

Parameter	Gender		*p-value**
	Male (n = 190)	**Female** (n = 158)	
Age (years)			
Median	12	11	0.08
IQR	7-17	6-15	
Range	5-20	5-17	
Height (cm)			
Median	134.4	133.7	0.436
p5-p95	109.7-165.8	110.6-158.5	
Weight (Kg)			
Median	28.9	28.4	0.418
p5-p95	18.5-51.1	19.3-48.0	
BMI (Kg/m^2)			
Median	16.4	16.1	0.309
p5-p95	13.0-19.8	13.7-19.9	

*****Mann-Whitney test, p5-p95: percentile 5 and percentile 95

RESULTS

Prevalence and distribution of asymptomatic *P. falciparum*

Plasmodium falciparum was the dominant species of Plasmodium in this study. Using blood smear we demonstrated the presence of *P. falciparum* in 195 individuals, giving an overall prevalence of APFI of 56.0% (195/3458).

Compared to adolescents, children presented a statistically significantly higher prevalence of APFI (61.5% *versus* 47.3%, p=0.007). In both, children and adolescentes, the prevalence of APFI was higher in boys than in girls (58.0% *versus* 54.7% for children, and 53.1% *versus* 39.1% for adolescents), although these differences were not statistically significant (Table 2). *Plasmodium malarie* infection was evidenced in 7 (seven) out of 348 samples, giving an overall prevalence of 2.0%. No sample was positive for either *P. ovale* or *P. vivax*.

Immunohematological indices of study subjects compared to age matched western countries normal reference standards

When compared with text books, describing normal reference values, children and adolescents from Calanga presented lower values of hemoglobin, hematocrite, median corpuscular volume MCV, but not mean corpuscular hemoglobin concentration in both genders. (Table 3 - compares the median and 95th percentiles of hematological indices for children and adolescents living in Calanga, with those obtained from western countries as available from text books or peer-reviewed scientific literature).

TABLE 2: Prevalence of asymptomatic Plasmodium *falciparum* infection in study subjects.

Characteristic	Asymptomatic *P. falciparum*	*p-value**
Age, years		
5.0 - 11.9	118/181 (65.2%)	
12.0 - 20.0	79/167 (47.3%)	**0.007**
Gender, < 12 years old		
Male	68/97 (58.0%)	
Female	52/95 (54.7%)	0.058
Gender, > 12 years old		
Male	52/98 (53.1%)	
Female	27/69 (39.1%)	0.076

* chi-square test

TABLE 3: Median and 90th percentile range for hemoglobin, mean corpuscular volume, hematocrit, mean corpuscular hemoglobin concentration indices of children and adolescents from Calanga: age and gender stratification.

Parameter	Study consensus immuno-hematologival values				Text book based immuno-hematologival values			
	Age (years) 5 - 11		Age (years) 12 - 20		Age (years) 6 - 10		Age (years) 11 - 15	
	F (n=93)	M (n=92)	F (n=65)	M (n=98)	F	M	F	M
Heamoglobin (Hgb), gr/dL								
Median	11.4	11.1	11.8	11.9	12.7	12.6	12.9	13.5
p5-p95	9.1-12.6	8.9-12.6	10.0-13.5	10.1-14.0	11.1-14.2	11.3-14.1	11.4-14.4	11.6-15.1
Mean corpuscular volume (MCV), fL								
Median	79.5	79	81.5	79.4	85	84	88	86
p5-p95	69.2-88.5	67-87.6	70.5-88.3	70.7-89.2	72-96	73-93	74-98	75-97
Hematocrit (Htc), %								
Median	33.3	32.3	34.4	34.7	37.7	37.1	39.2	40.6
p5-p95	27.9-36.3	26.5-36.2	30.7-38,2	29.2-40.4	33.1-42.7	33.7-42.3	34.0-44.7	34.2-46.3
Mean corpuscular hemoglobin concentration (MCHC), gr/dL								
Median	34.4	34.6	34.5	34.5	33.2	33.5	33.1	33.8
p5-p95	32.1-36.8	32.3	32.0-36.8	31.6-37.1	30.2-36.4	30.7-36.4	28.9-36.0	30.8-36.1

***** Mann-Whitney test

Children and adolescents living in Calanga presented higher total lymphocyte counts, higher CD4+ T cells absolute counts, higher CD8+ T cells absolute counts, higher CD4+ T cells percentage counts, higher total leukocyte counts and lower

CD8+ T cells percentage counts. (Table 4, compares the median and 95th percentiles of total leukocytes, total lymphocytes and lymphocyte subsets counts for children and adolescents living in Calanga with those obtained from western countries).

Remarkably, the median of CD4+ T cells absolute counts of children and adolescents living in Calanga was almost twice as high as the values described in text books or international literature.

DISCUSSION

Immunohematological reference values have never been established in Mozambique. The reference intervals of immunohamatological indices currently in use in Africa, including Mozambique, are derived from data collected from populations living in industrialized countries. To our knowledge this represents the first attempt to assess if immunohematological reference ranges reported in western countries are adequate for children and adolescents living in Mozambique, particularly in rural areas highly endemic for malaria. Overall, our data demonstrated that immunohematological indices of children and adolescents living in Calanga differed from the standard reference values described for age matched western population, confirming previous reports all over Sub-Saharan Africa (Onwukeme and Olomu, 1991; Embree *et al*., 2001; Lugada *et al*., 2004; Mekasha and Zerfu, 2009; Buchanan *et al*., 2010), and highlighting the need for the establishment of local reference ranges.

TABLE 4: Median and 90th percentile range for white blood cells, total lymphocytes and lymphocytes subsets indices of children and adolescents from Calanga: age and gender stratification.

Parameter	Study consensus immuno-hematologival values				Text book based immuno-hematologival values	
	Age (years) 5 - 11		Age (years) 12 - 20		Age (years)	
	F (n=93)	M (n=92)	F (n=65)	M (n=98)	6 - 11	12-18
WBC (10^3 cells /mm^3)						
Median	6.9	7.0	6.3	6.2	6.5	6.0
p5 – p95	4.2-11.4	4.81-11.6	4.4-9.5	4.4-9.4	4.4-9.5	4.4-8.1
LY (10^3 cells /mm^3)						
Median	3.9	3.8	3.3	3.0	2.7	2.2
p5 – p95	1.8-7.7	2.1-7.7	2.2-5.9	1.8-4.3	1.9-3.7	1.4-3.3
T CD4+ cells (cells /mm^3)						
Median	1619	1580	1425	1289	980	840
p5 – p95	712-3414	873-3261	903-2385	789-2377	650-1500	530-1300
T CD8+ cells (cells /mm^3)						
Median	793	958	778	788	680	530
p5 – p95	376-2020	412-2042	433-1523	385-1710	370-1100	330-920
T CD4+ cells (%)						
Median	42.6	41.3	42.8	42.0	37	41
p5 – p95	31.8-53.1	31.1-50.0	36.4-52.6	28.8-53.8	31-47	31-52
T CD8+ cells (%)						
Median	21.5	24.6	22.5	25.8	25	26
p5 – p95	14.2-32.9	16.6-33.9	15.4-32.0	17.1-35.2	18-35	18-35

***** Mann-Whitney test

In our study, *P. falciparum* accounted for most of malarial infections, as only a minor proportion of samples were positive for *P. malarie* and no sample was positive for either *P. vivax* or *ovale*. More than half of the study group presented parasitemia. These findings are in agreement with a country-wide study conducted by Mabunda *et al*. (2008). The real prevalence of APFI is probably even higher in Calanga as microscopic diagnosis of APFI under-diagnoses parasitemia (Steenkeste *et al*., n/d). Multiple studies in Africa have shown a higher prevalence of asymptomatic parasitaemia using PCR-based techniques (Bottius *et al*., 1996).

In our study, the prevalence of APFI in boys was higher when compared to girls. These findings are in accordance with data published by Baliraine *et al*., (2009) in a study conducted in Kenya. If these differences reflect biological differences, such as hormonal differences, or represent merely different pattern of exposure to the vector still remains to be clarified. Similarly to the report from Frederick *et al.* cited by Baliraine *et al* (2009), we demonstrated that APFI decreased with age. Several authors argue that the decrease of APFI with age is associated with acquisition of immunity to *P. falciparum*.

In our study, hemoglobin, hematocrit, MCV, but not MCHC, tended to be lower than age matched values described in western countries. These findings are similar to results of a previous study conducted by Lugada *et al* (2004) and by Buchanan *et al* (2010) among children with similar age in Uganda and Tanzania, respectively. Possible explanations for these differences include high prevalence and transmissibility of malaria, high burden of intestinal parasites, iron deficiency, higher levels of under nutrition, genetic factors, racial, dietetic and climatic differences, among others' (Williams, 1981; Bain *et al*.; 1984; Karazawa and Jamra, 1989; Siebers and Carter, 1991; Neuman *et al*., 2000; WHO and CDC, 2008).

In contrast, in our study, total leukocyte counts, total lymphocyte counts, T CD4+ absolute counts, T CD8+ absolute counts and T CD4+ percent counts - but not CD8+ T cells percent counts - were all higher compared to western countries derived indices. These differences might likely be due to a higher prevalence of parasitic infections and more frequent exposure to a wide spectrum of environmental antigens in our settings. Data from previous studies in Africa regarding CD4+ T cells counts are contrasting. While Lugada *et al* (2004) and by Buchanan *et al* (2010) found results similar to ours, studies conducted in Botswana and Malawi found lower values (Bussmann *et al*., 2004). These differences suggest that the use of western countries derived reference values in Mozambique might be misleading, resulting in inappropriate clinical management of patients.

The difference in the absolute number of CD4+ T cells counts was remarkable; children living in Calanga had almost twice as many CD4+ T cells when compared with children from western countries. This is particularly relevant in a time in which Sub-Saharan Africa carries the highest burden of the human immunodeficiency virus (HIV) infection rate, and where HAART and CD4+ T cells measurements by flow cytometry are being scaled up in many countries. CD4+ T cells counts represents the most important laboratory parameter for the clinical monitoring of HIV and HAART, and are widely used to assess the immune status, stage HIV disease, decide when to start HAART or prophylaxis for opportunistic infections, assess HAART efficacy and also perform HIV surveillance. The difference reported raises concern that the

international cut-offs of CD4+ T cells counts used for laboratory monitoring of HIV and HAART might not be adequate for older children and adolescents living in Sub-Saharan countries, particularly in places where malaria is highly endemic and underscores the importance of establishing and using locally appropriate laboratory reference intervals for CD4+ cells counts.

CONCLUSION

Reference intervals for immunohematological indices based on results from western individuals of the same age are not in agreement with the estimated values for children and adolescents living in rural areas highly endemic for malaria. Of remark, was the finding of high absolute CD4+ T cell counts, indicating that the use of western values in our settings would be misleading and have important implications for the management of HIV disease in children and adolescents, underscoring the need to develop local reference values. More than half of apparently healthy and asymptomatic children and adolescent living in Calanga carry *P. falciparum* in their bloodstream.

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