

Original article

Parasito-haematological features of acute *Plasmodium falciparum* and *P. vivax* malaria patients with and without HIV co-infection at Wonji Sugar Estate, Ethiopia

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Abstract

Background: Investigation to identify factors that determine the clinical outcome of malaria are essential for the improvement of patient care in severe malaria cases.

Objectives: To assess the parasitologic and haemato-immunological characteristics of malaria patients with and without HIV co-infection.

Methods: Immune cells were enumerated using the Flow cytometer method. Haematological values were determined using the Coulter Counter system. Malaria parasite detection was conducted using standard microscopy; and HIV testing was conducted using Determine, ELISA and Western blot methods.

Results: A total of 253 study participants, out of which 87 were control cases, and 166 were acute malaria patients (49.4% due to *P. falciparum*, 48.8 % due to *P. vivax*, 1.8% due to mixed infection) were included. Eight out of 166 (4.8%) of the malaria patients were found to be HIV positive. Asexual stage parasitemia was increased in HIV positive falciparum patients ($P=0.031$). However, the increase in parasitemia in HIV positive vivax malaria patients was not significant. HIV infection is seen to aggravate the decrease in Haemoglobin (Hgb), Haematocrit (Hct) and Platelet (Plt) levels. Lymphopenia, CD4+ lymphocytopenia, eosinopenia, monocytosis and thrombocytopenia were found to be higher in both malaria infections compared to the control ones ($P<0.05$).

Conclusion: Care should be taken when using immune cell counts for research or diagnostic purposes in malaria patients. The effects of HIV on parasitologic and haematologic values should be taken into account during the provision of care to HIV positive malaria patients. [Ethiop.J.Health Dev. 2005;19(2):132-139]

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Introduction

One hundred fifty million malaria related deaths, of which 90% occur in sub-Saharan Africa, were estimated to have occurred over the whole of the 20th century (1). Due to drug and insecticide resistance, malaria control is becoming more difficult (2). Out of the 39.4 million people living with HIV worldwide, 64.5 % are found in sub Saharan Africa (3). In Ethiopia, while 66% of the population is at risk of malaria (4), 1.5 million people are infected with HIV (3).

Due to the overlapping distribution of HIV and malaria, there is a theoretical possibility of concomitant infection, and the potential for immunological interactions between these two infections. However, there is inconsistency in the findings. Earlier studies done in Zaire (5), Burundi (6) and Zimbabwe (7) showed no interaction between these two infections. However, others have shown that *P. falciparum* antigens induced HIV-1 replication in vitro (8) and in vivo (9). HIV-1 infection was also found to reduce the immunity of pregnant women (10, 11) and increase the onset of malaria fever (12) in *falciparum* malaria cases.

Malaria and HIV are known to be the most severe of all infections in Ethiopia, but no study has yet been done which evaluates the association between malaria and HIV, and the clinical outcome of malaria is also not

universal, but it is dependent on local factors such as host genetics (13), parasite stages and strains (14) and levels of endemicity (15).

This study was aimed at assessing the parasitological and haematologic features of acute *falciparum* and *vivax* malaria cases, and the effect of HIV infection on these parameters. Abnormalities such as fever (axillary temperature ≥ 37.5 °C), leucopenia (WBC $< 4000/\mu\text{l}$), lymphopenia (lymphocytes $< 1400/\mu\text{l}$), CD4+ lymphopenia (CD4+ $< 300/\mu\text{l}$), monocytosis (monocytes $> 800/\mu\text{l}$), monocytopenia (monocytes $< 200/\mu\text{l}$), eosinopenia (eosinophils $< 40/\mu\text{l}$), neutrophilia (neutrophil $> 2500/\mu\text{l}$), anaemia (Hgb $< 12\text{g/dl}$) and thrombocytopenia (Platelets $< 150000/\mu\text{l}$) (16,17) were also evaluated in HIV negative malaria patients in relation to healthy donors.

Methods

This cross sectional study was undertaken from November 2002 to November 2003 at Wonji Sugar Estate, 114 km away from Addis Ababa. The average elevation of the study area is 1500 masl and its climate is characteristic of tropical lowlands. Total annual rainfall is around 8324 mms and 65 % of the total falls occur within the months of June to September. The topography and climatic conditions of the area create suitable conditions for malaria transmission (18).

As the malaria epidemic during the study period was very low due to intensive malaria control activities. The researches in this study recruited all adults of both sexes (age > 15 years) infected with either *P. falciparum* or *P. vivax* malaria that and who came to attend Wonji Hospital from November 2002 – November 2003. Informed written consent was obtained from all the participants. All malaria cases were treated according to the national standard drug regimens. Clinical and demographic data were recorded using a standard questionnaire. Age and sex proportions matched that for HIV negative and HIV positive cases but aparasitemic adult volunteers were included as controls from the same area.

The study was undertaken under the auspices of the Ethio-Netherlands AIDS Research Project (ENARP). It was ethically approved, nationally by the National Ethical Clearance Committee, NECC and institutionally by the Ethiopian Health and Nutrition Research Institute, EHNRI.

About 6–8 ml of venous blood was collected from each study subject by venepuncture into Ethylenediamine tetra acetate (EDTA) tubes. Whole blood samples, slide smears and filled questionnaires were sent daily to the ENARP laboratory at Addis Ababa.

Thick and thin blood films stained with 3% Giemsa were examined microscopically. At least 200 microscopic fields were scanned before labeling a smear as negative (19). Parasite number was counted against a 300 WBC count. Parasite density per micro litre of blood was calculated by multiplying the parasites counted by WBC/300 (19).

Absolute counts of WBC and levels of haemoglobin, platelets and haematocrits were obtained by using the Coulter counter T540 method (Coulter Electronics, Florida, USA).

HIV testing was conducted using Determine (Abbott Laboratories, Japan) rapid HIV1/2 test, Enzyme Linked Immunosorbant Assay (ELISA) (Vironostika-HIV UniForm II, Organon Teknika, the Netherlands) and Western Blot (WB) (Gee labs Diagnostics, Singapore) as screening, confirmatory and tiebreaker methods respectively.

Ten μl of the monoclonal antibodies, specific to the lymphocyte subsets (CD4+, CD8+, B and NK), were mixed with 50 μl of whole blood sample in a test tube and mixed by the vortexing method. This was followed by incubation of the samples for 15 minutes in the dark, and

at room temperature. To lyse the red blood cells, 450µl of fluorescent activated cell sorter (FACS) lysing solution (Becton Dickinson, San Jose, CA) was added. After vortexing, it was incubated for another 15 minutes at room temperature. This was followed by a three-color flow cytometry analysis done using the FACScan flow cytometer (Becton Dickinson, San Tase, CA), to enumerate the lymphocyte subsets.

Statistical analyses were performed by using STATA version 7.0 and SPSS version 10.0 soft ware. Results were compared between groups that use parametric or non-parametric statistical tools where appropriate. The degree of correlation between variables was evaluated by using the Spearman correlation analysis method. For all the statistical tests, a two-tailed P-value of < 0.05 was considered significant. For the purpose of this study, seasons were classified as rainy (June-August), post rainy (September-October) and dry (November-May).

Results

Demographic data, WBC and CD4+ cell counts of the study participants are summarized in Tables 1 & 2. A total of 253 adults of both sexes were included in the study. Out of these, 87 aparasitemic individuals (46 healthy and 41 HIV positive) were controls. The remaining 166 subjects (68.1% males) were malaria patients, of whom 82 (49.4 %) were falciparum, 81 (48.8 %) vivax malaria patients, and 3 (1.8 %) had mixed infection. Eight (4.8 %) of the malaria patients (3 falciparum and 5 vivax patients) were HIV-1 positives. There was no significant difference in the body temperature between falciparum and vivax malaria

patients. However, it was higher in HIV co-infected falciparum than vivax patients (P = 0.034).

Parasitological results

Although no significant difference was detected in the proportions of falciparum and vivax malaria cases during the study period, there was a marked seasonal variation between the two parasites (P < 0.0001) as well as within the onset of falciparum (P<0.0001) and vivax (P < 0.0001) infections. The emergence of both parasites was considerably related with rainfall (Fig. 1). In the dry months (January-May), almost all malaria infections were due to *P. vivax*. When rainfall started in March, an immediate onset of falciparum malaria was observed in the two months, up to June. The onset both falciparum and vivax malaria then increased up to the month of August, parallel with the increase in rainfall. However, when the rainfall gets maximum in July and August, an immediate decrease in both parasites occurred. An immediate increase of both parasites was observed from October to December, following the decrease in rainfall in September.

Monthly frequency of asexual and sexual parasite stages During *P. falciparum* infection, gametocyte stages started appearing in July, after about a month following the appearance of trophozoites, which occurred in June. However, gametocytes of *P. vivax* were observed throughout the year although they increased parallel with

Table 1: Characteristics of HIV negative falciparum (pf) and vivax (Pv) malaria patients and HIV negative controls. Mean (±SD) of Age, temperature (°C), WBC (x10³) and CD4+ counts (per µl of blood) in Wonji Sugar Estate, November 2002-2003.

	Study participants			Controls (n=46)	P value	
	Pf(n=79)	Pv (n=76)	Pf+pv (n=3)		a	b
Age (yrs)	36.2 ± 8.7	31.2 ± 9.1	29.3 ± 5.5	34.3 ± 5.7	0.152	0.042
Sex; Male (%)	61(77.2%)	43 (56.7 %)	3 (100 %)	39 (84.7%)	0.319	0.001
T (°C)	38.1±0.8	37.9 ± 0.65	39.0 ±0.45	NA		
WBC (x10 ³)	4.8 ±1.7†	5.5 ±2.0	6.3±1.8	5.7 ±1.8	0.015	0.957
CD4+ (per µl)	387 ±206	455 ±240	113±106	691±234	<0.0001	<0.0001

a = P value when pf compared with control; b= P value when pv compared with control † = Difference in WBC between pf and pv malaria patients (P = 0.031), using students t test T (°C) = Body temperature; NA = not available; n=number of participants

Table 2: Characteristics of HIV positive falciparum (pf) and vivax (pv) malaria patients and HIV negative controls. Mean (±SD) of Age, temperature (°C), WBC (x10³) and CD4+ counts (per µl of blood) in Wonji Sugar Estate, November 2002-2003.

	Study participants	P value
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	Pf +HIV(n=3)	Pv+HIV (n=5)	Controls (HIV+) (n=41)	a	b
Age (yrs)	34 ±5.3	33.4 ±4.9	36.9 ±6.0	0.413	0.209
T (°C)	38.7 ±0.6*	37.9 ±0.4	NA		
WBC (x10 ³)	2.0±0.91	4.5±2.3	5.4±1.4	0.0001	0.152
CD4+	78.6±20.9	225.2±116.5	274.0±196	0.023	0.929

a = P value when pf +HIV compared with control; b= P value when pv+HIV compared with control * = P value when pf+HIV compared with pv+HIV, (P = 0.034), using students t test.

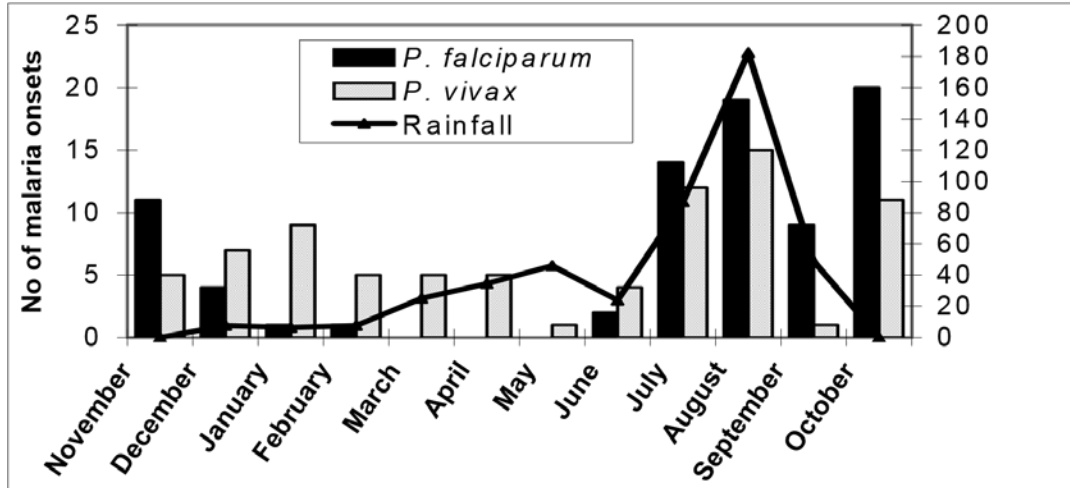


Figure 1: Monthly distribution of *P. falciparum* and *P. vivax* infections in relation to rainfall (per mm) (Rain fall data was secondary data from Wonji agricultural center, Wonji Sugar Estate) November 2002-2003.

the increase in rainfall and the frequency of trophozoite stages.

Asexual and sexual stage parasitic densities with and without HIV co-infection

The mean trophozoite density of *P. falciparum* was found to be higher than that of *P. vivax* ($P=0.0006$). But, the reverse was true in gametocyte density ($P < 0.0001$). A

significant increase in trophozoite density was observed in HIV positive falciparum patients ($P=0.031$). However, the increase in trophozoite and gametocyte densities in HIV positive vivax malaria patients was not significant (Table 3). Positive correlation was observed between trophozoite and gametocyte density in both *P. vivax* and *P. falciparum* infections, but this was significant only in vivax malaria patients ($r=0.33$; $P=0.023$; $n=77$).

Table 3: Mean and standard deviation (in parenthesis) of trophozoite and gametocyte densities (\log_{10} density/ μ l) of *P. falciparum* (pf) and *P. vivax* (pv) with and without HIV co-infection in Wonji Sugar Estate, November 2002-2003

Malaria patients	Trophozoite densities	Gametocyte densities
Pf (n=79)	3.9 (± 0.63) **	1.7 (± 0.49)
Pf + HIV (n=3)	4.9 (± 0.49) •	0
Pv (n= 76)	3.3 (± 0.62)	2.1 (± 0.53) ††
PV + HIV (n= 5)	3.6 (± 0.34)	2.1 (± 0.77)

** P = 0.0006, variation in trophozoite density between pf and pv; using Student t test
 †† P < 0.0001, variation in gametocyte density between pf and pv; using Student t test
 • P = 0.031 when trophozoites of pf compared with pf + HIV, using Wilcoxon rank-sum test
 pf+HIV= HIV positive falciparum patients; Pv+HIV= HIV positive vivax malaria patients
 n= number of subjects

Fever was positively correlated with trophozoite densities during *P. falciparum* malaria infection ($r=0.40$; $P=0.0003$; $n=79$). The same result was also found during *P. vivax* infection, although this, too, is not significant.

Haematological results

Hgb, Hct and Plt values decreased in falciparum ($P=0.001$, $P < 0.0001$, $P < 0.0001$) and vivax ($P=0.0003$, $P=0.0002$, $P < 0.0001$) malaria patients (Figure 2). However, no significant variation was found in any of the haematological values between the two malaria groups. No significant correlation was also observed between the decrease in all the haematological values and the density of trophozoites or gametocytes of either *P. falciparum* or *P. vivax*. No seasonal difference of haematological values was also found within falciparum or within vivax malaria cases, as well as between the two malaria groups.

HIV infection was seen to aggravate the decrease in the haematological levels of all the malaria patients, but this was significant only in the decrease of platelet levels in falciparum patients ($P=0.049$).

Haemato-immunological laboratory abnormalities Leucopenia cases were higher only during *P. falciparum* ($P=0.029$), but lymphopenia and CD4+ lymphocytopenia cases were higher in both falciparum

($P < 0.001$, $P < 0.001$) and vivax ($P < 0.001$, $P = 0.001$) malaria patients. Comparing the two patient groups, leucopenia was higher in falciparum than in vivax patients ($P = 0.032$), but no significant difference was observed in lymphopenia and CD4+ lymphocytopenia cases (Table 4).

Monocytosis was also higher in both falciparum ($P = 0.026$) and vivax ($P = 0.003$) malaria patients, but an increase in monocytopenia was observed only during P. vivax infection ($P = 0.015$). No significant difference was found between the two malaria groups.

Considering the granulocyte subsets, eosinopenia increased in both falciparum ($P < 0.001$) and vivax ($P = 0.001$) malaria patients, but the increase in neutrophilia and neutropenia cases in both malaria types was not significant. Neutrophilia on the other hand, was found to be higher in vivax than in falciparum patients ($P = 0.026$).

In the case of Haematological abnormalities, thrombocytopenia was found to be higher in falciparum ($P < 0.001$) and vivax ($P < 0.001$) malaria patients, but the increase in the proportion of anaemic cases in the two malaria groups was not significant. No difference was observed in thrombocytopenia, anaemia and febrile cases between falciparum and vivax malaria groups.

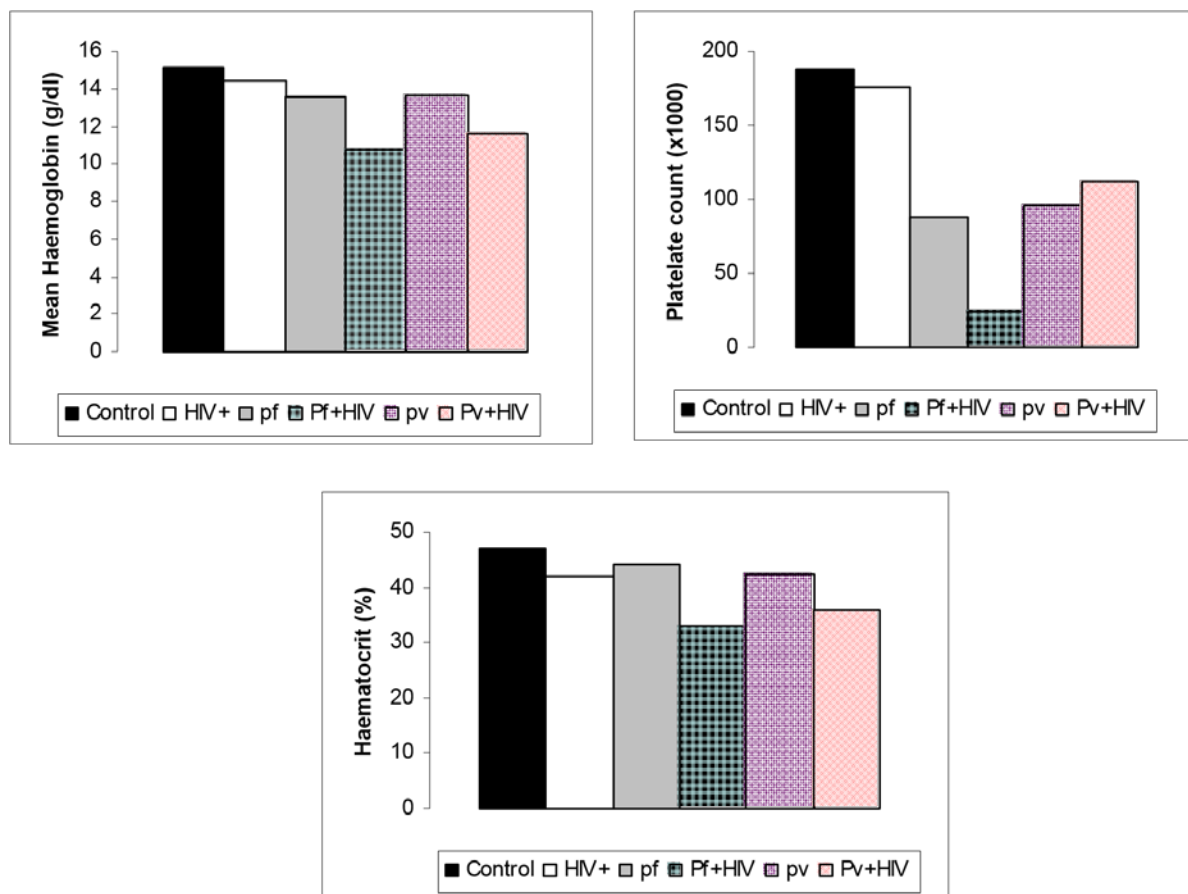


Figure 2: Mean values of haemoglobin, platelets and haematocrits in health controls (control), HIV positive but malaria negative controls (HIV+), HIV negative falciparum (pf) and vivax (pv) malaria patients, and in HIV positive falciparum (pf+HIV) and HIV positive vivax (pv+HIV) malaria patients in Wonji Sugar Estate, November 2002-2003.

Table 4: Numbers and proportions (in parenthesis) of haemato-immunological laboratory abnormalities in acute falciparum (pf) and vivax (pv) malaria patients and health donors. Cut of values of laboratory abnormalities are in parenthesis in Wonji Sugar Estate, November 2002-2003.

Laboratory abnormalities (cut off values)	pf (n= 79)	pv (n= 77)	Controls (n= 46)
Leucopenia (WBC < 4000/ μ l)	24 (30.4) [†]	12 (15.6)	6 (13.0)
Lymphopenia (Lymphocyte < 1400/ μ l)	55 (69.6)**	50 (65.8)**	12 (26.1)
CD4 lymphocytopenia (CD4+ < 300/ μ l)	29 (36.7)**	22 (27.6)**	2 (4.3)
Monocytopenia (monocytes < 200/ μ l)	5 (6.3)	9 (11.4) [*]	0
Monocytosis (monocytes > 800/ μ l)	8 (10.1) [*]	13 (17.1)**	0
Neutrophilia (neutrophil > 2500/ μ l)	38 (48.1)	51 (65.8) [†]	26 (56.5)
Neutropenia (neutrophil <2000/ μ l)	20 (25.3)	12 (15.8)	11 (23.9)
Eosinopenia (easinophil < 40/ μ l)	24 (30.4)**	16 (21.0)**	0
Thrombocytopenia (platelet < 150000)	64 (81.0)**	65 (84.2)**	10 (21.7)
Anaemia (Haemoglobin < 12g/dl)	15 (19.0)	8 (10.5)	3 (6.5)
Febrile (Temperature > = 37.5 °C)	62 (78.5)	60 (77.6)	NA

** = $P < 0.01$, * = $0.01 < P < 0.05$ when pf or pv compared with controls, using chi-square test

[†] = $P < 0.05$ when pf compared with pv, using chi-square test

NA= not available n= number of participant

Discussion

The overall yearly prevalence- 49.4 % of *P. falciparum* and 48.8% of *P. vivax* infection- as well as the seasonal variation between these two malaria species observed in this study was relatively similar to a previous study done in Nazareth town (20).

As relapsing is mainly a characteristic of the *P. vivax* species (21), almost all malaria cases occurring in the dry season (January - May) are expected to be due to *P. vivax* infection. The increase of both malaria species in the rainy (June-August) and post-rainy (September – November) seasons could be related to the emergence and increase in number of the female vector- *Anopheles* mosquito (22), where biting rates and the rate of transmission of malaria parasites are expected to increase (23). The observation of asexual stages of *P. falciparum* and *P. vivax* parasites in the blood film in June, while rainfall that starts in March could indicate the range of time the mosquito vector needs to complete its life cycle (22), plus the range of time between the first inoculation of malaria parasites (sporozoite) into the host and the first onset of the symptoms (21).

As is the case with the previous report (14, 24), HIV infection was found to cause an increase in asexual stage density in *falciparum* malaria patients ($P = 0.031$), and also aggravated the decrease in Hgb, Hct and Plt levels in both malaria infections. Bone marrow infection gastrointestinal bleeding and soluble factors in the serum which affect haematopoiesis are some of the mechanisms by which the virus affects Hgb and Plt values (25).

In agreement with the findings of this study other studies have reported a decrease in Hgb and Plt values (21) occurring during infection with the major species of malaria. The mechanical destruction of parasitized red blood cells, reduced RBC production in the bone marrow,

phagocytosis of uninfected red blood cells and autoimmune destruction of RBCs are some of the mechanisms known to cause anaemia during malaria (21). On the other hand, the decrease in Plt counts is related to the activation of Plt by the chemical adenosine diphosphate (ADP) under which Plts will have a shorter life span (26). One of the reasons believed to explain the absence of significant correlation between the decrease in haematological values and the trophozoite density in this study could be phagocytosis of uninfected RBCs by macrophages (21).

One of the reasons for the insignificant variation observed in the proportion of anaemia in *falciparum* and *vivax* malaria patients is the high proinflammatory to anti-inflammatory cytokines ratio that causes anaemic conditions (27). It could also be due to the short range of time existing between the onset of acute malaria symptoms and the time during which whole blood sample were taken from the patients for haematological analysis, so that most of the RBC are not haemolysed by the parasites.

Although Meek et al (1) reported leucopenia in *vivax* malaria patients, this was observed only in *falciparum* patients in the present study. The decrease in total lymphocyte counts (as observed in this study) could be one of the reasons for the occurrence of leucopenia. Similar to the current study, lymphopenia has also been reported in *falciparum* patients from other geographic locations (28). Depletion in the lymphocyte subsets through apoptosis (29) or due to sequestration of the cells in the lymph nodes or other body tissues (30) are some of the reasons behind the occurrence of lymphopenia in malaria patients.

Consistent with the findings of this study, an increase in monocytes and neutrophil counts (31)) and a decrease in eosinophil counts (32)) have been reported by others studies. Although the decrease in the number of eosinophils could be due to migration, sequestration or

destruction of the cells (17, 33), the increase or decrease in the monocyte and granulocyte subsets during acute infection is basically determined by factors such as the rate of input of the cells from bone marrow to the blood circulation; the proportion of circulating cells to cells attached on endothelial cells; and the rate at which the cells are leaving from the blood to the body tissue (17).

This study showed no significant difference in the proportion of *P. falciparum* and *P. vivax* infections over the study period. However, seasons have significant influence in the onset of both *falciparum* and *vivax* malaria. HIV co-infection seems to be associated with an increase in asexual stage parasite density in both *P. vivax* and *P. falciparum* infections. Both *P. falciparum* and *P. vivax* infections were associated with disrupted total WBC, lymphocyte and granulocyte subsets and monocyte counts, but in different ways. Both malaria species caused a decrease in haemoglobin, haematocrit and platelet levels. However, there was no significant correlation between the trophozoite and gametocyte densities of *P. falciparum* or *P. vivax* and the decreasing haematological values. HIV infection aggravated the decrease in haemoglobin, haematocrit and platelet levels in infections with both malaria species.

The impact of HIV infection on the decrease in haematological levels and the increase of asexual parasite densities should be considered during the management of malaria patients co-infected with HIV. For a better understanding of the existence of possible malaria–HIV interaction, however, a longitudinal study on a larger sample of participants is required. Disorders in WBC, lymphocyte, granulocyte and monocyte counts during acute malaria infections should also be accounted when enumerating these cells for diagnostic or research purposes, as optimal number of cells can not be found in the peripheral blood. The impact of distortions in the normal profile of lymphocyte, monocyte and granulocyte cells due to acute malaria infections on: a) the clinical outcome (severity) of malaria and b) increasing susceptibility of the host to be co-infected by other new infectious agents must be further investigated. As these immune cells could be important (effector) cells that could fight infectious organisms.

Acknowledgments

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