CHARACTERIZATION OF PERIPHERAL BLOOD LEUCOCYTE SUBSETS IN ACUTE *PLASMODIUM FALCIPARUM* AND *P. VIVAX* MALARIA INFECTIONS AT WONJI SUGAR ESTATE, ETHIOPIA

By
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A Thesis Submitted to The School of Graduate Studies
In Partial Fulfillment for the Degree of Master of Science in Biology
PARASITOLOGY (BIOMEDICAL SCIENCE)

Addis Ababa University
School of Graduate Studies
Department of Biology

June 2003

ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

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DECLARATION

I, the undersigned, declare that this thesis is my own work and it has not been presented in other Universities, Colleges or Institutions, seeking for similar degree or other purpose.

Desta Kassa Misgina _____ _____

Name Date of Submission Signature

ACKNOWLEDGMENTS

Above all, I would like to express my heartily thanks to my advisors Dr. Beyene Petros and Dr. Dawit Wolday for their invaluable support, encouragement and overall supervisions during proposal design, laboratory work and paper write-up; and for their very cheerful cooperation. I greatly acknowledged ENARP, EHNRI (Parasitology) and Wonji laboratory technicians for their assistance to teach me the high tech lab works; Ph.D. students and ENARP administrative staffs for their very friendly approach and making suitable environment for my laboratory works. Special thanks also to Wonji Agricultural Centre, in Wonji Sugar Estate, for the rainfall data.

I am also very thankful to Ato Mehari Woldu, Nages Tame, Kassa Moges, Fasika Demissie, Abate Asfaw, Abebe Alemu, Taddesse Bahta, Dagnew Negash, Hailemariam Moges, Wolday Hailu, G/Michael Halefe and their families, for their unforgettable assistance and encouragement.

I wish to thank ENARP for its invaluable support by covering the whole laboratory costs, and also the Biology Department of Addis Ababa University for financial and administrative supports.

I greatly acknowledged also all the patients who involved in this studyI am also very grateful to all my devoted instructors who enrolled me to the field of Biomedical Science and instilled me a love for immunology and parasitology.

Now, just being here, and recalling back the long road of my life I have been walking along, with all that my “bare foot”, I came to realize that I must also thank God.

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List of abbreviations

µl	Micro liter
AAU	Addis Ababa University
AIDS	Acquired Immunodeficiency Syndrome
ANOVA	Analysis of variance
CLA	Cutaneous lymphocyte antigen
CSF	Colony stimulating factor
CTL	Cytotoxic T lymphocyte
DNA	Deoxyribonucleic acid
ECAM-1	Endothelial cell adhesion molecule-1
EDTA	Ethylenediaminetetraacetate
EE	Exoerytrocytic
ELAM-1	Endothelial lymphocyte adhesion molecule-1
ELISA	Enzyme linked immunosorbant assay
ENARP	Ethio-Netherlands AIDS Research Project
ESTC	Ethiopian Science and Technology Commission
FACS	Fluorescence activated cell sorter
FITIC	Fulorescein isothiosyanate
g/dl	Gram per decilitre
GPI	Glycosulphotidylinositol
HIV	Human Immunodeficiency Virus
ICAM-1, 2	Intracellular adhesion molecule-1, 2
IFN-γ	Interferon-γ
IL-1	Interleukin-1
LECAM-1	Lymphocyte endothelia cell adhesion molecule-1
LFA-1	Lymphocyte functional association-1
MadCAM-1	Mucosal adhesion cell adhesion molecule-1
MHC-II	Major histocompatibility class-II
MoAb	Monoclonal antibody
NK	Natural Killer cell

PBMC	Peripheral blood mononuclear cell
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
pg/ml	Pictogram per millilitre
PSGL-1	P-selectin glycoprotein ligand-1
RBC	Red blood cell
RNA	Ribonucleic acid
rpm	Round per minute
SIV	Simian Immunodeficiency Virus
TCR	T cell receptor
Th	T helper cells
TMB	Tetramethylbenzidine
TNF-α	Tumour necrosis factor-α
VCAM-1	Vascular cell adhesion molecule-1
VLA-1	Very late antigen-1
WBC	White blood cell

Abstract

The aim of this study was to assess the absolute counts and percentages of lymphocyte and granulocyte subsets and monocytes in acute P. falciparum (pf) and P. vivax (pv) infections. Effect of HIV infection on haematological and Parasitological values was also assessed. Three-colour flow cytometry was used for enumerating the immune cells. Coulter Counter was used to determine haematological values. Parasite species were detected using light microscopy. HIV testing was done using Determine, ELISA and Western blot. Data was analysed using STATA and SPSS softwares. 166 acute malaria patients of whom 82 (49.4%) were infected with pf, 81 (48.8%) infected with pv, and 3 (1.8%) infected with both pf and pv were included in the study. 8 (4.8%) (3 pf and 5 pv patients) were HIV positives. As a control, 87 age-matched subjects (46 HIV negatives and 41 HIV positives) were included. Trophozoite density was increased in HIV positive falciparum patients than in the negatives (P=0.031). Moreover, although statistically insignificant, sexual and asexual parasitemia was higher in HIV positive pv patients than in the negatives. Haemoglobin (hgb), haematocrit (hct) and platelet (plt) levels were lower in pf and pv malaria patients (P<0.0001) than in controls. Plt level was lower in HIV positive falciparum patients than in the negatives (P=0.049). A decrease in hct and hgb levels in both pf and pv patients was also observed in the HIV infected. WBC count in pf patients was lower than in controls (P=0.015). Compared to healthy controls, significant decrease in the absolute counts of CD4+, CD8+, B, CD3+ and total lymphocytes was found in pf (P<0.0001) and pv (P<0.0001) malaria patients. However, NK cell count was not affected by pf or pv malaria parasite infections. No difference was found in CD4%, CD8% and CD3% in either pf or pv, whereas B and total lymphocyte percentages were lower in both pv (P=0.002 and P<0.0001 respectively) and pf (P=0.008 and P<0.0001 respectively) malaria patients. Monocyte (P=0.007) and neutrophil (P=0.035) counts were significantly increased only during pv infection. On the other hand, eosinophil and basophil counts were decrease in pf (P<0.05) but not during pv malaria. There was a negative correlation of CD4+, NK, CD3+, total lymphocytes and monocyte count with the asexual stage parasite densities of pf (P<0.05), while neutrophil counts in pf and pv malaria were positively correlated (P<0.05). Comparing the two malaria groups, CD4+, CD8+, CD3+, total lymphocytes, monocyte and basophil counts were lower in pf than in pv only in the rainy season (P<0.05). This study strongly suggests the need for special considerations when dealing with malaria patients. This includes: a) enumeration of immune cells for diagnostic or research purposes; b) studies done on peripheral blood to evaluate immune status of patients c) the effect of HIV on malaria parasitemia and haematological values.

1. INTRODUCTION

1.1. MALARIA PARASITES

The malaria parasites are protozoa belonging to the Order Coccidiida and family Plasmodiidae and the genus *Plasmodium*. Within the genus there are 100 species that are known to cause the disease in a wide range of vertebrates (Deans and Cohen, 1983). Among these, only four are known to infect humans. These are: *P. malariae*, Laveran, 1881; *P. vivax*, Grossi and Feletti, 1890; *P. falciparum*, Welch, 1897; and *P. ovale*, Stephens, 1922. Although all the four species occur in Ethiopia, over 90 % of the cases are due to *P. falciparum* (60-70%) and *P. vivax* (30-40%) (WHO, 2001).

Worldwide, malaria causes severe morbidity and mortality. 150 million deaths, out of which 90 % were in Sub-Saharan Africa, were estimated in the 20^{th} century (Meek *et al*., 1996). In Ethiopia, out of the 65 million people, 40 million are estimated to be at a risk of malaria infection (WHO, 2001).

Furthermore, because of increased drug and insecticide resistance malaria control is becoming more difficult causing an increase in epidemics (UNICEF, 2002).

Infection starts when the female anopheline mosquito inoculates motile sporozoites into the blood stream. Half an hour later most are destroyed by phagocytes from the blood but the few that enter in the liver parenchyma cells transform themselves to the next stage called the trophozoites. It divides to give rise to pre-erythrocytic schizonts. Six to sixteen days later, the schizonts rupture to release extra-erythrocytic merozoites in to the surrounding tissue and the

circulating system where they invade RBC. With a periodicity characteristic of the *Plasmodium* species, the erythrocytic schizonts mature and rupture 48 or 72 hrs, liberating 10-20 erythrocytic merozoites. The merozoites attach to specific receptors on the surface of red blood cell membranes to initiate further invasion. In a period of about ten days, the newly invading merozoites differentiate into male and female gametocytes through purely asexual replication. Then, sexual reproduction takes place following the ingestion of gametocytes into the gut of the appropriate species of *Anopheles* mosquito (Deans and Cohen, 1983; Tine and Paoletti, 1996).

There are many factors affecting malaria transmission. These include, the type and density of *Anopheles* species mosquito vector and the longevity of the vector to allow the sporozoite cycle to be completed. This in turn depends on physical factors such as altitude (<2000m above sea level) and temperature (about 28 ^{0}C) of the environment. The proportion of infected persons carrying the gametocyte in the community and the number of gametocytes in the infected person is also another factor, which determines malaria transmission (Greenwood, 1997a).

Epidemiologicaly, *P. falciparum* malaria covers 85% of all malaria cases worldwide. Almost all deaths are caused by this species. It occurs mainly in the hotter and more humid regions of the world, (Schofield, 2000). It is the most pathogenic of all the human malaria species. The following are believed to contribute to its malignancy: the high level of parasitemia as the result of invading young and old RBCs; erythrocytic schizogony that takes place in the capillary beds of the internal organs such as the spleen, bone marrow, brain, kidney, intestine, heart and placenta. It also causes changes on the surface of parasitized red blood cells and

form knobs, which cause sequestration of infected red blood cells with mature parasites in vital organs, particularly the brain and the heart. Symptoms of acute complications such as cerebral malaria, diarrhea, and symptoms of chronic complications such as tropical splenomegaly syndromes, falciparum recrudescence and latent malaria are also known during *P. falciparum* malaria (Parija, 1996).

P. vivax malaria is the second most important public health problem. It is more common in the temperate than in the tropics before its eradication (Gillers and Warrels, 1993; Parija, 1996). It is characterized by the occurrence of true malaria relapses due to the re-activation of hypnozoites in the liver cells. Although the complication of *P. vivax* malaria is relatively less, rupture of an enlarged spleen occasionally occurs and could be life threatening (Schofield, 2000). Unlike *P. falciparum*, all the life cycle stages can be found in the peripheral blood (Parija, 1996).

Fever is a key clinical manifestation and a characteristic feature of malaria infection. It usually occurs shortly after the rupture of malaria infected RBCs (Parija, 1996). When erythrocyte schizonts ruptured, they release parasites, erythrocytic debris, and especially protein toxins, glycosulphatidylinositol (GPI) of parasite origins, which activate tissue macrophages to produce proinflammatory cytokines such as IL-1 and TNF, which cause fever, chill, sweat and other pathological effects (Clark and Schofield, 2000). In *P. vivax* malaria, for example, fever occurs every 48 hours following erythrocytic raptures, while TNF rises shortly an hour or so before the onset of rigors (Greenwood, 1996). Children infected with *P. falciparum* and treated with anti-TNF monoclonal antibodies (MoAb) were known to have their fever reduced, showing that TNF to be the main causative agent of fever in malaria infection (Greenwood, 1996).

It is, therefore, practically as well as theoretically important to understand the relationship between malaria parasitemia and fever. Direct correlation of fever with *P. falciparum* parasitemia have been reported (Lisse *et al*., 1994; Greenwood, 1996; Rogier *et al.,* 1996). This is mainly due to the increase in TNF level, a factor strongly associated with high parasitemia (Kwiatkowski *et al.,* 1990). Attempts have also been made to identify the level of parasitemia that can be used to define the clinical episode of malaria. Using logistic regression methods, the threshold of trophozoite count was estimated to be in the range of 2-20000 parasites/µl of blood that has optimum sensitivity and specificity for the definition of a clinical episode of *P. falciparum* malaria (Greenwood, 1996). However, this approach is complicated by the fact that the threshold of trophozoite count to induce fever is age specific. In *P. falciparum* malaria, for example, the maximum threshold at one year of age was found to be 2-45 trophozoites per leucocytes, and a minimum of 0.5 trophozoites per leucocyte, at 60 years of age. When the parasite density of a person crosses the threshold level corresponding to his or her age, the individual's risk of fever was to be multiplied by 44% (Rogier *et al.,* 1996). Hence, the risk of fever considerably increases with parasitemia while it decreases with age. It seems likely then that frequent infection with malaria will result in down-regulation of cytokines, or the ability of cytokines to induce fever in a manner analogous to the repeated exposure to endotoxin, which is known to initiate TNF production. In this sense thresholds are also likely to vary with endemicity, showing a less pronounced age effect in areas where endemicity is low (Greenwood, 1996). This is because in non-immune individuals fever was observed to occur even when parasitemia is still very low, which is due to the absence of enough amount of neutralizing antibodies against the toxins which are responsible for an acute fever by inducing the production of cytokines such as TNF. However, in children living in highly endemic areas only a high parasitemia can induce fever,

since the high prevalence of high parasite loads maintains high titers of antibodies against the TNF-inducing toxins (Rogier *et al.,* 1996). On the other hand, in adolescents and adults, the progressive development of anti-parasite immunity results in a decreased parasite density, so that lower level of the short-lived antibodies against TNF-inducing toxins are expressed. This could explain the reason why lower threshold level is expressed in lower ages (Rogier *et al.,* 1996).

1.2. The effect of infectious agents on the circulation of leucocyte subsets

Normal peripheral blood is composed of three main cellular elements: Red blood cells (erythrocytes), White blood cells (Leucocytes), and platelets (Thrombocytes). These cells are suspended in an aqueous pale yellow fluid called plasma. Based on the nature of their nucleolus, leucocytes are subdivided as mononuclear cells, which include lymphocytes (CD4+, CD8+, B and NK cells) and monocyte/macrophages; and polymorphonuclear (PMN) cells, referred to as granulocytes, include basophils, eosinophils and neutrophils.

The physiology and migration of lymphocytes between various lymphoid and non-lymphoid tissues, and also their "homing" to their particular sites is highly regulated by means of various cell-surface adhesion molecules and receptors to these molecules (Picker and Butcher, 1992). The interaction of lymphocytes and endothelial cells involves at least three selectins (L-, P- and E- selectins); four integrins (LFA-1, MAC-1, VLA-4 and γ_4 β_7); three immunoglobulin-like molecules (ICAM-1, ICAM-2 and CD31) and four mucin-like molecules, (CD34, GlyCAM-1, MadCAM-1 and PSGL-1) (Hogg, 1997; Bragardo *et al.,* 1997).

Lymphocytes activated following exposure to pathogen have been observed to express distinct set of surface molecules, which are characteristic of their cell lineage, level of differentiation and their capacity to home to tissue compartments. This activation of lymphocytes will not only induce the emergence or disappearance of surface molecules which are important for cellular effector function, cellular cooperation, and homing to tissue; it might prompt also different movement of T cells out of the circulation and on to fixed

lymphoid organs and will result in the change in the composition of lymphocyte subsets in the peripheral blood (Grassman and Herberman, 1997). For example, while naïve ($CD45RA^+$ $CD6L^+$) T cells recirculate in L-selectin (CD62L)-dependent mechanism from blood to lymph node and then back to blood, the memory effector ($CD45RO^+$) T cells that develop subsequent to exposure to a pathogen, move through non-lymphoid tissues such as the liver and the lung thereby increasing the chance of encountering foreign microorganisms (McCune, 2001). Therefore, peripheral blood lymphocyte counts are known to be influenced by antigenic activation.

The cytokines TNF-α and IFN-γ, have also been observed to cause rapid depletion of lymphocytes from the blood by enhancing the rate at which lymphocytes move away from the peripheral blood and by reducing the rate at which they return to the peripheral blood. This is because these cytokines enhance gene expression, activation and secretion of the selectins, integrins, chemoatractant chemokines and Ig superfamily molecules found on lymphocytes and on the surfaces of vascular endothelial cells (Rosenberg *et al.,* 1998). As evidence to the cytokine influence on lymphocyte distribution, up on injection of TNF-α and IFN-γ in both man and experimental animals, rapid depletion of lymphocytes from blood has been observed (Wakelin, 1988). The loss and reappearance of CD4+ and CD8+ T cells in the blood of acute symptomatic primary HIV/SIV infections of human and monkeys was also observed to correspond precisely with TNF-α and IFN-γ levels (Rossenberg *et al.,* 1997). Cellular activation of T and B cells and distinct species-related changes in peripheral blood lymphocyte subsets during the course of the disease in *Shigella* infection was also reported (Islam *et al*., 1995).

Unlike lymphocytes, which can only stay in the circulation for only about 30 minutes and then adhere to specialized high endothelial cells in the nodules of lymphoid organs, granulocytes and monocytes are in continuous flow in the blood. For example, when phagocytotic process is activated during body tissue infection, the cells within the marginal granulocyte pool migrate to the source of infection and are replaced by cells from the circulating granulocyte pool; in turn cells in the circulation are replaced by cells from the maturation compartment in the bone marrow. Usually cells are randomly removed from the circulating granulocyte pool as the result of which their half-life in the pool is about seven hours (Rosenberg *et al.,* 1998).

However, like the alternation of absolute counts and compositions of lymphocyte subsets during acute infections, disorder of the granulocytes and monocytes is also common in the peripheral blood circulation. Three main factors are known to play a role: first, the rate of input of new cells from the storage pool (bone marrow) to the blood circulation pool; second, the proportion of circulating cells compared with marginating pool (cells attached on endothelial cells of body tissue); and third, the rate at which these cells leave the blood to the body tissue (Simmon, 1997) through diapedesis (Hogg, 1997). Neutropenia (neutrophil count < 2000/µl) for example, is common during overwhelming acute infections resulting from bacteria, viruses and protozoa, such as malaria and leishmaniasis, whereby a large number of neutrophils are in demand so that the bone marrow storage pool can become exhausted even in the face of increased production (Simmon, 1997).

The different life cycle stages of human malaria are known to act as potential antigens to initiate both innate and acquired immune responses. Thus, acquired immunity against malaria

infection involves both the humoral arm, which is basically mediated by antibody producing B cells; and by the cellular arm, which is mainly controlled by T cell (Kumaratilake *et al.,* 1991). For example, Th1 and Th2 subsets of CD4+ T cells are known to play a major role in protective immunity against the blood stage of malaria, whereby Th1 is active during early stages of the infection but a shift towards Th2 takes place later (Phillips *et al.,* 1997). CD8+ cells from mice immunized with irradiated sporozoites were also observed to kill exoerythrocytic (EE) parasites cultured in mouse hepatocytes. These appear to be involved in the destruction of EE parasites in the liver of immunized mice (Suhirbier, 1993). NK cells are also known to be involved in immunity against malaria by producing the cytokine IFN-γ, which in turn activates macrophages that are involved in controlling blood stages of malaria through phagocytosis (Phillips *et al.,* 1997).

However, the immunological background of *P. falciparum* and *P. vivax* infections have also its own effect on the absolute counts and proportions of peripheral blood mononuclear cells and granulocyte subsets in the peripheral blood. For example, the level of the cytokines TNF-α and IFN-γ, which are known to induce the expression of selectins, integrins and chemoatractant chemokines (Rosenberg *et al.,* 1998) have been observed to correlate with malaria severity caused by *P. falciparum* (Udomsongpetch *et al.,* 1997; Bate *et al.,* 1998;) and *P. vivax* (Elhasson *et al.,* 1994; Wijensekera *et al.,* 1996) infections. Furthermore, an increase in the plasma level of the adhesion molecules ICAM-1 and VCAM-1; and expression of ECAM-1 on the surface of endothelial cells was observed during malaria infection (Hviid *et al*., 1993; Elhasson *et al*., 1994). The activated T cells may also release inflammatory lymphokines, which cause additional augmentation of endothelial cell adhesion molecule expressions and will increase T cell adherence on body tissues (Elhasson *et al*., 1994). It has

also been shown that, although reactive T cells can be detected in a spleenic cell population at any time during and after infection, these cells were not detectable within the peripheral blood T cells during acute infections (Longhorn *et al.,* 1991). This may indicate the disappearance of T cells from the peripheral blood in acute malaria infections. This also shows that peripheral blood T cells may not necessarily be indicative of the immune status of a person especially during acute malaria infection, and this should be taken into account when evaluating immune responses from patients undergoing an active malaria infection. On the other hand, in studies done to quantify the circulating memory B-lymphocytes reacting against *P. falciparum* malaria antigens, antibody-secreting cells were detected in the peripheral blood in individuals infected with malaria 8 years ago. This suggested that the number of circulating antimalaria antibody secreting cells may not necessarily be decreased, since the maintenance of B cell memories depends on the persistence of stimulating antigens (Migot *et al.,* 1995).

Malaria infection is also known to induce apoptosis (Toure-Bolde *et al*., 1996). Evidence for this is that, the mean percentage of spontaneous apoptosis of mononuclear cells was found to be higher in patients with acute as well as chronic asymptomatic *P. falciparum* infection, compared to age and sex matched controls. Thus parasite-induced apoptosis would contribute to reducing the immune response directed towards critical antigens, by increasing the fragility of potential effector cells. Therefore, besides the cytokines (TNF-α and IFN-γ) and malaria antigen mediated activation-induced sequestration of the lymphocyte in the lymph nodes, which then cause depletion of these cells in the peripheral blood, malaria antigen-induced apoptosis could also play a role in altering the lymphocyte composition in the peripheral blood during malaria infections.

Several studies have been conducted to assess the practical occurrence of the alternation of the absolute counts and the composition of immune cells. Conditions such as lymphocytopenia (lymphocyte count < 1400/µl) have been reported for example during acute *P. falciparum* and *P. vivax* malaria infections (Jande, 1987). No response to antigen stimulation *in vitro* was observed, for example, in peripherally circulating cells in *P. falciparum* malaria infection (Ho *et al.,* 1986; Rilley *et al*., 1988). This unresponsiveness was suggested to be due to the disease episodes, which induce re-location of T cells away from the peripheral blood (Hviid *et al*., 1993). This hypothesis was supported by findings, which showed remarkable loss of T cells with high expression of LFA-1 (CD11/CD18) during acute *P. falciparum* malaria (Greenwood *et al.,* 1997b). In addition, the expression of membrane-bound IL-2R (CD25) and ICAM-1 (CD54) were not revealed on cells from malaria patients indicating the withdrawal of T cells, which recognize parasite antigens from the circulation (Hviid *et al.,* 1991; Chougent *et al*., 1992; Elhasson *et al*., 1994).

Depletion of CD4+ cells with high expression of LFA-1 antigens from the peripheral blood was also observed during acute *P. falciparum* malaria (Hviid *et al*., 1993; Elhasson *et al*., 1994). In Children with cerebral or uncomplicated malaria, the frequency and absolute number of peripheral T cells was also lower than normal and that the degree of disease-induced T cell outflow from the peripheral blood was correlated with disease severity (Hviid *et al.,* 1997). Worku *et al* (1997) have reported lower total leukocyte and lymphocyte counts but high number of activated cells in malaria patients with a distinct pattern observed between *P. falciparum* and *P. vivax* infections. Furthermore, in studies done in children aged 3-6 years who were infected with *P.falciparum*, lower CD4+ and CD8+ cell counts were observed in

those with acute malaria when compared with children with no parasitemia or in those with asymptomatic parasitemia; but no significance difference was observed in WBC count, percentage of CD4+ and CD8+ cells or in the ratio of CD4/CD8. Furthermore, while CD4+ percentage was inversely correlated with the density of malaria parasites, no correlation was observed with the CD8+ percentage or CD4/CD8 ratio. No effect of age, sex or season on the estimation of CD4+% was also observed (Lisse *et al.,* 1994). On the other hand, studies on *P. falciparum* patients showed a decline in WBC count after 11-12 days of infection and decline in NK, αβ and γδ T cell and B cell count was observed only in the period leading up to the acute stage of infection. This suggested sequestration of the immune cells to be the most likely explanation to their loss from the circulation (Rzepczyk *et al.,* 1996).

Thus, there is ample evidence to suggest that malaria infection is known to cause immunological abnormalities such as leucopoenia (WBC < 4000/µl of blood) and lymphocytopenia. But, how the lymphocytopenia phenomenon relates to the clinical course of malaria infection remains to be investigated. However, it has been reported that as the infection is gradually controlled through medication, inflammatory stimuli will be diminished allowing for the distribution of cells to return to the pre-infection state (Elhasson *et al*., 1994; Hviid *et al*., 1997).

In summary, malaria infection could result in the lowering of lymphocyte subset counts in the peripheral blood through two mechanisms. That is, by prompting different movements from the blood circulations and enhancing attachments of the lymphocyte subsets onto fixed lymphoid organs (sequestration) through antigenic activation of the cells and/or through the

induction of cytokines TNF-α and IFN-γ; and possibly through the induction of apoptosis mediated cell deaths.

However, unlike lymphocytes, monocyte count was not altered during experimental study in *P. falciparum* malaria (Rzepczyk *et al.,* 1996). In this case it can be said that, the amount of cells recruited to the site of inflammation site from the marginating pool are replenished by newly produced cells from the bone marrow. During acute infections, however, as the number of cells produced is sometimes more than the cells migrating from the circulating pool to the inflammed tissue, high number of cells (monocytosis, >800 cells/µl) could be found in the peripheral blood. Thus, monocytosis has been observed, for example, in *P. falciparum* malaria patients (El-Shoura, 1993). On the other hand, during chronic infections exhaustion of production of new cells from the bone marrow (monocytopenia, <200 cells/µl) could also take place (Simmon, 1997).

Besides the alternation of absolute counts and compositions of mononuclear cells (lymphocyte subsets and monocytes) during acute infections, disorder in the count of granulocyte subset is also common in the blood circulation. Three main factors are known to play a role in this: one, the rate of input of new cells from the storage pool (bone marrow) to the blood circulation pool; second, the proportion of circulating cells compared with marginating pool (cells attached on endothelial cells of body tissue); and third, the rate at which these cells leave the blood to the body tissue (Simmon, 1997) through diapedesis (Hogg, 1997). Thus it is possible that malaria infection would likewise influence the peripheral distribution of granulocytes.

MacDonald *et al* (2001) have reported, for example, the presence of *P. falciparum* translationally controlled protein (TCTP), which is a homolog of the mammalian histamine-releasing factor (HRF), which causes secretion of histamine from basophil and IL-8 from eosinophils, whereby histamine, IL-8 and eosinophil have been observed to be elevated in patients with falciparum malaria. Kurtzhals *et al* (1998) have also shown, an increase in eosinophil counts in asymptomatic *P. falciparum* patients, and a decrease in patients with cerebral malaria, or uncomplicated malaria. Like in *P. falciparum* malaria, eosinopenia (eosinophil count < 40/µl) (Simmon, 1997), have also been reported during *P. vivax* malaria (Lee *et al.,* 2001). However, a remarkable eosinophilic response has been observed after antimalarial therapy (Camacho *et al.,* 1999). The decrease of eosinophil count in the peripheral blood of malaria patients may occur due to migration of these cells to inflammatory sites (Simmon, 1997). However, others have explained the low eosinophil count to be due to tissue sequestration and destruction rather than decreased production. This is because, the level of cationic protein of eosinophil and eosinophil protein which are indicators of eosinophil activation have been observed to be higher in acute and severe malaria, despite the decrease of eosinophil cell count in the peripheral blood (Kurtzhals *et al.,* 1998).

Neutopenia (neutrophil count < 2000/µl), is also common during overwhelming acute infections resulting from bacteria, viruses and the protozoa such as *Plasmodium* and *Leshmania* species whereby a large number of neutrophils are in demand so that the bone marrow storage pool can become exhausted even in the face of increased production (Simmon, 1997). For this, an increase in polymorphonuclear cells has been observed, for example, in the liver of mice infected with live sporozoites of *P. yoelii* (Faure *et al.,* 1995).

Moreover, there is an occurrence of abnormal increase of the granulocytes in the circulating blood, which is usually evaluated relative to age, sex and race-matched individuals living in the same locality (Jande, 1987). Thus, while neutrophilia is usually occurs due to acute infections due to *P. falciparum* (El-Shoura, 1993), bacteria and viruses and also due to other non-microbial agents (Simmon, 1997), eosinophilia is caused due to tissue parasites (filariasis and malaria) and intestinal parasites (tape worms) and also during allergic conditions; but basophilia the principal cause of which is inflammation, is less common during variety of infections (Simmon, 1997).

It has also been shown that pathogenesis as well as the disease outcome of malaria is highly dependent on several factors. Local factors such as geographical location that determine the level of endemicity (Trape *et al.,* 1994); host genetic factors which include defects in the synthesis of α- and β-globin chains (Allen *et al.,* 1997), glucose-6-phosphate dehydrogenase (G6PD) deficiency (Martin, 1994), the HLA (Hill *et al.,* 1991) and TNF-α promoter region (McGuine *et al.,* 1994) genes, and MHC-II antigens (Jepson *et al.,* 1997) contribute to the outcome of malaria infection. Moreover, Parasite factors such as parasite species and strains, and stages of parasites (Chotivanich *et al.,* 2000; Ntoumi *et al.,* 1996), which have different virulence and disease patterns (Gupta *et al.,* 1994), due to their difference in the induction of pathogenic cytokines (Marsh, 1992) also play a role in malaria pathogenesis.

Therefore, this study was initiated to define the overall immunological, parasitological and haematological conditions in acute *P. falciparum* and *P. vivax* malaria infections around Wonji, which is a hyperendemic area.

2. OBJECTIVES

The general objective of the study was to assess the absolute counts and proportions of peripheral blood mononuclear cells (PBMCs) and granulocyte subset populations during acute *P. falciparum* and *P. vivax* malaria infections.

The specific objectives of the study are:

a) To assess and compare the absolute numbers and proportions of PBMCs (CD4+, CD8+, B, NK, T (CD3+), total lymphocytes and monocytes) and granulocytes (basophils, eosinophils and neutrophils) in the peripheral blood of acute *P. falciparum* and *P. vivax* malaria patients and healthy individuals.

b) To determine possible correlation between the absolute counts and proportions of PBMCs and granulocytes with gametocyte and asexual stage parasitemia of *P. falciparum* and *P. vivax*.

c) To determine asexual stage and gametocyte densities of *P. falciparum* and *P. vivax*, in patients with and without HIV co-infection.

d) To assess the haemoglobin, haematocrit and platelet levels in *P. falciparum* and *P. vivax* malaria patients, with and without HIV co-infection.

3. PATIENTS AND METHODS

3.1. The study area

The study was done at Wonji Sugar Estate, located along the Awash River at about 15 km from the city of Nazareth, East Oromia Zone and 114 km South East of Addis Ababa. The average elevation of the study area is 1500 meters above sea level and its climate characteristic of tropical low lands. The hottest months are March to May. Annual total rainfall is around 8324 mm and 65 % of the total falls within the months of June to September. The rest of the year is usually dry with some precipitation in April and May. The topography and climatic conditions of the area creates a suitable condition for malaria transmission (Mebrahtu, 1967). According to recent investigations carried out by the Wonji Hospital (May-June, 2001), malaria infection is still rampant despite the intense insecticide spraying. They have recorded a malaria prevalence of 8.2% (3.8% *P. falciparum* and 4.4% *P. vivax*) (unpublished data).

3.2. The study population

Adults of both sexes suspected of acute malaria symptoms and who came to attend the health centers; Wonji Hospital, Wonji Shoa and Wonji polyclinics were included in this study. As a criterion for inclusion, malaria positive subjects with either the asexual (asexual) or sexual (gametocyte) stages of *P. falciparum* (pf) and/or *P. vivax* (pv) malaria were included in the study (malaria group). Informed written consent was obtained from all participants. Clinical and demographic data were recorded for each patient by using a standard questionnaire. Individuals with temperature >= 37.5 ^{0}C were considered to be febrile. All malaria cases were treated with the standard drug regimens. Age and sex matched HIV negative and positive adult volunteers without detectable parasitemia and who are living in the same area were included as controls

3.3. Sample collection and processing

This study was done for one year from November 14 2001 to November 15 2002. Five ml venous blood was collected by venepuncture into Ethylendiaminetetraacetate (EDTA) tubes from the study subjects and controls. Thick and thin blood films for malaria were prepared by experienced microscopists. All the blood samples, the blood films and the record forms were brought to ENARP laboratory in Addis Ababa. The slides were cross-checked for species identification and parasite determination. If blood films prepared in the field were not good enough to read, new slides were prepared from the blood in EDTA tube to confirm findings. Aliquate of blood was taken from the whole blood sample for FACS and hematological analyses. The remainder of the whole blood was centrifuged at 1180 rpm/10 minutes to separate the plasma from the blood cells. This was followed by HIV screening. After the plasma was centrifuged again at 2500 rpm/10 minute to remove some debris, both the packed blood cells and the plasma were stored at -80 ^{0}C in deep freezer for further laboratory analyses.

For the detection of malaria infections and determination of parasite densities, blood samples were collected from finger prick. Thick (for parasite density count) and thin (for species identification) blood films were stained with 3% Giemsa and examined for the presence of *P. falciparum* and *P. vivax* malaria with a 100x oil immersion objective and 10x ocular. At least 200 microscopic fields were scanned before

regarding a smear as negative. Parasites were counted against 300 WBCs. The asexual stage and gametocyte density per microliter of blood was then calculated by multiplying the parasites counted by WBC/300 (Trape, 1985).

Moreover, the absolute counts of WBC per µl of whole blood, hemoglobin concentrations, platelet and haematocrit results were obtained by using a Coulter counter T540 (Coulter Electronics, Florida, USA) which dilutes 10 µl of whole blood and gives results in printouts.

Detection of antibodies for HIV infection in the plasma was done by using Determine (Abbott Laboratories, Tokyo, Japan). After removing the protective foil cover from the sample pad, 50µl plasma was added and then results were read after 15 to 60 minutes. Red bars on the patient and control windows indicate HIV positive results. Furthermore, Enzyme linked immunosorbant assay (ELISA) (Vironostika-HIV Uni-Form II, Organon Teknika, the Netherlands) was also used as a confirmatory test. After fitting the stripholder with the required number of microELISA strips, 100 µl specimen diluent was added in to the assigned wells. After incubation for 60±5 minutes at 37±2 ^{0}C, each well was washed and soaked six times with phosphate buffer. After adding 100 µl TMB substrate in to each well, strips were incubated again at 18-25 ^{0}C for 30 minutes. Reaction was stopped by adding 100 µl of sulpheric acid to each well. Finally, results were read by ELISA reader at 450±5 nm. Moreover, reactive samples were confirmed again by Western Blot (WB) (Gee labs Diagnostics, Singapore). After required number of strips were placed in to each well of the try, 2 ml of diluted wash buffer was added to each well and incubated for 5 minutes at room temperature. 2 ml of blotting buffer was added, followed by 20 µl of plasma and control to appropriate wells. The try was covered and incubated for one hour at room temperature on the rocking platform. After aspirating the mixture from the wells, each strip was washed with 2 ml of diluted wash buffer. Then, after adding 2 ml of working conjugate solution, it was incubated for 1 hour at room temperature. After washing, 2 ml of substrate solution was added and incubated again for 15 minutes. After rinsing the strips with water to stop the reaction, they were removed onto paper towels and let to dry. Then, results were observed as bands on the strips.

Immunophenotyping to identify and differentiate subpopulations of leucocytes, based on their surface antigens was done by flow cytometry. That is, whole blood samples were stained with a combination of three fluorochrome-labeled antibodies, such as CD3FITIC/CD4PE/CD45PerCP. To 10 µl of the MoAbs in test tubes, 50µl of whole blood was added and mixed by vortexing. Samples were then incubated for 15 minutes in the dark at room temperature. To lyse the red blood cells, 450µl of fluorescent activated cell sorter (FACS) lysing solution (50% diethylene glycol and 15% formadehyde; Becton Dickinson, San Jose, CA) was added, and after vortexing, it was incubated for another 15 minutes at room temperature.

To enumerate the absolute counts and percentages of the lymphocyte subsets in the peripheral blood, three-color flow cytometry analysis panel was applied using the FACScan flow cytometry (Becton Dicknson, San Tase, CA). Data was analyzed by using the software Paint-A-Gate which is used to calculate the percentages of lymphocytes, monocytes, neutrophils, eosinophils and basophils; followed by cell quest MultiSET that enumerates absolute counts and percentages of lymphocyte subsets. As a quality control, it was checked if the total sum of the percentages of CD4+ and CD8+ T cells are within the range of the ±10% of the average percentage of CD3+ cells. Moreover, total lymphocyte count was also maintained within the range of ±5 of 100%. However, absolute counts of monocytes and granulocyte subsets were recalculated by multiplying the proportions of monocytes and granulocyte subsets by the total count of leucocytes.

3.4. Data analysis

All FACScan, haematological and parasitological results are presented as medians, percentiles or means with their standard deviation, or as proportions (percentages). Statistical analyses of the data were performed by using STATA (Stata Corporation, Texas) and SPSS (SPSS Inc. Chicago, IL, USA) softwares. Results were compared between groups by using the non-parametric statistics (Wilcoxon rank-sum test), Student's t test, Chi-square or ANOVA as found appropriate. The degree of correlation between variables was evaluated by the non-parametric Spearman correlation analysis. For all statistical tests, a two-tailed P value < 0.05 was considered significant.

3.5. Ethical consideration

Specimens obtained under the Ethio-Netherlands AIDS Research Project (ENARP) HIV-1 cohort study were both institutionally (Ethiopian Health and Nutrition Research Institute, EHNRI, and Biology Department, Addis Ababa University) and nationally (National Ethical Clearance Committee, Ethiopian Science and Technology Commission, ESTC) cleared.

4. RESULTS

4.1. Characterization of the study participants

A total of 253 adults of both sexes were included in the study. Among the study participants 166 subjects had acute malaria infections, of which 82 (49.4 %) were *P. falciparum* (pf) malaria patients, 81 (48.8 %) were *P. vivax* (pv) patients and 3 (1.8 %) were infected by both *P. falciparum* and *P. vivax*. The mean body temperature was not significantly different in *P. falciparum* (38.1 ^{0}C) and in *P. vivax* (37.9 ^{0}C) malaria patients (Table 1). Furthermore, 8 (4.8 %) of the malaria patients were infected by HIV (3 by *P. falciparum* and 5 by *P. vivax*). Mean body temperature was not significantly different in pf and HIV co-infected (38.7±0.6) than in pv and HIV co-infected patients (37.9±0.4) (Table 2). 46 adult healthy individuals of both

sexes (Table 1), and 41 HIV positive malaria negative adult individuals of both sexes (Table 2) were included as controls.

Table 1. Baseline characteristics and mean values (± SD) of haemoglobin (Hgb), Haematocrit (Hct) and platelet (Plt) in HIV negative (HIV-) malaria patients (pf = falciparum, pv = vivax) and HIV- controls.

Participant Characteristics	Study participants				P value	
	Pf (n=79)	Pv (n=76)	pf + pv* (n=3)	Controls (n=46)	a	b
Age	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 (^{0}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
Hgb (g/dl)	13.6±2.2	13.7±2.1	13.7±3.4	15.2±2.1	<0.0001	<0.0001
Hct (%)	41.0±6.6	42.4±7.5	41±11.5	47.2±5.6	<0.0001	<0.0001
Plt (x10^3)	87.6±57.3	96.4±47.4	78.3±27.1	188±54.9	<0.0001	<0.0001

Key:

a= p value, when pf compared with controls; b= when pv compared with controls

• Significant difference of WBC between pf and pv (P = 0.031), using Student t test

T (^{0}C)= Body temperature; NA = not available

* Due to small sample size, pf+pv samples were not compared with controls or malaria group

Table 2. Patient characteristics and mean values (±SD) of Haemoglobin (Hgb), Haematocrit (Hct) and Platelet (Plt) in HIV positive malaria patients (pf = falciparum, pv = vivax) and HIV positive controls.

Participant characteristics	Study participants			P value	
	Pf (n=3)	Pv (n=5)	Control (n=41)	a	b
Mean age	34 ±5.3	33.4 ±4.9	36.9 ±6.0	0.413	0.209
T (^{0}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	0.001	0.152
CD4+ (per µl)	78.6±20.9	225.2±116.5	274.0±196	0.023	0.929
Hgb (g/dl)	10.6±4.5	11.6±2.8	13.9±1.7	0.234	0.069
Hct (%)	33±13.5	35.8±7.8	42.2±7.1	0.213	0.062
Plt (x10^3)	24±12.7	112±61.5	175.2±59.7	0.018	0.048

a= P value, when pf compared with controls; b= when pv compared with controls

T (^{0}C)= Body temperature; NA = not available

Pf and pv were compared with controls using Wilcoxon rank-sum test

4.2. Parasitological findings

The onset of malaria transmission was related considerably with rainfall. However, no significant difference was found between *P. falciparum* (50.3%) and *P. vivax* malaria (49.7%). From the 166 total *P. falciparum* and *P. vivax* malaria cases, 16.8 % occurred in the dry season (January – May), of which 89.3 % was due to *P. vivax,* 7.1 % due to *P. falciparum,* and 3.6% was due to mixed infections due to both *P. falciparum* and *P. vivax* species. During the rainy season (June – August), the overall malaria infection rate went up to (40.4 %), of which 53.7 % was due to *P. falciparum* and 46.3 % due to *P. vivax*. During the post-rainy season (September – December), 42.8 % of the total malaria infection was recorded, of which 62.0 % was *P. falciparum,* 35.2 % P. *vivax* and 2.8% was due to infection by both parasite species. In all cases, there was significant seasonal variation between the two malaria

infections (P<0.001), and within the onset of *P. falciparum* (P < 0.0001) and within *P. vivax* (P < 0.001) infections.

Asexual and sexual stage parasitemia of *P. falciparum* and *P. vivax* infections have been also assessed in this study. In HIV negative subjects, the mean asexual stage parasite density/µl of *P. falciparum* was significantly higher (P=0.0006) than that of *P. vivax*. Whereas the gametocyte densities of *P. vivax* was higher than that of *P. falciparum* (P< 0.0001). A significant increase in asexual stage parasitemia was observed among individuals co-infected by both *P. falciparum* and HIV when compared with those infected with *P. falciparum* alone (P = 0.031). On the other hand, the increase in asexual and sexual parasitemia in the HIV positives than HIV negative *P. vivax* malaria patients was not significant. Also, no variation was found in asexual and sexual parasitemia between HIV positive *P. falciparum* and *P. vivax* malaria patients (Table 3).

Taking seasonal effects in to account, asexual stage parasitemia was observed to be higher in *P. falciparum* than in *P. vivax* in the rainy season (pf= 25069 vs pv= 6072/µl of blood; P=0.017) and post-rainy season (pf=13471 vs pv=5914/µl of blood; P=0.003), with no variation in the dry season. However, gametocyte density was higher in the rainy (pv= 87 vs pf= 7/µl of blood; P=0.012) and post-rainy seasons (pv=169 vs pf= 15/µl of blood; P=0.0007) in *P. vivax* than in *P. falciparum*.

Table 3. Mean values (±SD) of the asexual stage and gametocyte densities (log10 density/µl of blod) of *P. falciparum* (pf) and *P. vivax* (pv) with and without HIV co-infection

Patients	Parasite density	
	Trophozoites	Gametocytes
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)	2.6 (± 0.77)

** P = 0.0006, variation in asexual 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 asexual stage of pf compared with pf + HIV; using Wilcoxon rank-sum test;

n= number of subjects

4.3. Absolute counts and proportions of PBMCs and granulocytes in P. falciparum *and* P. vivax *malaria patients and healthy controls*

When compared with their age and sex matched controls, the mean count of total WBC was significantly lower in *P. falciparum* malaria patients (P = 0.015) but not in *P. vivax* malaria patients. Moreover, total WBC count was lower in *P. falciparum* than in *P. vivax* malaria patients (P=0.031) (Table 4).

Almost two-fold decrease in the absolute counts of all CD4+, CD8+, B cells, T (CD3+) and total lymphocytes was found in falciparum patients when compared with the controls (P<0.0001). Likewise, a significant decrease in CD4+, CD8+, B cells, T (CD3+) and total lymphocytes was also observed in *P. vivax* malaria patients (P<0.0001). The CD4/CD8 ratio was higher in falciparum patients (P=0.044) but showed no difference in *P. vivax* infection. The only lymphocyte subset which has no significant difference in both falciparum and vivax malaria groups, when compared to healthy controls, was the absolute counts of NK cells (Table 4).

Comparison of *P. falciparum* and *P. vivax* malaria groups showed no variation in the absolute counts of any of the lymphocyte subsets, except in B cell counts, which was lower during *P. falciparum* infection (P=0.028).

It is only the proportion of B cells and total lymphocytes that were significantly lower than that of the healthy controls in both *P. falciparum* (P=0.008, P<0.0001) and *P. vivax* (P=0.002, P<0.0001) malaria infections. No difference was found in the CD4+, CD8+ and CD3+

proportions. On the other hand, NK%, was increased in falciparum patients (P=0.005) but was not significantly different during *P. vivax* malaria.

No significant difference in the proportion of CD4+, CD8+ and total lymphocytes was found between *P. falciparum* and *P. vivax* infections, whereas an increase in NK % (p=0.030) and a decrease in CD3+ % (p=0.029) during *P. falciparum* malaria was observed when compared to *P. vivax* was.

The picture of monocyte count following malaria infection was different from that of lymphocytes. That is, monocyte count was increased significantly in the peripheral blood of *P. vivax* malaria patients (P=0.007) than in controls, while in *P. falciparum* malaria patients the increase was insignificant. However, percentage composition of monocytes was significantly increased in both *P. falciparum* (p=0.0002) and *P. vivax* (p=0.0006) infected individuals than in the controls.

However, neither in the absolute count nor in the percentage composition of monocyte was variation found between the two malaria groups (Table 4).

Table 4. Mean values (±SD) of the absolute counts/µl of blood (n) and proportions (%) of the total WBC (x 10^3) and PBMC populations in *P. falciparum* (pf) and *P. vivax* (pv) malaria patients and healthy controls.

Cell types	Study participants		
	P. falciparum (N=71)	*P. vivax* (N=70)	Controls (N=46)
Total WBC (n)	4.8 (±1.7) †*	5.5 (±2.0)	5.7 (±1.8)
Lymphocytes (n)	940 (±472)**	1078 (±583)**	1815 (±729)
(%)	20.9 (±11.5)**	20.4 (±9.2)**	33.1 (±9.7)
Monocytes (n)	482 (±236)	549 (±300)**	420 (±133)
(%)	10.3 (±4.3)**	10.6 (±5.2)**	7.7 (±2.0)
CD4+ (n)	387 (±206)**	455 (±240)**	691 (±234)
(%)	41.9 (±12.1)	43.1 (±8.7)	40.3 (±9.7)
CD8+ (n)	297 (±203)**	336 (±200)**	643 (±482)
(%)	29.6 (±10.8)	32.6 (±9.8)	33 (±11.5)
CD19+ (n)	61 (±39) †**, (N=26)	86 (±56)**, (N=43)	192 (±98)
(%)	8.2 (±3.7)**,(N=26)	7.9 (±4.2)**, (N=43)	10.7 (±3.6)
CD16+56 (n)	156 (±157), (N=26)	180 (±176), (N=43)	222 (±141)
(%)	17.6 (±9.5) †**, (N=26)	13.4 (±8.9), (N=43)	12.3 (±5.9)
T (CD3+) (n)	701 (±378)**	819 (±404)**	1379 (±607)
(%)	73.3 (±15) †	77.9 (±7.8)	75.7 (±6.9)
CD4/CD8	1.67 (±1.09)*	1.49 (±0.68)	1.32 (±0.55)

When pf or pv compared with controls: ** = $p<0.01$ (highly significant); * = $0.01<p<0.05$ (Significant difference), using Student t test

When pf compared with pv: † = Significant difference, using Student t test; and Wilcoxon rank-sum test for CD19+ and CD16+56 comparisons

N = number of subjects

However, variation in lymphocyte and monocyte cell counts between *P. falciparum* and *P. vivax* malaria patients was observed only when seasons are taken into account. Thus, a significant decrease in the mean values of CD4+ (P=0.032), CD8+ (P=0.036), CD3+ (P=0.024), total lymphocytes (P=0.018) and monocytes (P=0.003) in *P. falciparum* malaria than in *P. vivax* malaria patients was found in the rainy season but not in the dry and post-rainy seasons.

Using one-way ANOVA, the absolute counts of CD4+, CD8+, B cells, CD3+, total lymphocytes and monocytes were found to be lowest in the rainy season in *P. falciparum* patients (P=0.006; 0.005; 0.021; 0.005; 0.0001 and 0.007). However, in vivax malaria, it was only the total WBC and CD3+ cell count that was found to be lowest in the rainy season (P=0.043; 0.032). Seasonal variation of cell counts was higly related to the level of rain fall and asexual parasitemia, which were very high in the rainy season. Mean rainfall level was 97.7, 14.1 and 23.9 mm in the rainy, post-rainy and dry seasons. (Rainfall data was from Wonji agricultural reserch center, at Wonji Sugar Estate).

Effect of *P. falciparum* and *P. vivax* malaria infections on the absolute counts and proportions of granulocyte cells has been also assessed in this study. In *P. falciparum* and *P. vivax* infections, the picture of the absolute counts of granulocyte subsets in the peripheral blood was quite unique. That is, while eosinophil and basophil counts were decreased in *P.*

falciparum patients (p=0.0001; 0.004), no variation was found during *P. vivax* malaria infection when compared to healthy controls. On the other hand, neutrophil count was increased in both *P. falciparum* and *P. vivax* infections, although the difference was significant only in *P. vivax* infection (p=0.035) (Table 5).

However, the picture of proportion of granulocyte subsets was similar in both *P. falciparum* and *P. vivax* infections. Thus, relative to healthy controls, there was an increase in the proportion of neutrophils, and a decrease in eosinophils in *P. falciparum* (P=0.0001; <0.0001) and *P. vivax* (P<0.0001; 0.0006) infections. Although statistically insignificant, a decrease in basophil composition was also detected in both malaria infections (Table 5).

No variation was found in the absolute counts and proportions of granulocyte subsets between *P. falciparum* and *P. vivax* malaria infections. However, taking seasons in to account, basophil count was lower in *P. falciparum* than in *P. vivax* malaria patients (P=0.018) in the rainy but not in the dry and post-rainy seasons. Eosinophil count was lower for *P. falciparum* infection only in the rainy season (P=0.046), but not in *P. vivax* malaria patients.

Table 5. Mean values (±SD) of the absolute counts/µl of blood (n) and proportions (%) of granulocyte subsets in *P. falciparum* (pf) and *P. vivax* (pv) malaria patients and healthy controls of both sexes.

Cell types	Study participants		
	P. falciparum (N=70)	*P. vivax* (N=66)	Control (N=46)

Neutrophil (n)	3134 (±1605)	3490 (±1613)*	2897 (±1243)
(%)	62.2 (±16.5)**	62.7 (±14.1)**	50.4 (±11.8)
Eosinophil (n)	144 (±234)**	282 (±617)	458 (±379)
(%)	3.3 (±5.7)**	4.1 (±5.6)**	7.8 (±4.8)
Basophil (n)	34 (±16)**	39 (±23)	46 (±29)
(%)	0.8 (±0.35)	0.7 (±0.36)	0.9 (±0.49)

When pf or pv compared with controls; ** = P<0.01; * = 0.01<P<0.05, using Student t test
N = number of subjects;

4.4. Analysis of relationships between leucocyte populations and P. falciparum *and* P. vivax *parasite densities*

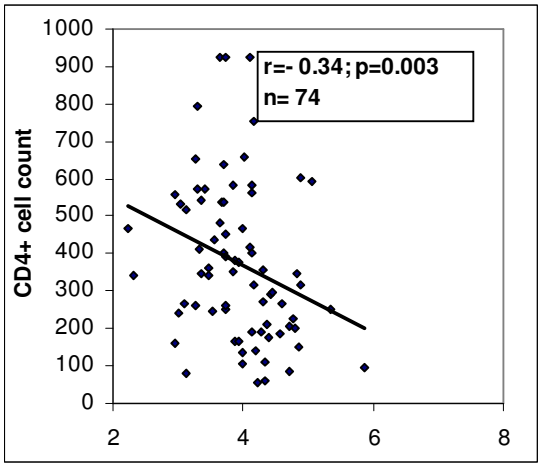
4.4.1. The relationship of absolute counts of WBC, PBMCs and granulocytes and asexual stage parasitemia

The decrease in the absolute counts of CD4+, T (CD3+) and total lymphocytes in the peripheral blood, in acute *P. falciparum* infections (Table 4), was found to be negatively correlated (P =0.003; 0.011 and 0.005) with the density of asexual stage parasitemia (Fig.1). Moreover, although the absolute counts of NK cells and monocytes in the peripheral blood of acute *P. falciparum* infections were not significantly different from that of healthy controls (Table 4), there was a negative correlation (P=0.026; P=0.034) with asexual stage parasitemia (Fig.1C & 2). Therefore, as the asexual stages of *P. falciparum* in the blood increases, there was a decrease in the absolute counts of lymphocyte subsets and monocytes in the peripheral blood of acute *P. falciparum* patients. On the other hand, B cells showed no decrease in their

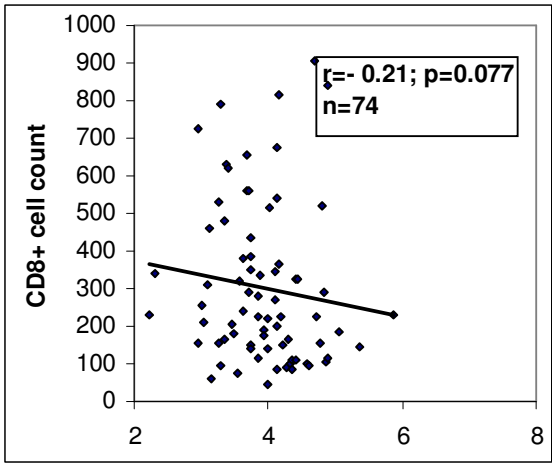
absolute number in *P. falciparum* infected individuals in relation to the asexual stage parasite densities (Fig. 1D).

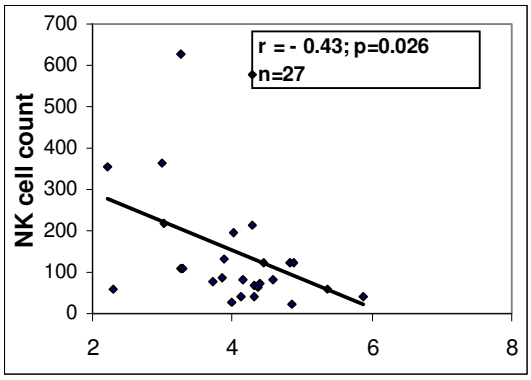
In *P. vivax* infections, a decrease in the absolute counts of CD4+, CD8+, B cells and CD3+ and increase in monocytes was observed (Table 4), although there was no significant correlation with the density of asexual stage in the blood. However, although insignificant, there was an inverse relationship between the asexual stage parasitemia and NK ($r = - 0.18$; $P=0.210$; $n=74$) and total lymphocyte ($r = -0.12$; $P= 0.320$; $n= 74$) counts.

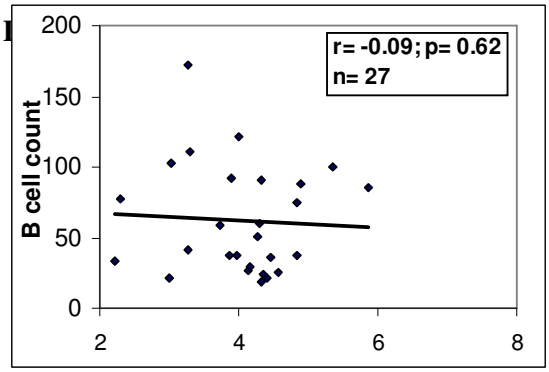
A

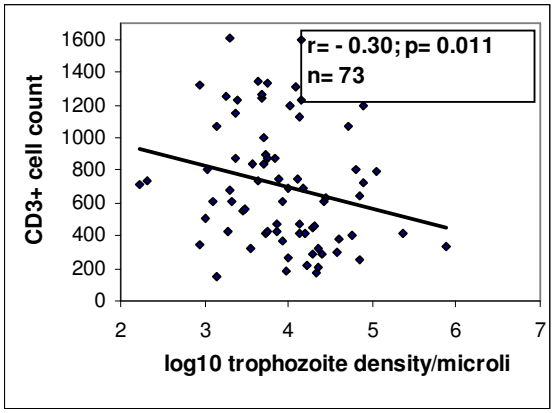

CD4+ cell count
r=- 0.34; p=0.003
n= 74

B


CD8+ cell count
r=- 0.21; p=0.077
n=74


NK cell count
r = - 0.43; p=0.026
n=27


B cell count
r= -0.09; p= 0.62
n= 27


CD3+ cell count
r= - 0.30; p= 0.011
n= 73
log10 trophozoite density/microli

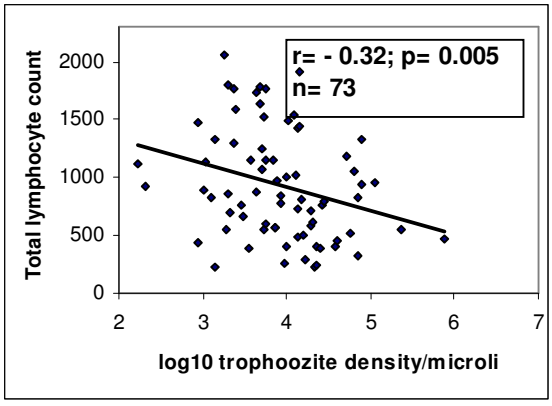

Total lymphocyte count
r= - 0.32; p= 0.005
n= 73
log10 trophoozite density/microli

Figure 1. The relationship of: (A) CD4+, (B) CD8+, (C) NK, (D) B cells, (E) CD3+ and (F) total lymphocytes with asexual stage parasitemia/µl of blood in *P. falciparum* infections. The correlation coefficient (r) and its statistical measure (p) and number of subjects (n) are indicated**.**

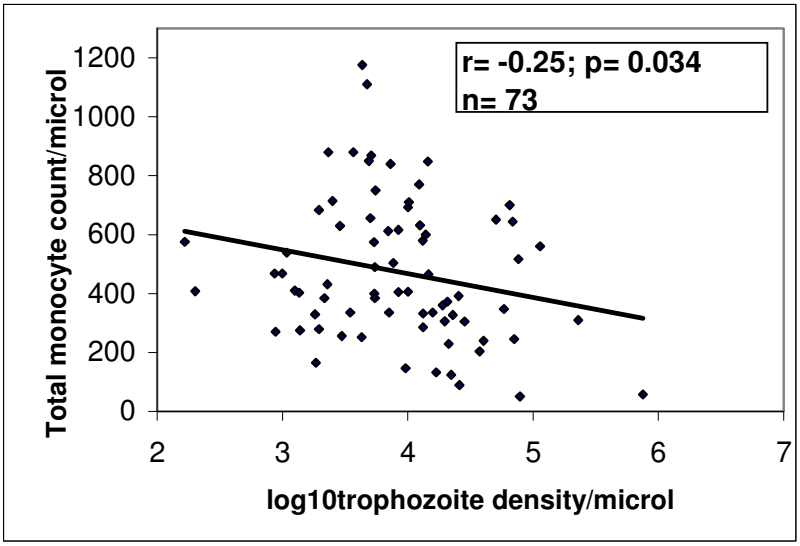


Figure 2. The relationship of monocyte counts with asexual stage parasitemia/µl of blood in *P. falciparum* infections.

Moreover, assessment of association of the absolute counts of granulocyte subset with the asexual stage parasitemia showed, the absolute counts of neutrophils to be positively correlated with the increase in the number of asexual stage parasitemia in the blood in both acute *P. falciparum* (P= 0.037) and *P. vivax* (P=0.001) infections (Fig. 3). On the other hand,

although insignificant, an inverse correlation was found with eosinophils (r = -0.15; P = 0.210; n= 73) and basophils (r = -0.12; P = 0.310; n= 73) in *P. falciparum* malaria infections. In *P. vivax* malaria infection, no correlation was observed between the asexual stage parasite parasitemia and the absolute counts of basophils while there was insignificant negative correlation with eosinophils (r = -0.16; P = 0.20; n= 74).

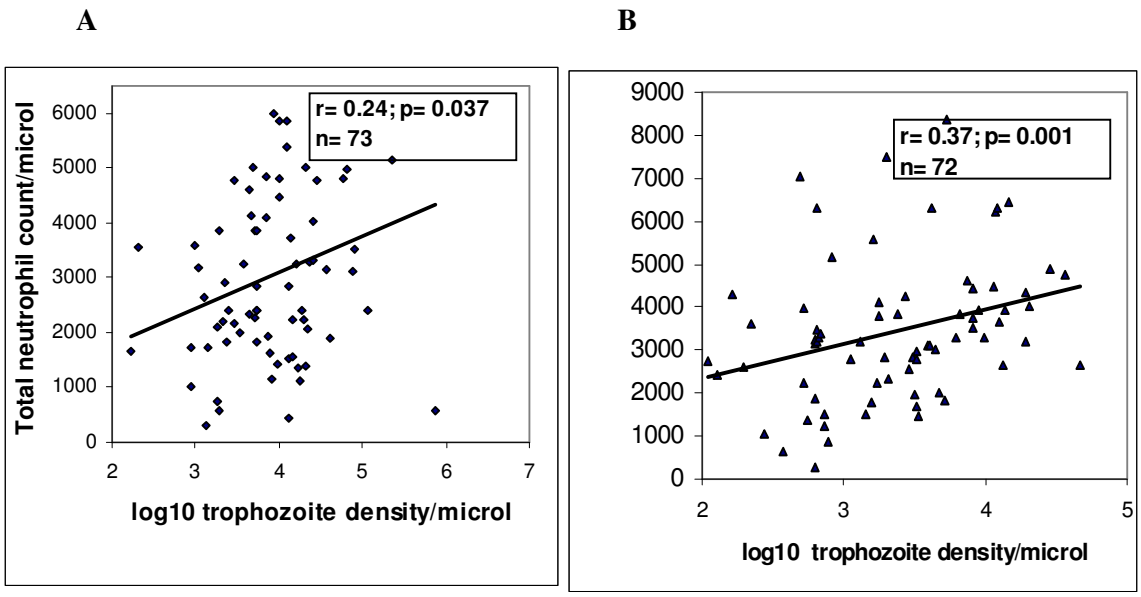


Figure 3. The relationship of total neutrophil counts with asexual stage parasitemia/µl of blood in: (A) *P. falciparum* and (B) *P. vivax* patients.

4.4.2. The relationship of proportions of PBMCs and granulocyte subsets and asexual stage parasite densities

The percentage composition of CD4+, CD8+ and CD3+ lymphocyte subsets in the peripheral blood had no correlation with asexual stage parasite density of *P. falciparum*. However, the decrease of the mean percentage of total lymphocytes in acute *P. falciparum* malaria infections was found to be inversely correlated (r= -0.37; P= 0.001) with the asexual stage parasitemia. Similarly, an inverse correlation was observed between monocyte count and the

asexual stage parasite densities (r = -0.43; P = 0.0002). Although not significant, similar inverse correlation was observed in the percentages of NK cells (r= -0.24; P= 0.23) (Table 6).

In *P. vivax* malaria, no correlation was found between percentage values of CD4+, CD8+ and B cells and asexual parasitemia. However, the significant decrease of the proportion of total lymphocyte count (Table 4), was negatively correlated with asexual stage parasitemia (r = -0.31; P = 0.007). Similar pattern was observed for NK percentages (r = -0.34; P = 0.020), although the mean percentage of NK in *P. vivax* infections was not significantly different from healthy individuals (Table 4). On the other hand, while the mean percentage of monocytes was significantly higher than that of the healthy individuals, it was found to decrease as the asexual stage parasitemia increases (Table 6).

In the correlation analysis of the granulocyte percentage composition with blood parasitemia, neutrophil proportion was positively and significantly correlated with asexual densities, in both *P. falciparum* (P=0.001) and *P. vivax* (P=0.007) infections. However, no correlation was observed with basophil and eosinophil proportions in both malaria groups (Table 6).

Table 6. Spearman correlation analysis (r and P values) between proportions (%) of PBMC and granulocyte subpopulations and asexual stage parasitemia/µl of blood in acute *P. falciparum* and *P. vivax* infections.

Cell types	r and P values	
	P. falciparum (n = 73)	*P. vivax* (n = 74)
CD4+	0.012 (0.204)	0.14 (0.250)
CD8+	0.046 (0.698)	0.03 (0.796)
CD19+	0.33 (0.085), (n=27)	-0.06 (0.688), (n=46)
CD16+56	-0.24 (0.226), (n=27)	-0.34 (0.021), (n=46)
T (CD3+)	0.13 (0.284)	0.24 (0.042) *
Total lymphocytes	-0.37 (0.001) **	-0.31 (0.007) **
Monocytes	-0.42 (0.0002) **	-0.15 (0.205)
Neutrophil	0.34 (0.001) **	0.32 (0.007) **
Eosinophil	-0.12 (0.310)	-0.23 (0.057)
Basophil	-0.15 (0.180)	0.02 (0.803)

** = P<0.01 (highly significant)

* = 0.01<P<0.05 (significant)

n= number of participants

4.4.3. The relationship of absolute counts of PBMCs and granulocyte subsets and gametocyte densities

Although non-significant, *P. falciparum* malaria gametocyte count showed an inverse relationship with PBMCs and granulocyte subsets (Table 7). Whereas in acute *P. vivax* infection, there was no correlation of gametocyte densities with CD3+ and total lymphocytes, and an inverse pattern of association, which is not statistically significant, was observed for

CD4+, CD8+, B and NK cells. A significant positive correlation was found only with the absolute numbers of neutrophils (P=0.029) and basophils (P=0.045) (Table 7).

Table 7. Spearman correlation analysis (r and P values) between absolute counts//µl of blood of the PBMC and granulocyte subpopulations and gametocyte densities//µl of blood in acute *P. falciparum* and *P. vivax* infections.

Cell types	r and P values	
	Falciparum (n = 11)	Vivax (n = 42)
CD4+	-0.24 (0.466)	-0.15 (0.330)
CD8+	-0.24 (0.466)	0.14 (0.371)
CD19+	-0.37 (0.264)	-0.13 (0.511)
CD16+56	-0.39 (0.236)	-0.28 (0.135)
T (CD3+)	-0.31 (0.355)	0.01 (0.930)
Total lymphocytes	-0.36 (0.355)	-0.04 (0.785)
Monocytes	-0.29 (0.385)	0.12 (0.454)
Neutrophils	-0.23 (0.501)	0.34 (0.029)*
Eosinophils	-0.31 (0.340)	0.24 (0.139)
Basophils	-0.20 (0.545)	0.32 (0.045)*

* = P<0.05 (significant)
n= number of participants

4.5. Haematological profiles in P. falciparum *and* P. vivax *infections with and without HIV*

When compared with healthy donors, haemoglobin (Hgb), haematocrit (hct) and platelet (plt) levels were decreased in patients infected with *P. falciparum* (P=0.001; P<0.0001; P<0.0001) and in *P. vivax* infections (P=0.0003; P=0.0002; P<0.0001), respectively. However, no

significant difference was observed between the two malaria infected groups in all haematological values. The decrease in the level of haematological values in both *P. falciparum* and *P. vivax* infections had no significant correlation with either asexual or sexual stage parasitemia.

On HIV and malaria co-infection, Hgb, plt and hct levels were lower in HIV positive *P. falciparum* malaria patients than in the HIV negative *P. falciparum* malaria patients. However, the difference was significant only in the case of plt ($p = 0.049$). Also, a non-significant decrease in Hgb, hct and plt levels in HIV positive than in HIV negative *P. vivax* malaria patients was observed (Table 2).

4.6. Fever and its association with asexual and sexual stage parasitemia

Fever was positively correlated with asexual stage parasitemia during *P. falciparum* malaria ($r= 0.40$; $P=0.0003$) (Figure 4). Although not significant, the same pattern was also observed in *P. vivax* infection ($r= 0.11$; $P=0.360$). On the other hand, there was a significant positive correlation between gametocytemia and fever in *P. vivax* malaria ($r=0.32$; $P=0.039$; $n=41$), while no such correlation was found in *P. falciparum* malaria infection.

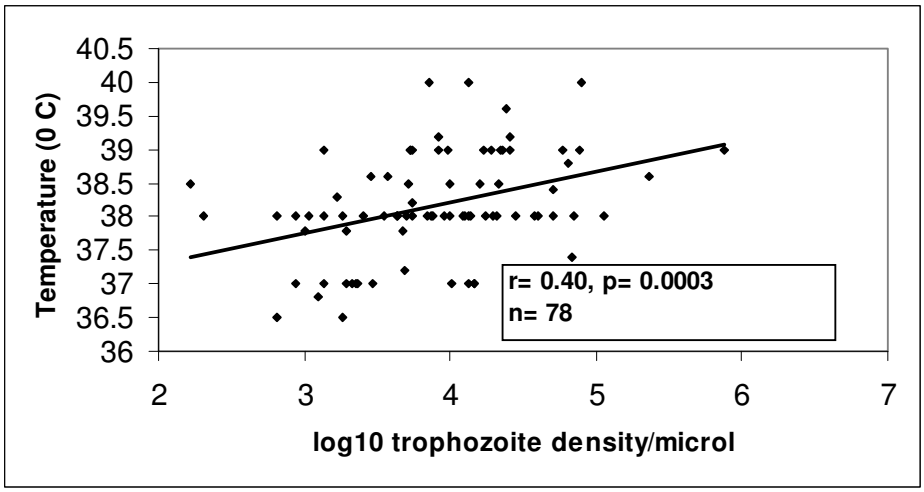


Figure 4. The relationship of fever with asexual stage parasite densities of *P. falciparum* malaria patients.

5. DISCUSSION

Out of the total microscopically diagnosed 166 malaria positive blood films, 82 (49.4 %) of the infections were due to *P. falciparum* infection, 81 (48.8 %) was due to *P. vivax* and 3 (1.8) due to mixed infection. Previous studies at Nazareth, which is 10 km from Wonji, have also showen 48.7 % *P. falciparum* and 51.3 % *P. vivax* infections (Worku *et al.,* 1997).

The 8 (4.8 %) of confirmed HIV positives among patients who presented with acute malaria is lower than that has been determined for the factory workers of the Wonji ENARP cohort (n=816) population which was 7.1% according to the 1997 survey (de Wit *et al.,* 2002), but was higher than values obtained by recent survey done in the general population of the Wonji town among the ages of 18-30, which was 2.1% (Neway Gesesse, EHNRI).

The seasonal variation in the prevalence of *P. falciparum* infection determined in this study was in agreement with pervious results from Nazareth, which showed high rate of *P. falciparum* infection in the post-rainy season (September – December) (Yohannes and Petros, 1996), but it was inconsistence with the infection rate of *P. vivax,* which was higher in the rainy season (June-August) in this study. This difference in the seasonal prevalence of *P. vivax* malaria between the two closeby locations (Nazareth and Wonji) could be due to difference in environmental factors (rain fall, temperature) or due to the difference in malaria control activities in the two locales.

The seasonal increase in the prevalence of *P. falciparum* and *P. vivax* infections in the rainy and post-rainy seasons can be related to the emergence and increase of the density of the female *Anopheles* mosquito vectors. This is because the female mosquitoes which usually mate only once but produce eggs through out their life need water to lay its eggs and for hatching and development of its larvae (Molyneux, 1994). Therefore, when the number of mosquito increases following the rainfall, the biting rate (number of mosquito bites per a given period of time) increases and the rate of transmission of malaria parasites also increases (Greenwood, 1997a). This could be the reason why *P. falciparum* and *P. vivax* infection increases in relation to rainfall. As relapsing mainly is the characteristic of *P. vivax* malaria (Parija, 1996), almost all malaria cases in the dry season were due to *P. vivax* and not *P. falciparum.*

The frequency of asexual stage and gametocyte parasites was also affected by season, which were high in the rainy and post-rainy seasons in both malaria types. While asexual stage and gametocytes were found throughout the year during *P. vivax* infection, almost no parasite stages of *P. falciparum* were observed in the rainy season. The observation of asexual stage of *P. falciparum* and *P. vivax* parasites in June, while rainfall started in March, indicates the range of time which the mosquito vector needs to develop from egg to adult, which takes about 7-13 days under a good condition (Molyneux, 1994). It also accounts for the duration of time between the malaria parasite inoculation up to the first disease symptom and the observation of the asexual and or gametocyte stages in the blood (Parija, 1996).

As reported previously from Nazareth (Worku *et al.*, 1997), asexual stage parasite densities of *P. falciparum* was higher than in *P. vivax* ($P = 0.0006$), however, at a higher magnitude of

parasitemia in Wonji. Comparing the two malaria species, asexual densities in the rainy and post-rainy seasons were higher in *P. falciparum* than in *P. vivax* malaria. The increase of asexual stage parasite densities in the rainy and post-rainy seasons is in agreement with a similar finding from Sri Lanka where a high transmission of *P.viax* during the rainy season (Ramasamy *et al.,* 1992) was reported. Reports from Kenya have also shown a positive association between exposure to transmission and subsequent density of asexual parasitemia (Jones *et al.,* 1997). Moreover, experimental studies done in Malawi have shown that, in seasons with high malaria transmission there was high mosquito infectivity rates, which result in high gametocytemic rates (Toure *et al.,* 1998). Another possible explanation which could be given for the increase of parasitemia in the rainy and post-rainy seasons is that when the rate of inoculation of new sporozoites increases, probably new strain of parasites (Ntoumi *et al.,* 1996) will be inoculated into the host, whereby the immune responses developed against the pervious parasite strains might not affect these new strains, so that they will successfully invade the liver and RBCs and will result in high parasitemia in the peripheral blood.

The asexual stage parasite density in HIV positive *P. falciparum* malaria patients was higher than in HIV negatives (P = 0.031), a finding similar to reports from other geographical locations. For example, a study done on pregnant women in Malawi had shown the prevalence and geometric mean density of parasitemia of *P. falciparum* at enrollment and at delivery to be higher in HIV positive than HIV negative women (Steketee *et al.,* 1996). Moreover, reports from Uganda have shown the risk of having parasitemia to increase with decreasing CD4+ counts, and this risk was significantly higher for HIV positive individuals (French *et al.,* 2001). Another study done in Kenya also has shown a marked increase of malaria infection, high parasite density, and an increase in fever in HIV positive women

compared to HIV-negative women (van Eijk *et al.,* 2003). This suggests that HIV infection increases predisposition to or aggravation of malaria infection.

The possible explanation which could be given for the increase of parasitemia in HIV positive than HIV negative malaria patients could be due to the depletion of CD4+ cells which are the target cells of HIV and which are known to play a major role in immunity against blood stage malaria parasites (Phillips *et al.,* 1997). Although the findings consistently showed that HIV infection seems to be associated with increase in asexual stage density in both *P. falciparum* and *P. vivax* malarias and gametocyte density in vivax malaria, a conclusive understanding of such possible interaction should be based on a longitudinal study by using a large sample size.

Contrary to the reports from other geographical locations was the finding that fever was not significantly associated with HIV and malaria co-infections in the present study. A study done in Uganda which involved adult male and female subjects had shown an increase in clinical malaria (fever) in HIV positive *P. falciparum* malaria patients, and this was associated with advanced immunosupression as measured by CD4 counts (Whitworth *et al*., 2000). A recent study done in Uganda again confirmed an increased rate of *P. falciparum* malaria fever with decreasing CD4 T cell counts in HIV positive adults (French *et al*., 2001). The disparity of the results could be due to the small sample of dually infected study participants in the present study.

The finding that total WBC count significantly decreases during *P. falciparum* malaria infection when compared to the healthy controls ($P = 0.015$), was in agreement with an earlier report from Nazareth (Worku *et al*., 1997). Similar findings have also been reported from

other geographical locations (Rzepczyk *et al.,* 1996; Church *et al.,* 1997; Oh *et al.,* 2001). Likewise, the lack of reduction in WBC count during *P. vivax* malaria was also in agreement with the report of Worku *et al* (1997) from Nazareth. This, together with the lower leucocyte count in *P. falciparum* than in *P. vivax* malaria patients may indicate that immunopathogenesis is more important in disease due to *P. falciparum* than in *P. vivax* malaria, since falciparum and *P. vivax* malaria infection act differently towards the components of WBC subsets.

A study done at Nazareth, by Worku *et al* (1997), which showed a significant decrease in CD8+, T (CD3+) and total lymphocytes in acute falciparum patients, was in agreement with this study. However, contrary to the present findings, they found no change in the absolute counts of CD4+, B and NK cell and in the percentages of NK, B cells and total lymphocyte. The difference could be due to the difference in the asexual stag parasitemia, which were 7299 [647-76461] and 5500 [2900-12700] (median and 95% percentile) per micro liter of blood at Wonji and Nazareth, respectively. This may be true because the results of this study have shown that the higher the asexual stage parasite densities the lower are the lymphocyte subset cell counts in the peripheral blood. Other workers have also reported lower absolute counts of CD4+, CD8+, CD3+ and total lymphocytes (Lsse *et al.,* 1994; Hviid *et al*., 1997; Lee *et al.*, 2001) as well as lower B and NK cells (Rzepczyk *et al.,* 1996; Lee *et al.,* 2001) during acute *P. falciparum* malaria. Different from this study, a decrease in the percentage of T (CD3+), CD4+ and CD8+ cells have also been reported elsewhere (Hiviid *et al.,* 1997). Therefore, even if there is a general consistency in the findings that malaria infection alters the lymphocyte cell counts in the peripheral blood, the extent of decrease and type of the cells to be altered however varies in different geographical locations, which could be due to the

difference in the immune status of the study subjects because of difference in the level of malaria endemicity (Trape *et al.,* 1994); and possible difference in the strains of the parasites (Chotivanich *et al.,* 2000), which may cause difference in the activation of the immune system (Marsh, 1992). The variations could also be due to the difference in the baseline values of the absolute counts of the immune cells. For example, it has been documented that the CD4+ count of healthy Ethiopians is lower than that of the Dutch (Tsegaye *et al.,* 1999). This study strongly suggests that while interpreting lymphocyte subset counts in acute *P. falciparum* malaria patients, geographical locations should be taken into account. In support of this suggestion is the report of Kassu *et al* (2001), which demonstrated differences in some lymphocyte subsets in two closeby geographical locales, Wonji and Akaki, which are only about 90 kms apart.

One explanation which could be given to the absence of significant change in the percentages of lymphocyte subsets in *P. falciparum* and *P. vivax* malaria patients compared to healthy controls, is the similarity in the rate of decrease of both the numerator (lymphocyte subset counts) and the denominator (total lymphocyte counts) at the same rate. For example, due to the insignificant change in the absolute counts of NK cells, and due to the significant decrease of total lymphocyte counts in falciparum malaria patients, NK% was significantly higher in *P. falciparum* malaria patients than in the controls (P=0.005). The lack of variation in the absolute counts of NK cells in both *P. falciparum* and *P. vivax* malaria infections, when compared to healthy individuals, has been explained by the rare exit of NK cells from the peripheral blood into lymph nodes or payers patches despite their expression of several adhesion molecules like the other lymphocyte subsets (Rosenberg *et al.,* 1998). An additional evidence to this effect is that, while significant increase in the number of monocyte/macrophages and CTL, cells that readily exit peripheral blood, was observed in the

intravillous space of placentas of acute malaria patients, which is related to the severity of infection, a complete absence of NK cells was also found in all placentas (Ordi *et al.,* 2001).

The increase in NK% in falciparum patients than in *P. vivax* malaria patients could be due to the decrease of total lymphocytes in *P. falciparum* than in *P. vivax* malaria patients, or it could be also the two malaria parasites act differently towards NK cells. Although no variation in the percentages of CD4+, CD8+ and total lymphocytes between *P. falciparum* and *P. vivax* malaria patients have reported by Worku *et al* (1997), CD3% was decreased in *P. falciparum* than in *P. vivax* malaria patients in this study ($P = 0.029$). This could be due to the relative decrease of the absolute counts of CD4+, CD8+ and total lymphocytes, which is known to take place during *P. falciparum* infection.

The seasonal variation seen on the lymphocyte subset counts between *P. falciparum* and *P. vivax* malaria infections, could be due to the variation of asexual density, which was highest in falciparum malaria patients in the rainy season, or may be due to other environmental factors. This is most likely, because there are monthly (van Rood *et al.,* 1991) and even daily (Jande, 1987) variabilities reported in immune cell counts. These findings therefore suggest that seasons should be taken into account when enumerating immune cells in malaria patients.

Except with B cells, an inverse correlation of the CD4+, CD8+, T (CD3+), and total lymphocytes and NK cell counts with the asexual parasitemia of *P. falciparum* was found ($P<0.05$). This is consistent with the report of Lisse *et al* (1994) who showed an inverse association of CD4% with *P. falciparum* asexual stage density. An inverse correlation of the CD4+ and CD8+ cell counts with the blood stage parasitemia has also been observed in BALB/C mice infected with *P. chabaudi* (Helmby *et al.,* 2000). The exceptional case in B cell

correlation in this study has also been supported by an experimental study conducted in mice, which showed that while the number of CD4+ and CD8+ T cells decreased in the peripheral blood 6-9 days after infection, and then increased in 12-15 days, B cell count was continually increased starting day 5 up to days 12-15 (Haemby *et al*., 2000). Moreover, in studies done to quantify the circulating memory B-lymphocytes reacting against *P. falciparum*, antibody secreting cells were detected in the peripheral blood in individuals infected 8 years ago, suggesting that the number of antimalaria antibody secreting cells may not necessarily be decreased, since the maintenance of B cell memory depends on the persistence of stimulating antigens (Migot *et al.,* 1995). Thus, findings of this study have indicated unique characteristics of B and NK cells during malaria infections, which need further investigation.

The effect of gametocyte parasitemia on the decrease of leucocyte counts or proportions was not as strong as with the asexual stage densities in *P. falciparum* malaria. This could be related to the production and the level of cytokines TNF-α and IFN-γ, which is related to the rupture of parasitized erythrocytes (Grau *et al.,* 1989) and the level of parasitemia (Bate *et al.,* 1998; Elhasson *et al.,* 1994). Hence, as RBCs that harbor gametocytes are not usually ruptured as the RBCs infected by asexual stagess, optimal cytokines might not be produced due to the effect of gametocytes. Therefore the decrease of the immune cells due to the effect of gametocytes cannot be similar to the effect of asexual stages. This is because cytokines TNF-α and IFN-γ, have been reported to induce the expression of adhesion molecules on lymphocytes and endothelial cells of lymph nodes (Udomsongpetch *et al.,* 1997) where they play role in the sequestration of the lymphocytes in the lymph nodes, and results in the decrease of the cell counts in the peripheral blood.

During *P. vivax* infection, no significant correlation of the absolute counts of lymphocyte subsets with asexual stage parasitemia in the blood, except for the inverse correlation of NK% (P=0.021) and total lymphocyte percentages (P=0.007), has been found. The absence of significant correlation in vivax malaria might indicate that the asexual stage parasitemia can be one among the other factors, which cause the depletion of the cell counts in the peripheral blood.

Therefore these findings indicate that although both *P. falciparum* and *P. vivax* infections cause significant decrease in lymphocyte cell counts, the rate or degree of influence of the asexual parasitemia is more strong in *P. falciparum* malaria than in *P. vivax*, which might be related to the level of asexual stage densities, or due to the antigenic variation of the two malaria asexual stages, which might activate the immune system differently.

There probably are two main possible potential mechanisms, which could explain the depletion of lymphocyte subsets from the peripheral blood in acute *P. falciparum* and *P. vivax* malaria patients: 1) sequestration of cells by trapping into the lymph nodes; 2) abnormal death of the cells through apoptosis.

In support of the sequestration hypothesis, several physiological and immunological evidences have been suggested. First, the repetitive cycle of migration of lymphocytes from blood to body tissue and back to the blood (Ebnet *et al.,* 1996), for example, is highly regulated by adhesion molecules and their receptors that are expressed on the surface of lymphocytes (Hogg, 1997; Bragardo *et al.,* 1997). Therefore, the emergence and disappearance of these molecules might prompt different movements of the cells from blood to lymphoid organs and can result in the alternation in the composition and absolute counts of

immune cells in the peripheral blood (Grassman and Herberman, 1997). Furthermore, the cytokines TNF-α and INF-γ have been observed to cause rapid depletion of lymphocytes from the blood through induction of expression of adhesion molecules and their receptors (Rossenberg *et al.,* 1998).

Thus, physiological and immunological mechanisms that occur during malaria infections, which could cause sequestration of lymphocyte subsets in the lymph nodes are known. For example, the level of cytokines (TNF-α and IFN-γ) which are known to induce the expression of selectins, integrins and chemoatractant chemokines (Rosenberg *et al.,* 1998) have been observed to correlate with malaria severity caused by *P. falciparum* malaria infection (Udomsogpetch *et al.,* 1997; Bate *et al.,* 1998;) and *P. vivax* (Elhasson *et al.,* 1994; Wijensekera *et al.,* 1996). Furthermore, an increase in the plasma level of the adhesion molecules, ICAM-1 and VCAM-1; and expression of ECAM-1 on the surface of endothelial cells were reported during malaria infection (Hviid *et al*., 1993; Elhasson *et al*., 1994). T cells have also been shown to recognize and to be activated by malaria antigens and then express surface integrins, which will increase their adhesiveness on endothelial cells (Prober *et al*., 1986).

In line with the immunological and physiological evidence, which support the lymphocyte sequestration theory during malaria infection, there are additional evidences which strengthen the hypothesis that the sequestration of these cells takes place in the lymph nodes. No response to antigen stimulation *in vitro* was observed for example in peripheral circulating cells in *P. falciparum* malaria infection (Ho *et al.,* 1986; Riley *et al*., 1988) and this unresponsiveness was suggested to be due to the disease episode which induces reallocation

of T cells away from the peripheral blood (Hviid *et al*., 1993). This hypothesis was supported by findings which show remarkable loss of T cells with high expression of LFA-1 (CD11/CD18) during acute *P. falciparum* malaria (Greenwood *et al.,* 1997b). Besides this, the expression of membrane-bound IL-2R (CD25) and ICAM-1 (CD54) were not revealed on cells (Hviid *et al.,* 1991; Chougent *et al*., 1992; Elhasson *et al*., 1994) in both cases indicating the withdrawal of T cells, which recognize parasite antigens from the circulation.

Depletion of CD4+ cells with high expression of the LFA-1 antigens from the peripheral blood was also observed during acute *P. falciparum* malaria (Hviid *et al*., 1993; Elhasson *et al*., 1994). Although reactive T cells can be detected in a splenic cell population at any time during and after infection, these cells were not detectable within the peripheral blood T cells during acute infection (Longhorne *et al.,* 1991). An experimental study done in BALB/C mice infected with *P. chabaudi* (Halmby *et al.*, 2000) has shown that when the number of CD4+ and CD8+ cells decreased in the spleen, a parallel increase of these cells in the blood was observed. Thus, when the number of CD4+ and CD8+ cells increase 3-5 fold in the spleen, 6-9 days after infection, their number was decreased in the peripheral blood. And then when CD4+ and CD8+ count decreased by 50 % in days 12-15 in the spleen, their number increases by 2-3 fold in the peripheral blood. The increase and decrease of these cells in either the blood or the spleen was opposite to the increase or decrease of the blood stage parasitemia. Therefore, this alternative increase and decrease of T cells in this experimental study may reflect migration of the cell to the peripheral blood leaving the lymph node (spleen) where they have been sequestered. This can also be related to the decrease of TNF-α level following the decrease of the blood-stage parasitemia. This is because, the level of TNF-α has been correlated with parasite densities and with severity of the illness during *P. falciparum* malaria

(Grau *et al.,* 1989). Moreover, the loss and reappearance of CD4+ and CD8+ T cells in the blood of acute symptomatic primary HIV/SIV infection in humans and monkeys was also found to correlate with the level of TNF-α and IFN-γ (Rosenberg *et al.,* 1997). Upon injection of TNF-α and IFN-γ in both man and experimental animals, rapid depletion of lymphocytes has also been observed (Wakelin, 1988).

The second hypothesis, which probably could explain the depletion of the lymphocyte subsets in acute malaria, is apoptosis, the programmed cell death. Supporting this hypothesis, earlier reports have shown the mean percentage of spontaneous apoptosis of mononuclear cells to be higher in patients with acute as well as chronic asymptomatic *P. falciparum* infection compared to age and sex matched controls (Toure-Bolde *et al.,* 1996). Moreover, in an experimental study done using the *Macaca* monkey model system, infected with *P. coatneyi* (Matsumoto *et al.,* 2000), peripheral blood CD4+ and CD8+ T cells were markedly decreased whereby parallel to the decrease of the T cells, the fragmentation of chromosomal DNA in peripheral blood mononuclear cells were detected during the terminal period of the infection. Moreover, soluble Fas ligands in the sera have also been increased. These and other pieces of evidence suggest apoptotic cell death to be responsible, at least in part, for the depletion of the CD4+ and CD8+ T cells. Furthermore, apoptotic processes during malaria infection at lymph nodes (the spleen) have been determined to be different from the apoptotic condition which takes place in the peripheral blood. In an experimental study done in BALB/C mice infected with *P. chabaudi* (Helmby *et al.,* 2000), starting on day 9 after the infection, the number of apoptotic cells, the majority of which are B cells and macrophages, was increased in the spleen, but also apoptosis of CD4+ and CD8+ cells was slightly increased. Furthermore, spleenic Fas-ligand expression was peaked at day 9 coinciding with the peak of apoptotic

death of the cells. Fas and Fas-ligand positive CD4+, CD8+, B cells and macrophages were also found. Thus, both the number and frequency of apoptotic cells in the spleen were higher during peak parasitemia of the blood-stage malaria parasites.

The mechanisms or major types of apoptotic cell death types so far known, which are activation induced apoptosis (Fas-Fas ligand mechanism) (Ju *et al.,* 1995), TNF-α (Albin *et al.,* 1993) and nitric oxide (Buttke *et al.,* 1994) mediated apoptosis, may be relevant to the decrease of the absolute counts of lymphocyte subsets in acute malaria infection. This is more likely since the level of the molecules TNF-α and nitric oxide has been observed to increase particularly during acute *P. falciparum* malaria (Grau *et al.,* 1989; Mendis & Carter, 1995).

The inverse correlation of the absolute lymphocyte cell counts with asexual stage parasite densities of *P. falciparum* could be related to the production of malaria toxins and parasite antigens, which in turn will activate more monocyte/macrophages to secrete more TNF-α (Grau *et al.,* 1989; Clark and Schofield, 2000) is to be expected. Thus, due to increase in TNF-α, more adhesion molecules and their receptors will be expressed on lymphocyte surfaces and endothelial cells of lymph nodes, such as the spleen, through the action of TNF-α (Udomsongpetch *et al.,* 1997; Bate *et al.,* 1998). It has also been reported that adhesion and receptor molecules can also be induced through antigenic activation by the asexual parasitemia (Elhasson *et al.,* 1994). The final outcome of this will be that more lymphocytes will remain trapped or sequestered in the lymph nodes, opposite to the increase in asexual stage densities.

The decrease of lymphocyte subset cell counts while asexual stage density increases during *P. falciparum* malaria, could also be due to the higher immune activation of the lymphocyte

subsets, so that more cells might die through Fas-Fas ligand apoptotic mechanism. Furthermore, more TNF-α and nitric oxide might also be produced, which are usually increased during severe *P. falciparum* malaria (Grau *et al.,* 1989; Clark and Schofield, 200), and more cells might die then through TNF-α and/or nitric oxide mediated apoptosis mechanisms (Albin *et al.,* 1993; Buttke *et al.,* 1994).

The distribution of monocytes is different from that of lymphocytes as reported by Rzepczyk *et al* (1996). However, in an experimental study done in BALB/C mice infected with *P. chabaudi* the number of monocytes was slightly increased in the peripheral blood during the acute phase of the infection (Helmby *et al.,* 2000). As monocytes do not recirculate like lymphocytes, but are recruited to the inflammatory sites when there is infection, the increase in monocytes in the peripheral blood in this study implies the input of cells from the storage pool (bone marrow) to the circulation (blood) is higher than the out put of the cells from the circulating pool to the marginating pool and then to the body tissue (Simmon, 1997). For instance, the increase of monocytes in falciparum patients was not as high as in vivax malaria patients, which indicates the severity of the disease in falciparum patients, so that when the disease is so acute more cells will leave the circulation which then will be replaced from the bone marrow, but ends up in the exhaustion of the stored cells in the bone marrow. This is supported by findings in this study that monocyte count of *P. falciparum* malaria patient in the rainy season is lower than in the dry and post-rainy ($p = 0.003$), and this was correlated with the highest asexual parasitemia in the rainy season, and the reverse was true in the vivax malaria even if it was not significant.

The decrease in eosinophil count observed in falciparum patients was also reported from other geographical locations (Rzepczyk *et al.,* 1996; Simmon, 1997; Kurtzhals *et al.,* 1998).

However, Lee *et al* (2001) have reported an increase in eosinophil numbers in *P. falciparum* malaria patients. The possible reason for the decrease of the number of eosinophils in the peripheral blood of malaria patients could be due to migration of the cells to inflammatory sites (Simmon, 1997), or it could be due to sequestration or destruction of the cells (Kurtzhals *et al.,* 1998).

On the other hand, while neutrophil counts were increased in both *P. falciparum* and *P. vivax* infections, it was significant only during *P. vivax* malaria ((P= 0.035). In agreement with this finding an increase in neutrophil count in malaria patients was reported by EL-Shoura (1993). Basically, the increase or decrease of the granulocyte subsets during acute infection is determined by factors such as rate of input of cells from bone marrow to the circulation; proportion of circulating cells to the cells attached on endothelial cells; and the rate of these cells leaving the blood to the body tissue (Simmon, 1997). For example, Faure *et al* (1995) have shown an increase in polymorphonuclear cells in the liver of the mice infected with live *P. yoelii* sporozoites . Thus, during overwhelming infection, since the cells will be in great demand, their number will increase in the blood circulation. The significant and positive correlation of neutrophil counts with asexual stage densities in both *P. falciparum* ($r = 0.24$; $p = 0.037$) and *P. vivax* (0.37; $p = 0.001$) malaria supports this argument, showing that when asexual parasitemia are increased in the blood, more neutrophils are needed to fight the infection, so that their number will increase in the peripheral blood. However, if this condition is continued, the storage or source in the bone marrow will be exhausted, so that the number of cells in the peripheral blood will be decreased.

Fever, was positively correlated with asexual stage densities of *P. falciparum* malaria (r=0.40; P=0.0003). In agreement with this study, other workers have shown direct correlation of fever

with *P. falciparum* asexual parasitemia (Lisse *et al.,* 1994; Greenwood, 1996; Roger *et al.,* 1996). The onset and increase of fever with asexual stage densities is associated with the blood stage of the parasite, and the rupture of the infected RBC (Parija, 1996). This is because when the infected RBC rupture, they release parasite antigens, erythrocytic debris and especially the protein toxin, glycosulphatidylinositol (GPI) which activates monocyte/macrophages to release the cytokine TNF which is the main causative agent of fever during *P. falciparum* and *P. vivax* infections (Greenwood, 1996; Clark and Schofield, 2000). Thus, the level of TNF has been known to be associated very strongly with asexual parasitemia parasitemia (Kwiatkowski *et al.,* 1990).

Haematological values, such as the level of haemoglobin (Hgb), haematocrit (hct) and platelet (plt) were significantly lower in both *P. falciparum* and *P. vivax* malaria patients compared to controls ($P < 0.0001$). But no difference was found between the two malaria groups. Other studies have shown anaemic conditions during *P. falciparum* and *P. vivax* malarias (Parija, 1996; McElory *et al.,* 2000), and also a decrease in platelet count during *P. falciparum* infection (Rzepczyk *et al.,* 1996). Even if the pathogenesis of anaemia in malaria is multifactorial and incompletely understood, factors such as mechanical destruction of the parasitized red cells, reduced RBC production in the bone marrow due to persistent low-grade parasitemia and cytokine production, phagocytosis of uninfected red cells, or due to auto-immune destruction are known to be involved (Parija, 1996).

The decrease in platelet is considered to relate with haemolysis of parasitized RBC, which then release adenosine diphosphate (ADP) which in turn activates the platelets, which will then be functional but will have short life span (Church *et al.,* 1997). Others have speculated

platelet-associated IgG and macrophage colony-stimulating factors to be associated with the decrease in platelet count (Yamaguchi *et al.,* 1997).

No seasonal variation in Hgb values was found in this study which is in agreement with the report by Baird *et al* (2002), although Rogerson *et al* (2000) have shown severe decrease in Hgb levels in the rainy season. This inconsistency in seasonal variation of the haematological results might indicate other factors such as life style for example, than the asexual parasitemia could be involved.

Haematological results in *P. falciparum* and *P. vivax* malaria patients with and without HIV co-infection has also been evaluated in this study. The decrease in the level of Hgb, hct and plt in malaria and HIV co-infection was in line with the report of Ayisi *et al* (2003) who showed that HIV positive women co-infected with malaria were two-fold as likely to have anaemia compared to HIV-negative women with or without malaria. The possible mechanism of the decrease of Hgb during HIV infection among other factors is through the bone marrow destruction of RBC progenitor cells by the virus and through gastrointestinal bleeding (Northfelt, 1994). Thus, the absence of significant decrease in the level of all haematological values during HIV co-infection may be due to the small sample size used. More samples should be included in the study to get more significant information on the outcome of co-infection of malaria and HIV.

No significant correlation was also found between the decrease of Hgb, hct and plt levels with asexual stage densities of *P. falciparum* and *P. vivax* parasites. Thus, parasite densities should be consider as one of the factors, which probably cause the decrease of haematological values during malaria. Parija (1996) has explained for example why Hgb decrease is not correlated with asexual parasitemia is because of phagocytosis of uninfected RBC by macrophages.

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

Only *P. falciparum* infection causes a decrease in total leucocyte count but not *P. vivax* malaria. Moreover, leucocyte count is lower in *P. falciparum* than in *P. vivax* malaria patients.

Both malaria parasites cause depletion in CD4+, CD8+, B, CD3+ and total lymphocytes, but cause no change in NK cell counts.

Although not highly significant, the effect of *P. falciparum* malaria on lymphocyte subset cell counts were higher than due to vivax malaria. However, B cell counts were significantly lower during *P. falciparum* malaria infection than during *P. vivax*. Seasonal variations in cell counts of CD4+, CD8+, NK, CD3+ and total lymphocytes were observed in *P. falciparum* malaria but not in *P. vivax* malaria. Both *P. falciparum* and *P. vivax* malaria had no influence on percentages of CD4+, CD8+, CD3+ cells, while B cell and total lymphocyte percentages were reduced in both malaria infections.

There is an inverse correlation between asexual stage parasitemia and the absolute counts of CD4+, CD8+, CD3+, NK and total lymphocytes in *P. falciparum* patients. On the other hand, only B cell counts did not have any association with asexual stage counts. However, asexual stage parasitemia of *P. vivax* did not have any association with the decrease of the lymphocyte subsets. Asexual parasitemia of both *P. falciparum* and *P. vivax* malaria did not have association with the proportions of all lymphocyte subsets except for an inverse correlation with lymphocyte proportions in both malaria infections. Gametocyte densities of *P.*

falciparum and *P. vivax* infections did not have significant association with the absolute counts or proportions of lymphocyte subsets.

Entrapment or sequestration of the cells in the deep cappilaries and apoptosis, are mechanisms that possibly account for the loss of lymphocytes from the peripheral circulation.

There is a negative correlation between ring/trophozite parasite densities of *P. falciparum* and the absolute counts and proportions of monocytes. *P. falciparum* and *P. vivax* malaria infections have different effects on the monocyte counts, which could be related to the severity of the disease which causes difference in the flow of cells from bone marrow to peripheral circulation and then to the body tissue.

P. falciparum and *P. vivax* infections disrupt the granulocyte subset counts, but in a different way. That is, while *P. falciparum* malaria causes a significant decrease in eosinophil and basophil counts, a significant increase in neutrophil count was found during *P. vivax* malaria only. There was positive correlation between asexual parasitemia and neutrophil counts in the peripheral blood in both malaria infections.

Based on the parasitological results of this study it can be concluded that, the overall annual prevalence of *P. falciparum* infection was not different from that of *P. vivax* infection. In *P. falciparum* infection, HIV co-infection was associated with increase in asexual stage density. Therefore, HIV infection may contribute to disease severity in malaria infections, and even may increase malaria transmission by contributing to the increase of gametocyte densities.

Both *P. falciparum* and *P. vivax* malaria infections cause a very significant decrease in haemoglobin (Hgb), haematocrit (hct) and platelet (plt) levels. Trophozoite and gametocyte

densities of *P. falciparum* and *P. vivax* had no significant association with the level of Hgb, hct and plt levels. However, HIV infection was associated with the decrease in Hgb, hct and plt levels in both malaria infections. This implies that treatment of HIV and malaria co-infected patients should consider all aspects relating to both malaria and HIV infection.

6.2. Recommendations

Since *P. falciparum* and *P. vivax* malaria infections cause a depletion of lymphocyte cell counts in the peripheral blood, great caution should be taken during enumeration of lymphocytes for diagnostic or research purposes. This should also be considered in studies aimed at evaluating the immune status of individuals or assessing immune responses to natural or artificial immunizations, in peripheral circulation. Granulocyte and monocyte disorders during *P. falciparum* and *P. vivax* malaria infections should also be considered while enumerating leucocytes for diagnostic or research purposes. The effect of HIV infection on haematological and parasitological values should also be considered during management of malaria patients co-infected with HIV.

As *P. falciparum* and *P. vivax* infections deplete lymphocyte subsets and also distort the normal profile of monocytes and granulocytes in the peripheral blood, the impact of these on co-infection by other infectious agents and on the clinical consequence of the concomitant infections must be investigated. To adequately establish the role of apoptosis in malaria-associated depletion of lymphocytes, its effect on the parasite and the host should be evaluated. Possible sequestration of the immune cells in the deep capillaries, and its effect on severity of *P. falciparum* and *P. vivax* malaria infections is another point that must be investigated. The clinical impaction of the inverse correlation of lymphocyte cell counts with

asexual parasitemia in *P. falciparum* but not in *P. vivax* infection should be explored. For a better understanding of the clinical effect of malaria–HIV interaction, a longitudinal study on a larger sample of study participants will be required.

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8. Appendices

Appendix 1. Parasitological and haematological results of malaria patients and controls.

NO	Lab_ID	HIV	Tem	Pf	Pv	pf+pv	Trophozoite density	Gametocyte density	Hgb	PLT	Hct
1	MAL2015	0	38	1	0	0	9125	0	-	-	-
2	MAL2036	0	38	0	1	0	164	27	12.9	179.0	40.0
3	MAL2013	0	38	0	1	0	1043	471	12.2	39.0	-
4	MAL0002	0	38	1	0	0	1350	0	-	-	-
5	MAL2007	0	38	1	0	0	655	0	-	-	-
6	MAL1004	0	37	1	0	0	0	205	-	-	-
7	MAL1005	0	37	0	1	0	205	0	-	-	-
8	MAL1006	0	38	0	1	0	226	0	-	-	-
9	MAL1007	0	37	1	0	0	647	0	15.5	94.0	48.0
10	MAL2010	0	38	1	0	0	1656	0	13.0	101.0	39.0
11	MAL2009	0	38	0	1	0	174	1056	12.6	114.0	38.0
12	MAL2011	0	38	0	1	0	654	634	12.5	123.0	-
13	MAL0003	0	38	0	1	0	651	0	12.4	86.0	40.0
14	MAL0004	0	38	0	1	0	219	0	11.3	204.0	36.0
15	MAL2041	0	38	0	1	0	6542	0	14.4	69.0	44.0
16	MAL1023	0	38	1	0	0	13253	0	10.4	102.0	30.0
17	MAL0010	0	38	1	0	0	1082	0	13.9	86.0	42.0
18	MAL2016	0	38	0	1	0	1212	0	11.0	81.0	34.0
19	MAL0011	0	39	1	0	0	18941	0	13.9	54.0	42.0
20	MAL1029	0	37	0	1	0	654	59	12.8	77.0	38.0
21	MAL1030	0	38	1	0	0	28393	0	14.2	32.0	44.0
22	MAL2050	1	38	0	1	0	28381	1018	10.6	79.0	32.0
23	MAL2051	0	38	0	1	0	18927	0	14.2	55.0	43.0
24	MAL2013A	0	38	0	1	0	829	0	-	-	65.0
25	MAL1031	0	38	1	0	0	19680	0	15.1	119.0	46.0
26	MAL1032	0	39	1	0	0	229170	0	16.3	47.0	48.0
27	MAL2052	0	38	1	0	0	999	0	14.3	110.0	40.0
28	MAL2053	0	39	1	0	0	76461	0	13.5	93.0	40.0
29	MAL2054	0	38	0	1	0	12041	905	13.3	56.0	38.0
30	MAL2055	0	38	0	1	0	8111	239	13.8	78.0	41.0
31	MAL2014	0	39	0	1	0	720	64	14.0	93.0	44.0
32	MAL2056	0	39	1	0	0	5394	0	8.6	112.0	26.0
33	MAL1033	0	38	0	1	0	2880	83	15.9	115.0	48.0
34	MAL2057	0	38	0	1	0	1448	0	9.1	73.0	27.0
35	MAL1034	0	39	0	1	0	1298	0	11.2	216.0	34.0
36	MAL1035	0	39	1	0	0	25374	0	13.2	83.0	40.0
37	MAL1036	0	38	1	0	0	20699	0	15.2	162.0	46.0
38	MAL2018	0	39	1	0	0	166	0	13.5	92.0	42.0
39	MAL1037	0	39	0	1	0	6193	0	13.4	85.0	39.0

40	MAL2058	0	37	0	1	0	3916	0	13.9	72.0	42.0
41	MAL2060	0	38	1	0	0	1851	59	10.2	59.0	30.0
42	MAL2059	0	38	0	1	0	1182	33	14.2	88.0	41.0

Appendix 1. (Continued)

43	MAL0014	0	38	0	1	0	9673	616	13.0	70.0	40.0
44	MAL2019	0	39	0	1	0	1750	707	13.1	12.0	39.0
45	MAL1038	0	38	1	0	0	7299	0	-	-	-
46	MAL1039	0	38	1	0	0	1958	0	16.0	32.0	48.0
47	MAL1040	0	39	1	0	0	22910	0	16.0	101.0	47.0
48	MAL1041	0	38	0	1	0	9050	0	13.3	96.0	40.0
49	MAL2061	0	37	1	0	0	68931	0	18.9	113.0	55.0
50	MAL1008	0	37	0	1	0	665	111	12.3	86.0	38.0
51	MAL2062	0	37	0	1	0	3252	0	15.4	110.0	44.0
52	MAL1042	0	39	1	0	0	9594	0	14.4	72.0	44.0
53	MAL1043	0	38	0	1	0	617	0	14.2	72.0	44.0
54	MAL1044	0	39	1	0	0	21200	0	17.3	94.0	53.0
55	MAL0005	0	38	0	1	0	128	0	16.3	67.0	51.0
56	MAL2020	0	37	1	0	0	1822	16	13.0	136.0	41.0
57	MAL0015	0	38	1	0	0	37464	0	14.7	92.0	45.0
58	MAL2065	0	37	0	1	0	3316	0	14.4	77.0	41.0
59	MAL2066	0	38	0	1	0	1914	49	12.0	181.0	35.0
60	MAL2067	0	37	1	0	0	10233	0	14.4	104.0	42.0
61	MAL2068	0	37	0	1	0	3146	0	15.0	141.0	45.0
62	MAL1046	0	38	1	0	0	40199	209	16.4	28.0	50.0
63	MAL0006	0	38	0	1	0	627	0	15.2	148.0	49.0
64	MAL1047	0	39	0	1	0	368	0	14.3	62.0	42.0
65	MAL2071	0	39	1	0	0	1378	0	13.1	47.0	37.0
66	MAL2072	0	38	0	1	0	1120	0	14.0	109.0	43.0
67	MAL2073	0	39	0	1	0	2074	103	14.3	47.0	42.0
68	MAL1050	0	39	1	0	0	15808	0	13.9	66.0	42.0
69	MAL1010	0	36	0	1	0	655	42	11.3	215.0	37.0
70	MAL2074	0	37	1	0	0	2156	0	14.2	78.0	42.0
71	MAL0016	0	39	1	0	0	58405	0	16.9	31.0	51.0
72	MAL2075	0	38	1	0	0	5024	0	12.4	72.0	38.0
73	MAL2076	0	37	0	1	0	12498	0	19.1	-	55.0
74	MAL0017	0	38	0	1	0	620	0	13.4	36.0	38.0
75	MAL2021	0	39	0	1	0	194	81	12.5	123.0	41.0
76	MAL0018	0	38	0	1	0	8091	53	18.4	62.0	54.0
77	MAL2077	0	39	1	0	0	8415	0	13.3	47.0	40.0
78	MAL1051	0	37	1	0	0	2973	0	11.3	86.0	32.0
79	MAL1052	0	38	0	1	0	4679	0	12.9	91.0	38.0
80	MAL2078	0	39	0	1	0	1780	0	13.5	100.0	43.0
81	MAL1012	0	38	0	1	0	8175	44	12.5	74.0	40.0
82	MAL0019	0	39	0	1	0	14608	0	16.7	77.0	50.0

83	MAL2079	0	38	1	0	0	5544	0	14.4	91.0	44.0
84	MAL1053	0	39	1	0	0	10043	0	12.0	102.0	35.0
85	MAL2080	0	37	1	0	0	870	0	11.6	77.0	34.0
86	MAL2081	0	38	1	0	0	6974	0	15.0	28.0	45.0
87	MAL2082	0	40	1	0	0	13272	0	14.5	72.0	42.0

Appendix 1. (Continued)

88	MAL1011	0	38	0	1	0	277	14	14.3	86.0	45.0
89	MAL2084	0	37	1	0	0	1362	0	10.6	27.0	31.0
90	MAL0020	1	38	1	0	0	50613	0	15.2	-	46.0
91	MAL2085	0	38	1	0	0	2513	203	11.0	98.0	34.0
92	MAL1054	0	38	1	0	0	4326	0	17.0	-	51.0
93	MAL2086	0	39	1	0	0	2874	0	17.2	-	52.0
94	MAL2023	0	38	1	0	0	7665	14	10.5	193.0	32.0
95	MAL2087	0	40	1	0	0	23868	0	13.8	44.0	42.0
96	MAL2024	0	39	1	1	1	2966	0	16.4	75.0	50.0
97	MAL2088	0	38	1	0	0	51104	0	13.9	25.0	40.0
98	MAL1013	0	38	0	1	0	13860	396	13.7	97.0	43.0
99	MAL0020	0	38	1	0	0	17652	0	15.2	375.0	46.0
100	MAL1055	0	39	1	1	1	27619	77	15.0	107.0	45.0
101	MAL2089	0	38	0	1	0	47250	0	13.5	118.0	41.0
102	MAL1009	0	38	0	1	0	2003	39	15.8	100.0	51.0
103	MAL2090	0	39	1	0	0	25819	0	13.9	40.0	43.0
104	MAL1056	0	38	1	0	0	4731	0	14.2	143.0	45.0
105	MAL1057	0	38	0	1	0	4172	0	13.7	103.0	41.0
106	MAL2091	0	39	1	0	0	3681	0	13.9	155.0	41.0
107	MAL1058	0	38	1	0	0	12495	0	13.1	131.0	40.0
108	MAL2030	1	38	0	1	0	552	20	14.5	76.0	46.0
109	MAL2092	0	38	0	1	0	11794	69	13.4	30.0	40.0
110	MAL1060	0	39	1	0	0	64871	23	13.5	90.0	42.0
111	MAL0007	0	38	0	1	0	525	0	15.3	58.0	46.0
112	MAL2094	0	40	1	0	0	78421	0	14.0	131.0	42.0
113	MAL2094-2	0	37	1	0	0	2285	63	13.1	137.0	40.0
114	MAL2095	0	40	0	1	0	20531	111	13.0	48.0	40.0
115	MAL2096	0	37	0	1	0	2735	35	14.4	113.0	45.0
116	MAL2097	0	37	0	1	0	7390	0	13.6	61.0	42.0
117	MAL022	0	39	1	0	0	22302	0	10.2	53.0	31.0
118	MAL2012	0	36	0	1	0	1003	286	18.0	46.0	57.0
119	MAL1063	0	39	1	0	0	5120	0	13.5	83.0	40.0
120	MAL2097-2	0	40	1	0	0	7094	0	14.5	58.0	44.0
121	MAL1064	0	38	1	0	0	4297	20	11.9	53.0	35.0
122	MAL023	0	38	1	0	0	10025	0	14.0	4.0	43.0
123	MAL2022	0	38	0	1	0	726	13	18.1	46.0	-
124	MAL1065	0	39	1	0	0	5365	0	10.7	87.0	32.0
125	MAL2098	0	39	0	1	0	11443	236	10.2	181.0	32.0

126	MAL2099	0	37	1	0	0	4896	0	15.4	153.0	47.0
127	MAL1066	0	38	0	1	0	1724	209	14.0	63.0	44.0
128	MAL1067	0	38	1	0	0	13946	0	14.0	138.0	43.0
129	MAL1014	0	38	0	1	0	5304	62	12.0	122.0	38.0
130	MAL1070	0	37	0	1	0	689	0	14.8	133.0	44.0
131	MAL2100	0	39	0	1	0	19432	476	11.9	94.0	38.0
132	MAL1071	0	37	1	0	0	14436	0	13.3	93.0	42.0

Appendix 1. (Continued)

133	MAL1072	0	38	0	1	0	3063	83	12.9	52.0	38.0
134	MAL1073	0	37	0	1	0	764	9	4.0	15.0	11.0
135	MAL2028	0	38	0	1	0	655	491	14.6	111.0	46.0
136	MAL1074	0	38	1	0	0	881	0	9.0	27.0	26.0
137	MAL1076	0	38	1	0	0	3468	0	10.4	34.0	30.0
138	MAL1078	0	38	1	0	0	113686	0	9.0	24.0	27.0
139	MAL1079	0	38	1	0	0	5482	0	15.4	135.0	45.0
140	MAL2103	1	37	0	1	0	4390	149	11.6	111.0	34.0
141	MAL2104	0	37	1	0	0	1956	0	12.9	27.0	39.0
142	MAL2105	0	39	0	1	0	4035	339	15.1	156.0	46.0
143	MAL2106	0	37	1	0	0	2326	0	12.5	101.0	39.0
144	MAL2107	0	39	1	1	1	0	0	9.9	53.0	28.0
145	MAL2108	1	39	1	0	0	16855	0	10.6	33.0	34.0
146	MAL2109	0	39	1	0	0	8435	0	13.6	182.0	42.0
147	MAL1080	0	37	1	0	0	1256	13	14.2	65.0	43.0
148	MAL2110	0	37	1	0	0	13205	0	15.1	33.0	45.0
149	MAL2111	0	39	1	0	0	5477	0	13.4	33.0	39.0
150	MAL2033	0	39	0	1	0	111	0	13.0	237.0	62.0
151	MAL2025	0	38	0	1	0	2416	138	14.0	114.0	45.0
152	MAL2035	0	38	0	1	0	517	443	13.2	94.0	43.0
153	MAL2038	0	38	0	1	0	3243	0	14.2	98.0	45.0
154	MAL2042	0	37	0	1	0	1606	0	13.1	99.0	40.0
155	MAL0008	1	39	1	0	0	752247	0	6.2	15.0	19.0
156	MAL2039	0	39	0	1	0	1584	177	13.8	136.0	44.0
157	MAL2029	1	38	0	1	0	617	26	7.5	219.0	26.0
158	MAL2017	0	38	1	0	0	202	118	8.5	228.0	27.0
159	MAL2043	0	38	0	1	0	3302	0	18.5	54.0	60.0
160	MAL1016	0	37	1	0	0	14638	0	14.3	52.0	44.0
161	MAL2046	0	38	0	1	0	491	82	14.3	44.0	44.0
162	MAL1020	1	38	0	1	0	13439	599	14.0	75.0	41.0
163	MAL2048	0	38	0	1	0	37022	259	13.2	97.0	39.0
164	MAL1061	0	38	1	0	0	12354	0	16.5	119.0	50.0
165	MAL1017	0	38	1	0	0	70859	0	16.1	151.0	51.0
166	MAL1019	0	38	0	1	0	5141	51	13.7	98.0	41.0
167	W7350-T	1	0	0	0	0	0	0	15.4	78.0	48.0
168	W7856-S	0	0	0	0	0	0	0	16.4	82.0	50.0

169	WP682-N	0	0	0	0	0	0	0	14.6	215.0	44.0
170	W7731-A	1	0	0	0	0	0	0	13.5	98.0	43.0
171	W7630-J	1	0	0	0	0	0	0	11.4	324.0	37.0
172	W7722-S	1	0	0	0	0	0	0	13.4	162.0	42.0
173	W7709-U	0	0	0	0	0	0	0	15.3	175.0	47.0
174	W7849-P	1	0	0	0	0	0	0	11.2	184.0	37.0
175	WP349-W	1	0	0	0	0	0	0	13.3	227.0	42.0
176	W7118-N	1	0	0	0	0	0	0	14.3	202.0	43.0
177	W7862-G	0	0	0	0	0	0	0	17.9	194.0	56.0

Appendix 1. (Continued)

178	W7860-K	0	0	0	0	0	0	0	16.3	198.0	49.0
179	WP651-C	0	0	0	0	0	0	0	13.2	155.0	42.0
180	W7793-U	1	0	0	0	0	0	0	14.5	280.0	45.0
181	W7013-N	0	0	0	0	0	0	0	10.6	55.0	34.0
182	W7067-J	0	0	0	0	0	0	0	15.1	233.0	46.0
183	W7089-G	0	0	0	0	0	0	0	14.8	125.0	45.0
184	W7092-J	0	0	0	0	0	0	0	16.6	169.0	51.0
185	W7102-C	1	0	0	0	0	0	0	14.7	199.0	46.0
186	W7114-D	1	0	0	0	0	0	0	14.4	177.0	45.0
187	W7139-H	0	0	0	0	0	0	0	15.6	237.0	48.0
188	W7140-T	1	0	0	0	0	0	0	10.5	278.0	14.0
189	W7152-S	1	0	0	0	0	0	0	14.1	154.0	42.0
190	W7168-U	1	0	0	0	0	0	0	14.7	137.0	47.0
191	W7173-A	1	0	0	0	0	0	0	15.7	40.0	49.0
192	W7184-Y	0	0	0	0	0	0	0	14.1	159.0	43.0
193	W7216-R	0	0	0	0	0	0	0	15.5	186.0	48.0
194	W7248-X	1	0	0	0	0	0	0	17.4	157.0	52.0
195	W7264-V	1	0	0	0	0	0	0	14.3	157.0	46.0
196	W7278-L	1	0	0	0	0	0	0	13.9	162.0	40.0
197	W7288-V	1	0	0	0	0	0	0	13.1	184.0	41.0
198	W7294-J	1	0	0	0	0	0	0	13.6	50.0	43.0
199	W7303-B	1	0	0	0	0	0	0	12.8	253.0	42.0
200	W7304-D	1	0	0	0	0	0	0	13.1	220.0	41.0
201	W7305-Y	0	0	0	0	0	0	0	13.3	225.0	44.0
202	W7325-G	1	0	0	0	0	0	0	10.2	100.0	30.0
203	W7346-L	0	0	0	0	0	0	0	14.3	207.0	44.0
204	W7373-L	1	0	0	0	0	0	0	15.8	145.0	47.0
205	W7400-M	1	0	0	0	0	0	0	15.4	154.0	47.0
206	W7401-C	1	0	0	0	0	0	0	14.6	182.0	43.0
207	W7410-B	1	0	0	0	0	0	0	15.0	180.0	46.0
208	W7437-H	1	0	0	0	0	0	0	13.4	163.0	41.0
209	W7452-G	1	0	0	0	0	0	0	15.2	176.0	48.0
210	W7489-T	0	0	0	0	0	0	0	17.4	155.0	54.0
211	W7503-G	1	0	0	0	0	0	0	14.0	195.0	_

212	W7511-D	0	0	0	0	0	0	0	15.4	164.0	50.0
213	W7557-S	1	0	0	0	0	0	0	14.6	177.0	44.0
214	W7620-N	0	0	0	0	0	0	0	14.6	172.0	45.0
215	W7002-H	0	0	0	0	0	0	0	14.8	219.0	46.0
216	W7621-P	1	0	0	0	0	0	0	14.1	125.0	45.0
217	W7649-Y	0	0	0	0	0	0	0	16.1	215.0	50.0
218	W7669-F	0	0	0	0	0	0	0	16.2	190.0	53.0
219	W7689-C	1	0	0	0	0	0	0	10.4	218.0	32.0
220	W7693-U	1	0	0	0	0	0	0	14.6	219.0	44.0
221	W7716-S	1	0	0	0	0	0	0	15.3	203.0	25.0
222	W7727-Q	0	0	0	0	0	0	0	12.0	82.0	35.0

Appendix 1. (Continued)

223	W7742-P	0	0	0	0	0	0	0	15.1	124.0	44.0
224	W7753-D	0	0	0	0	0	0	0	16.2	223.0	52.0
225	W7775-G	1	0	0	0	0	0	0	14.1	205.0	44.0
226	W7003-Y	0	0	0	0	0	0	0	8.8	291.0	38.0
227	W7004-D	0	0	0	0	0	0	0	16.2	258.0	50.0
228	W7818-S	0	0	0	0	0	0	0	14.7	247.0	46.0
229	W7823-Q	1	0	0	0	0	0	0	15.2	192.0	48.0
230	W7831-C	0	0	0	0	0	0	0	16.7	169.0	50.0
231	W7842-Q	0	0	0	0	0	0	0	15.3	239.0	47.0
232	W7853-M	1	0	0	0	0	0	0	16.0	108.0	48.0
233	W7854-P	0	0	0	0	0	0	0	17.0	252.0	50.0
234	W7861-J	0	0	0	0	0	0	0	17.0	145.0	52.0
235	W7864-H	0	0	0	0	0	0	0	21.2	169.0	62.0
236	W7866-A	0	0	0	0	0	0	0	10.1	137.0	34.0
237	W7869-J	0	0	0	0	0	0	0	17.8	179.0	52.0
238	W7870-G	0	0	0	0	0	0	0	14.4	321.0	45.0
239	WP188-P	0	0	0	0	0	0	0	14.0	236.0	44.0
240	W7014-H	0	0	0	0	0	0	0	15.6	190.0	47.0
241	WP208-W	0	0	0	0	0	0	0	14.0	290.0	_
242	WP211-W	0	0	0	0	0	0	0	14.3	158.0	43.0
243	WP275-S	0	0	0	0	0	0	0	16.0	138.0	49.0
244	WP400-N	0	0	0	0	0	0	0	14.6	192.0	45.0
245	WP793-V	1	0	0	0	0	0	0	10.2	248.0	32.0
246	W7019-P	0	0	0	0	0	0	0	16.1	181.0	49.0
247	W7027-E	0	0	0	0	0	0	0	18.4	170.0	57.0
248	W7028-R	1	0	0	0	0	0	0	16.5	133.0	48.0
249	W7048-L	0	0	0	0	0	0	0	15.4	142.0	48.0
250	W7051-P	1	0	0	0	0	0	0	13.5	109.0	44.0
251	W7066-R	1	0	0	0	0	0	0	15.7	229.0	46.0
252	W7090-F	0	0	0	0	0	0	0	14.7	139.0	45.0
253	W7091-V	0	0	0	0	0	0	0	17.2	248.0	52.0

Note:

Lab-id = Subject id; Lab-id started with MAL = malaria patients; Lab-id started with W = controls; Tem = Temperature (^{0}C); pf = *P. falciparum*; pv = *P. vivax*; pf + pv = double infection; Hgb = Haemoglobin; PLT = Platelet; Hct = haematocrit; HIV= 1 is positive; pf = 1 is *P. falciparum* patient; pv=1 is *P. vivax* patient; 0= negative for malaria or HIV; - = not done

Appendix 2. Absolute counts and proportions (%) of peripheral blood mononuclear cells in HIV positive and HIV negative malaria patients and controls.

NO	Lab_ID	Pf	HIV	Mon%	Lym%	CD3%	CD4%	CD8%	B%	NK%	CD4+	CD8+	B	NK	CD3+	lymp	Mon	Ratio
1	MAL2015	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	MAL2036	0	0	10.0	33.0	61.0	32.0	34.0	7.0	25.0	877	941	191	688	1800	2739	830	0.9
3	MAL2013	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	MAL0002	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	MAL2007	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	MAL1004	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	MAL1005	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	MAL1006	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	MAL1007	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	MAL2010	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	MAL2009	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	MAL2011	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	MAL0003	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	MAL0004	0	0	7.0	16.0	83.0	39.0	37.0	-	-	373	354	-	-	794	960	420	1.1
15	MAL2041	0	0	10.0	9.0	75.0	39.0	55.0	9.0	16.0	148	136	35	62	287	384	480	1.1
16	MAL1023	1	0	9.0	13.0	85.0	40.0	41.0	6.0	9.0	193	199	27	41	409	481	333	1.0
17	MAL0010	1	0	11.0	23.0	71.0	47.0	19.0	9.0	19.0	531	212	103	217	804	1127	539	2.5
18	MAL2016	0	0	-	18.0	72.0	50.0	21.0	-	-	486	207	-	-	703	972	-	2.4
19	MAL0011	1	0	10.0	16.0	50.0	33.0	16.0	9.0	40.0	190	92	51	576	287	576	360	2.1
20	MAL1029	0	0	15.0	23.0	47.0	25.0	19.0	3.0	48.0	353	270	42	682	669	1426	930	1.3
21	MAL1030	1	0	5.0	13.0	79.0	37.0	41.0	4.0	16.0	296	323	36	123	627	793	305	0.9
22	MAL2050	0	1	12.0	21.0	81.0	11.0	71.0	2.0	14.0	205	1274	40	250	1472	1806	1032	0.2
23	MAL2051	0	0	18.0	16.0	81.0	50.0	31.0	7.0	11.0	549	336	79	120	879	1088	1224	1.6
24	MAL2013A	0	0	7.0	21.0	66.0	41.0	25.0	-	-	690	418	-	-	1129	1701	567	1.6
25	MAL1031	1	0	9.0	21.0	62.0	38.0	23.0	8.0	30.0	270	165	60	214	443	714	306	1.6
26	MAL1032	1	0	5.0	9.0	74.0	45.0	26.0	18.0	11.0	250	145	100	59	415	558	310	1.7
27	MAL2052	1	0	9.0	17.0	57.0	27.0	29.0	2.0	41.0	241	256	21	364	504	884	468	0.9
28	MAL2053	1	0	11.0	20.0	77.0	64.0	12.0	9.0	13.0	603	116	88	124	726	940	517	5.2
29	MAL2054	0	0	2.0	11.0	77.0	46.0	31.0	15.0	8.0	395	269	126	70	662	858	156	1.5
30	MAL2055	0	0	7.0	16.0	82.0	31.0	48.0	6.0	12.0	307	465	57	113	800	976	427	0.7
31	MAL2014	0	0	17.0	31.0	83.0	46.0	37.0	-	-	551	452	-	-	1006	1209	663	1.2
32	MAL2056	1	0	8.0	11.0	75.0	47.0	28.0	11.0	14.0	250	151	59	78	414	550	400	1.7
33	MAL1033	0	0	14.0	29.0	75.0	34.0	39.0	7.0	14.0	536	603	113	222	1174	1566	756	0.9
34	MAL2057	0	0	15.0	25.0	79.0	47.0	29.0	14.0	11.0	319	197	92	73	529	675	405	1.6
35	MAL1034	0	0	10.0	21.0	81.0	45.0	34.0	11.0	7.0	466	348	108	73	834	1029	490	1.3
36	MAL1035	1	0	8.0	8.0	74.0	45.0	28.0	5.0	19.0	175	110	21	73	292	392	392	1.6
37	MAL1036	1	0	6.0	10.0	75.0	57.0	16.0	15.0	11.0	356	99	91	68	464	620	372	3.6
38	MAL2018	1	0	16.0	31.0	63.0	42.0	21.0	3.0	32.0	467	232	34	355	707	1116	576	2.0
39	MAL1037	0	0	4.0	18.0	82.0	37.0	44.0	6.0	13.0	297	345	44	103	647	792	176	0.9
40	MAL2058	0	0	18.0	26.0	71.0	34.0	33.0	9.0	19.0	547	531	152	304	1143	1612	1116	1.0
41	MAL2060	1	0	11.0	37.0	-	47.0	28.0	8.0	20.0	261	157	42	109	423	555	165	1.7
42	MAL2059	0	0	20.0	32.0	71.0	53.0	17.0	3.0	23.0	814	260	48	348	1096	1536	960	3.1
43	MAL0014	0	0	14.0	8.0	86.0	51.0	30.0	7.0	7.0	217	128	29	29	365	424	742	1.7

44	MAL2019	0	0	10.0	11.0	79.0	35.0	38.0	17.0	4.0	215	235	101	26	483	612	510	0.9
45	MAL1038	1	0	24.0	16.0	77.0	30.0	40.0	7.0	15.0	168	224	37	86	430	560	840	0.8
46	MAL1039	1	0	10.0	64.0	89.0	44.0	44.0	6.0	6.0	795	788	111	107	1604	1792	280	1.0

Appendix 2. (Continued)

47	MAL1040	1	0	8.0	10.0	78.0	51.0	27.0	6.0	16.0	209	111	24	64	320	410	328	1.9
48	MAL1041	0	0	11.0	17.0	82.0	45.0	33.0	4.0	13.0	440	323	43	130	793	969	627	1.4
49	MAL2061	1	0	7.0	9.0	77.0	42.0	35.0	9.0	15.0	349	288	75	125	639	828	644	1.2
50	MAL1008	0	0	15.0	24.0	81.0	47.0	34.0	12.0	5.0	635	453	161	70	1084	1344	840	1.0
51	MAL2062	0	0	16.0	14.0	82.0	43.0	41.0	2.0	17.0	267	253	15	105	503	616	704	1.1
52	MAL1042	1	0	7.0	12.0	73.0	53.0	18.0	15.0	12.0	134	44	37	29	183	252	147	3.0
53	MAL1043	0	0	12.0	30.0	82.0	59.0	22.0	5.0	12.0	621	228	57	127	856	1050	420	2.7
54	MAL1044	1	0	10.0	10.0	74.0	27.0	43.0	8.0	19.0	62	98	19	43	171	230	230	0.6
55	MAL0005	0	0	13.0	21.0	83.0	46.0	34.0	9.0	9.0	417	309	81	82	753	903	559	1.4
56	MAL2020	1	0	7.0	43.0	61.0	32.0	26.0	8.0	30.0	654	528	172	628	1254	2064	330	1.2
57	MAL0015	1	0	4.0	8.0	73.0	46.0	24.0	6.0	20.0	188	98	26	83	299	408	204	1.9
58	MAL2065	0	0	17.0	29.0	71.0	45.0	24.0	18.0	11.0	402	213	160	97	643	899	527	1.9
59	MAL2066	0	0	14.0	19.0	85.0	37.0	45.0	6.0	8.0	322	399	48	71	744	874	644	0.8
60	MAL2067	1	0	10.0	21.0	80.0	44.0	35.0	8.0	13.0	659	517	122	197	1198	1491	710	1.3
61	MAL2068	0	0	10.0	34.0	80.0	45.0	31.0	-	-	808	566	-	-	1441	2862	530	1.4
62	MAL1046	1	0	8.0	15.0	82.0	59.0	22.0	-	-	268	97	-	-	375	450	240	2.8
63	MAL0006	0	0	3.0	7.0	77.0	49.0	32.0	8.0	15.0	132	86	22	40	204	266	114	1.5
64	MAL1047	0	0	9.0	17.0	85.0	58.0	20.0	-	-	89	31	-	-	130	153	81	2.9
65	MAL2071	1	0	12.0	10.0	64.0	35.0	26.0	-	-	79	60	-	-	146	230	276	1.3
66	MAL2072	0	0	18.0	28.0	66.0	33.0	28.0	-	-	540	453	-	-	1086	1624	1044	1.2
67	MAL2073	0	0	10.0	11.0	79.0	41.0	33.0	-	-	139	114	-	-	269	341	310	1.2
68	MAL1050	1	0	8.0	12.0	81.0	34.0	44.0	-	-	143	223	-	-	410	504	336	0.6
69	MAL1010	0	0	11.0	36.0	79.0	43.0	31.0	8.0	10.0	981	703	182	231	1824	2304	704	1.4
70	MAL2074	1	0	11.0	20.0	87.0	59.0	24.0	-	-	410	166	-	-	611	700	385	2.5
71	MAL0016	1	0	6.0	9.0	77.0	43.0	30.0	-	-	226	156	-	-	403	522	348	1.5
72	MAL2075	1	0	16.0	26.0	79.0	50.0	27.0	-	-	538	288	-	-	843	1066	656	1.9
73	MAL2076	0	0	8.0	20.0	82.0	54.0	29.0	-	-	525	280	-	-	809	980	392	1.9
74	MAL0017	0	0	10.0	11.0	86.0	41.0	45.0	-	-	222	241	-	-	463	539	490	0.9
75	MAL2021	0	0	9.0	31.0	71.0	51.0	19.0	12.0	15.0	777	289	176	228	1081	1519	441	2.7
76	MAL0018	0	0	12.0	16.0	87.0	41.0	36.0	-	-	356	315	-	-	669	864	648	1.1
77	MAL2077	1	0	15.0	31.0	44.0	20.0	23.0	-	-	168	188	-	-	371	837	405	0.9
78	MAL1051	1	0	8.0	21.0	83.0	54.0	26.0	-	-	364	178	-	-	560	672	256	2.0
79	MAL1052	0	0	20.0	25.0	73.0	43.0	27.0	-	-	507	314	-	-	864	1175	940	1.6
80	MAL2078	0	0	21.0	13.0	65.0	42.0	33.0	-	-	273	280	-	-	560	858	1386	1.0
81	MAL1012	0	0	2.0	16.0	90.0	61.0	27.0	5.0	3.0	428	34	34	24	632	704	88	2.3
82	MAL0019	0	0	3.0	6.0	78.0	43.0	28.0	-	-	222	145	-	-	402	516	238	1.5
83	MAL2079	1	0	15.0	23.0	76.0	34.0	38.0	-	-	392	436	-	-	876	1150	750	0.9
84	MAL1053	1	0	7.0	7.0	65.0	26.0	34.0	-	-	106	139	-	-	264	406	406	0.8
85	MAL2080	1	0	12.0	38.0	89.0	38.0	49.0	-	-	557	723	-	-	1324	1482	468	0.8
86	MAL2081	1	0	9.0	17.0	75.0	50.0	24.0	-	-	582	278	-	-	873	1156	612	2.1
87	MAL2082	1	0	11.0	28.0	65.0	55.0	11.0	-	-	401	83	-	-	474	728	286	4.8
88	MAL1011	0	0	14.0	29.0	75.0	45.0	29.0	8.0	16.0	273	177	50	99	458	609	294	1.6
89	MAL2084	1	0	13.0	43.0	79.0	39.0	35.0	-	-	520	461	-	-	1063	1333	403	1.1

90	MAL0020	1	1	21.0	38.0	91.0	7.0	77.0	-	-	86	903	-	-	1069	1178	651	0.1
91	MAL2085	1	0	14.0	31.0	78.0	39.0	39.0	-	-	573	622	-	-	1234	1581	714	0.9
92	MAL1054	1	0	21.0	31.0	77.0	53.0	22.0	-	-	925	381	-	-	1343	1736	1176	2.4
93	MAL2086	1	0	10.0	12.0	72.0	45.0	27.0	-	-	342	203	-	-	552	756	630	1.7
94	MAL2023	1	0	12.0	23.0	77.0	40.0	35.0	10.0	14.0	383	336	92	131	746	966	504	1.1

Appendix 2. (Continued)

95	MAL2087	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
96	MAL2024	1	0	10.0	8.0	66.0	31.0	31.0	8.0	23.0	211	206	56	154	444	674	840	1.0
97	MAL2088	1	0	-	-	-	-	-	-	-	207	224	-	-	-	-	-	0.9
98	MAL1013	0	0	9.0	17.0	84.0	38.0	34.0	10.0	9.0	519	458	134	122	1139	1360	720	1.1
99	MAL0020	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
100	MAL1055	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
101	MAL2089	0	0	18.0	24.0	78.0	54.0	22.0	-	-	614	247	-	-	876	1128	846	2.5
102	MAL1009	0	0	7.0	20.0	83.0	33.0	50.0	5.0	11.0	777	1200	119	261	1974	2380	833	0.7
103	MAL2090	1	0	2.0	17.0	81.0	38.0	42.0	-	-	290	323	-	-	613	765	90	0.9
104	MAL1056	1	0	15.0	22.0	76.0	33.0	40.0	-	-	540	653	-	-	1242	1628	1110	0.8
105	MAL1057	0	0	1.0	7.0	92.0	44.0	46.0	-	-	216	229	-	-	454	490	70	0.9
106	MAL2091	1	0	16.0	21.0	77.0	38.0	28.0	-	-	439	319	-	-	837	1155	880	1.4
107	MAL1058	1	0	8.0	13.0	72.0	41.0	26.0	-	-	419	272	-	-	744	1027	632	1.5
108	MAL2030	0	1	12.0	31.0	78.0	29.0	48.0	4.0	19.0	275	459	39	185	742	961	372	0.6
109	MAL2092	0	0	5.0	4.0	70.0	29.0	39.0	-	-	85	116	-	-	207	294	350	0.7
110	MAL1060	1	0	10.0	15.0	76.0	19.0	19.0	-	-	199	519	-	-	801	1050	700	0.4
111	MAL0007	0	0	4.0	14.0	75.0	30.0	42.0	13.0	9.0	210	286	93	59	518	689	212	0.7
112	MAL2094	1	0	1.0	26.0	91.0	24.0	64.0	-	-	318	842	-	-	1195	1326	51	0.4
113	MAL2094-2	1	0	9.0	27.0	67.0	27.0	37.0	-	-	348	478	-	-	873	1293	432	0.7
114	MAL2095	0	0	6.0	15.0	78.0	45.0	27.0	-	-	374	226	-	-	650	840	336	1.7
115	MAL2096	0	0	6.0	7.0	77.0	55.0	17.0	-	-	206	62	-	-	285	371	318	3.3
116	MAL2097	0	0	5.0	11.0	84.0	57.0	25.0	-	-	337	150	-	-	496	594	270	2.2
117	MAL022	1	0	5.0	10.0	83.0	45.0	35.0	-	-	112	87	-	-	205	250	125	1.3
118	MAL2012	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
119	MAL1063	1	0	14.0	20.0	81.0	32.0	45.0	-	-	400	560	-	-	998	1240	868	0.7
120	MAL2097-2	1	0	6.0	10.0	85.0	63.0	20.0	-	-	351	113	-	-	474	560	336	3.1
121	MAL1064	1	0	4.0	14.0	83.0	55.0	27.0	-	-	481	242	-	-	733	882	252	2.0
122	MAL023	1	0	9.0	13.0	69.0	47.0	22.0	-	-	466	218	-	-	694	1001	693	2.1
123	MAL2022	0	0	15.0	46.0	59.0	39.0	20.0	7.0	31.0	693	362	123	565	1053	1800	600	2.0
124	MAL1065	1	0	14.0	37.0	59.0	30.0	25.0	-	-	454	384	-	-	895	1517	574	1.2
125	MAL2098	0	0	11.0	22.0	76.0	48.0	29.0	-	-	750	446	-	-	1192	1566	781	1.7
126	MAL2099	1	0	10.0	21.0	70.0	36.0	31.0	-	-	640	562	-	-	1262	1785	850	1.1
127	MAL1066	0	0	12.0	25.0	77.0	33.0	37.0	-	-	308	343	-	-	711	925	440	0.9
128	MAL1067	1	0	10.0	24.0	86.0	39.0	47.0	-	-	563	675	-	-	1227	1440	600	0.8
129	MAL1014	0	0	1.0	8.0	86.0	39.0	41.0	8.0	6.0	294	308	58	48	641	752	94	1.0
130	MAL1070	0	0	15.0	30.0	76.0	54.0	20.0	-	-	1128	411	-	-	1575	2070	1035	2.7
131	MAL2100	0	0	9.0	29.0	89.0	56.0	29.0	-	-	894	459	-	-	1416	1595	495	1.9
132	MAL1071	1	0	16.0	36.0	83.0	39.0	43.0	-	-	753	817	-	-	1591	1908	848	0.9
133	MAL1072	0	0	12.0	27.0	69.0	50.0	17.0	-	-	654	230	-	-	936	1350	600	2.9
134	MAL1073	0	0	14.0	22.0	90.0	43.0	54.0	-	-	131	166	-	-	278	308	196	0.8
135	MAL2028	0	0	4.0	10.0	81.0	26.0	48.0	2.0	17.0	321	595	30	208	1000	1240	496	0.5

136	MAL1074	1	0	15.0	24.0	80.0	37.0	36.0	-	-	162	154	-	-	345	432	270	1.1
137	MAL1076	1	0	12.0	14.0	82.0	63.0	19.0	-	-	246	75	-	-	321	392	336	3.3
138	MAL1078	1	0	14.0	24.0	83.0	62.0	19.0	-	-	594	183	-	-	795	960	560	3.2
139	MAL1079	1	0	10.0	36.0	75.0	52.0	20.0	-	-	926	349	-	-	1332	1764	490	2.7
140	MAL2103	0	1	9.0	19.0	75.0	8.0	60.0	-	-	69	515	-	-	643	855	405	0.1
141	MAL2104	1	0	12.0	15.0	79.0	67.0	11.0	-	-	573	94	-	-	675	855	684	6.1
142	MAL2105	0	0	11.0	30.0	76.0	41.0	34.0	-	-	734	606	-	-	1374	1800	660	1.2

Appendix 2. (Continued)

143	MAL2106	1	0	16.0	32.0	65.0	31.0	36.0	-	-	541	631	-	-	1147	1760	880	0.9
144	MAL2107	1	0	3.0	7.0	84.0	38.0	39.0	-	-	130	133	-	-	290	343	147	1.0
145	MAL2108	1	1	7.0	15.0	76.0	19.0	53.0	-	-	55	152	-	-	217	285	133	0.4
146	MAL2109	1	0	8.0	10.0	78.0	49.0	23.0	-	-	375	177	-	-	604	770	616	2.1
147	MAL1080	1	0	10.0	20.0	73.0	32.0	38.0	-	-	264	312	-	-	604	820	410	0.8
148	MAL2110	1	0	20.0	57.0	79.0	41.0	38.0	-	-	585	542	-	-	1123	1425	580	1.1
149	MAL2111	1	0	11.0	17.0	72.0	44.0	23.0	-	-	262	138	-	-	428	595	385	1.9
150	MAL2033	0	0	6.0	22.0	74.0	31.0	32.0	3.0	37.0	385	408	38	466	765	1260	402	0.9
151	MAL2025	0	0	12.0	19.0	81.0	50.0	28.0	12.0	5.0	571	322	133	53	920	1140	720	1.8
152	MAL2035	0	0	21.0	26.0	70.0	36.0	34.0	3.0	23.0	522	490	51	338	1023	1456	1176	1.1
153	MAL2038	0	0	14.0	16.0	77.0	39.0	38.0	11.0	15.0	161	158	44	64	318	416	364	1.0
154	MAL2042	0	0	4.0	11.0	78.0	38.0	38.0	10.0	10.0	284	279	70	73	577	737	268	1.0
155	MAL0008	1	1	5.0	39.0	72.0	20.0	49.0	18.0	9.0	95	230	85	40	334	468	58	0.4
156	MAL2039	0	0	8.0	42.0	81.0	39.0	37.0	3.0	16.0	673	635	43	280	1401	-	-	1.1
157	MAL2029	0	1	16.0	65.0	92.0	23.0	68.0	2.0	6.0	386	1142	40	101	1545	1690	416	0.3
158	MAL2017	1	0	8.0	18.0	81.0	37.0	37.0	8.0	6.0	340	338	77	59	739	918	408	1.0
159	MAL2043	0	0	12.0	33.0	73.0	49.0	24.0	14.0	13.0	931	460	272	247	1414	1914	696	2.0
160	MAL1016	1	0	15.0	26.0	86.0	39.0	46.0	4.0	10.0	318	367	29	81	691	806	465	0.9
161	MAL2046	0	0	5.0	9.0	73.0	47.0	24.0	13.0	6.0	350	176	98	45	551	656	410	2.0
162	MAL1020	0	1	9.0	22.0	91.0	21.0	66.0	3.0	4.0	191	593	31	40	821	902	369	0.3
163	MAL2048	0	0	2.0	10.0	78.0	51.0	23.0	2.0	7.0	286	127	67	560	438	560	112	2.3
164	MAL1061	1	0	10.0	20.0	85.0	60.0	22.0	-	-	924	346	-	-	1305	1540	770	2.7
165	MAL1017	1	0	3.0	4.0	78.0	46.0	31.0	12.0	7.0	151	103	38	23	258	328	246	1.5
166	MAL1019	0	0	16.0	33.0	87.0	58.0	24.0	5.0	8.0	744	313	59	101	1116	1287	624	2.4
167	W7350-T	0	1	12.0	22.0	75.0	3.0	68.0	13.0	12.0	18	419	80	74	462	610	336	0.0
168	W7856-S	0	0	11.0	35.0	81.0	48.0	34.0	7.0	8.0	353	250	51	59	595	735	231	1.4
169	WP682-N	0	0	8.0	41.0	82.0	36.0	40.0	8.0	9.0	590	656	131	148	1345	1640	370	0.9
170	W7731-A	0	1	10.0	36.0	88.0	13.0	75.0	6.0	6.0	239	1377	110	110	1616	1836	510	0.2
171	W7630-J	0	1	15.0	13.0	84.0	23.0	58.0	5.0	6.0	144	362	31	37	524	624	720	0.4
172	W7722-S	0	1	7.0	21.0	86.0	13.0	67.0	6.0	6.0	147	760	68	68	975	1134	378	0.2
173	W7709-U	0	0	9.0	28.0	85.0	42.0	40.0	6.0	7.0	835	795	119	139	1690	1988	639	1.1
174	W7849-P	0	1	8.0	49.0	92.0	5.0	83.0	1.0	5.0	164	2725	33	164	3020	3283	536	0.1
175	WP349-W	0	1	6.0	26.0	81.0	25.0	53.0	9.0	10.0	306	648	110	122	990	1222	282	0.5
176	W7118-N	0	1	8.0	24.0	86.0	7.0	78.0	2.0	10.0	144	1610	41	206	1775	2064	688	0.1
177	W7862-G	0	0	7.0	26.0	79.0	51.0	26.0	8.0	11.0	517	264	81	112	802	1014	273	2.0
178	W7860-K	0	0	7.0	34.0	68.0	28.0	39.0	15.0	15.0	724	1008	388	388	1757	2584	532	0.7
179	WP651-C	0	0	5.0	25.0	75.0	43.0	28.0	13.0	10.0	452	294	137	105	788	1050	210	1.5
180	W7793-U	0	1	7.0	22.0	82.0	37.0	42.0	4.0	12.0	545	616	59	177	1209	1474	469	0.9
181	W7013-N	0	0	9.0	31.0	85.0	56.0	28.0	5.0	10.0	399	200	36	71	606	713	207	2.0

182	W7067-J	0	0	7.0	37.0	73.0	27.0	42.0	11.0	15.0	939	1461	383	522	2539	3478	658	0.6
183	W7089-G	0	0	7.0	42.0	78.0	8.0	67.0	4.0	15.0	242	2026	121	454	2359	3024	504	0.1
184	W7092-J	0	0	8.0	32.0	68.0	30.0	35.0	11.0	18.0	432	504	158	259	979	1440	360	0.9
185	W7102-C	0	1	10.0	51.0	77.0	20.0	55.0	5.0	17.0	561	1543	140	477	2160	2805	550	0.4
186	W7114-D	0	1	10.0	34.0	73.0	19.0	54.0	10.0	15.0	220	624	116	173	844	1156	340	0.4
187	W7139-H	0	0	7.0	23.0	66.0	40.0	23.0	11.0	22.0	754	434	207	415	1245	1886	574	1.7
188	W7140-T	0	1	13.0	45.0	90.0	29.0	61.0	5.0	5.0	639	1345	110	110	1985	2205	637	4.9
189	W7152-S	0	1	9.0	38.0	84.0	32.0	51.0	8.0	8.0	876	1395	219	219	2298	2736	648	0.6
190	W7168-U	0	1	5.0	41.0	89.0	23.0	62.0	7.0	3.0	575	1551	175	75	2226	2501	305	0.4

Appendix 2. (Continued)

191	W7173-A	0	1	12.0	43.0	94.0	11.0	81.0	4.0	2.0	199	1463	72	36	1698	1806	504	0.1
192	W7184-Y	0	0	10.0	20.0	77.0	50.0	23.0	12.0	10.0	550	253	132	110	847	1100	550	2.2
193	W7216-R	0	0	7.0	37.0	68.0	40.0	26.0	5.0	25.0	962	625	120	601	1635	2405	455	1.5
194	W7248-X	0	1	7.0	30.0	70.0	32.0	36.0	12.0	17.0	643	724	241	342	1407	2010	469	0.9
195	W7264-V	0	1	4.0	27.0	70.0	28.0	40.0	10.0	20.0	559	799	200	400	1399	1998	296	0.7
196	W7278-L	0	1	9.0	34.0	92.0	20.0	69.0	3.0	4.0	347	1196	52	69	1595	1734	459	0.3
197	W7288-V	0	1	6.0	22.0	53.0	4.0	46.0	5.0	42.0	61	698	76	638	805	1518	414	0.1
198	W7294-J	0	1	7.0	29.0	84.0	15.0	66.0	4.0	10.0	265	1168	71	177	1486	1769	427	0.2
199	W7303-B	0	1	12.0	24.0	73.0	8.0	67.0	8.0	21.0	115	965	115	302	1051	1440	720	0.1
200	W7304-D	0	1	11.0	31.0	77.0	11.0	65.0	6.0	15.0	191	1128	104	260	1337	1736	616	0.2
201	W7305-Y	0	0	11.0	38.0	74.0	45.0	30.0	17.0	6.0	872	581	329	116	1434	1938	561	1.5
202	W7325-G	0	1	8.0	21.0	82.0	4.0	76.0	14.0	8.0	49	926	171	97	999	1218	464	0.1
203	W7346-L	0	0	7.0	44.0	77.0	47.0	30.0	7.0	15.0	889	568	132	284	1457	1892	301	1.6
204	W7373-L	0	1	6.0	19.0	83.0	26.0	48.0	4.0	12.0	316	584	49	146	1009	1216	384	0.5
205	W7400-M	0	1	8.0	36.0	88.0	19.0	70.0	2.0	9.0	369	1361	39	175	1711	1944	432	0.3
206	W7401-C	0	1	8.0	53.0	66.0	7.0	60.0	1.0	32.0	178	1526	25	814	1679	2544	384	0.1
207	W7410-B	0	1	9.0	23.0	88.0	14.0	67.0	6.0	6.0	190	909	81	81	1194	1357	531	0.2
208	W7437-H	0	1	10.0	14.0	70.0	17.0	46.0	11.0	18.0	93	251	60	98	382	546	390	0.4
209	W7452-G	0	1	13.0	32.0	87.0	18.0	69.0	5.0	8.0	300	1148	83	133	1448	1664	676	0.3
210	W7489-T	0	0	5.0	26.0	82.0	42.0	38.0	8.0	8.0	753	682	144	144	1471	1794	345	1.1
211	W7503-G	0	1	9.0	28.0	66.0	12.0	52.0	7.0	27.0	228	990	133	514	1257	1904	612	0.0
212	W7511-D	0	0	7.0	22.0	66.0	41.0	25.0	12.0	20.0	541	330	158	264	871	1320	420	1.6
213	W7557-S	0	1	10.0	25.0	67.0	17.0	45.0	7.0	26.0	221	585	91	338	871	1300	520	0.4
214	W7620-N	0	0	8.0	35.0	90.0	41.0	49.0	3.0	6.0	732	875	54	107	1607	1785	408	0.8
215	W7002-H	0	0	8.0	36.0	70.0	42.0	26.0	9.0	21.0	892	552	191	446	1487	2124	472	1.6
216	W7621-P	0	1	15.0	41.0	81.0	8.0	72.0	5.0	15.0	121	1092	76	228	1229	1517	555	0.1
217	W7649-Y	0	0	11.0	38.0	69.0	33.0	34.0	9.0	21.0	527	543	144	335	1101	1596	462	1.0
218	W7669-F	0	0	6.0	34.0	73.0	42.0	29.0	17.0	8.0	671	463	272	128	1167	1598	282	1.4
219	W7689-C	0	1	7.0	16.0	89.0	8.0	78.0	3.0	6.0	68	661	25	51	755	848	371	0.1
220	W7693-U	0	1	7.0	21.0	54.0	9.0	43.0	15.0	30.0	138	659	230	460	828	1533	511	0.2
221	W7716-S	0	1	6.0	23.0	65.0	31.0	31.0	9.0	26.0	506	506	147	425	1061	1633	426	1.0
222	W7727-Q	0	0	8.0	51.0	85.0	8.0	76.0	4.0	9.0	192	1822	96	216	2037	2397	376	0.1
223	W7742-P	0	0	8.0	17.0	65.0	39.0	25.0	8.0	24.0	451	289	92	277	751	1156	544	1.6
224	W7753-D	0	0	7.0	32.0	84.0	39.0	42.0	11.0	4.0	649	699	183	67	1398	1664	364	0.9
225	W7775-G	0	1	10.0	21.0	82.0	18.0	61.0	4.0	-	170	576	38	0	775	945	450	0.3
226	W7003-Y	0	0	16.0	50.0	72.0	43.0	26.0	12.0	15.0	409	247	114	143	684	950	304	1.7
227	W7004-D	0	0	9.0	32.0	76.0	45.0	28.0	15.0	9.0	720	448	240	144	1216	1600	450	1.6

228	W7818-S	0	0	9.0	37.0	69.0	35.0	30.0	12.0	20.0	570	488	195	326	1123	1628	396	1.2
229	W7823-Q	0	1	6.0	29.0	95.0	15.0	82.0	2.0	1.0	165	904	22	11	1047	1102	228	0.2
230	W7831-C	0	0	6.0	26.0	80.0	41.0	38.0	11.0	9.0	586	543	157	129	1144	1430	330	1.1
231	W7842-Q	0	0	6.0	60.0	88.0	38.0	49.0	8.0	3.0	1072	1382	226	85	2482	2820	282	0.8
232	W7853-M	0	1	6.0	25.0	86.0	25.0	58.0	7.0	6.0	388	899	109	93	1333	1550	372	0.4
233	W7854-P	0	0	8.0	28.0	77.0	36.0	38.0	13.0	10.0	897	947	324	249	1919	2492	712	0.9
234	W7861-J	0	0	9.0	35.0	73.0	42.0	26.0	12.0	13.0	500	309	143	155	869	1190	306	1.6
235	W7864-H	0	0	6.0	30.0	61.0	36.0	23.0	12.0	26.0	864	552	288	624	1464	2400	480	1.6
236	W7866-A	0	0	8.0	20.0	69.0	46.0	22.0	14.0	17.0	515	246	157	190	773	1120	448	2.1
237	W7869-J	0	0	7.0	23.0	82.0	39.0	39.0	10.0	7.0	807	807	207	145	1697	2070	630	1.0
238	W7870-G	0	0	7.0	24.0	71.0	44.0	27.0	12.0	15.0	792	486	216	270	1278	1800	525	1.6

Appendix 2. (Continued)

239	WP188-P	0	0	5.0	53.0	85.0	31.0	53.0	11.0	5.0	1462	2500	519	236	4009	4717	445	0.6
240	W7014-H	0	0	6.0	23.0	71.0	40.0	27.0	18.0	9.0	782	528	352	176	1388	1955	510	1.5
241	WP208-W	0	0	9.0	22.0	82.0	61.0	20.0	10.0	9.0	604	198	99	89	812	990	405	0.0
242	WP211-W	0	0	6.0	38.0	75.0	50.0	23.0	14.0	11.0	836	385	234	184	1254	1672	264	2.2
243	WP275-S	0	0	8.0	36.0	69.0	40.0	26.0	13.0	17.0	706	459	229	300	1217	1764	392	1.5
244	WP400-N	0	0	9.0	32.0	83.0	51.0	28.0	8.0	8.0	996	547	156	156	1620	1952	549	1.8
245	WP793-V	0	1	10.0	30.0	78.0	10.0	63.0	13.0	5.0	114	718	148	57	889	1140	380	0.2
246	W7019-P	0	0	4.0	24.0	79.0	44.0	33.0	14.0	7.0	570	428	181	91	1024	1296	216	1.3
247	W7027-E	0	0	9.0	24.0	80.0	40.0	35.0	10.0	9.0	691	605	173	156	1382	1728	648	1.1
248	W7028-R	0	1	11.0	45.0	88.0	20.0	64.0	3.0	9.0	297	950	45	134	1307	1485	363	0.4
249	W7048-L	0	0	8.0	37.0	76.0	40.0	28.0	16.0	6.0	710	497	284	107	1350	1776	384	1.4
250	W7051-P	0	1	10.0	35.0	74.0	7.0	61.0	13.0	13.0	69	598	127	127	725	980	280	0.1
251	W7066-R	0	1	10.0	34.0	85.0	23.0	62.0	9.0	6.0	297	801	116	78	1098	1292	380	0.4
252	W7090-F	0	0	6.0	33.0	67.0	43.0	18.0	11.0	18.0	837	350	214	350	1304	1947	354	2.4
253	W7091-V	0	0	6.0	54.0	79.0	51.0	26.0	13.0	8.0	964	491	246	151	1493	1890	210	2.0

Note:

Lab-id started with MAL = malaria patients; Lab-id started with W = controls; pf =1 refers falciparum patients; pf = 0 vivax patients; HIV= 1 is positive and HIV = 0 is negative for the virus; - = not done; Mono = monocyte; lymh = total lymphocytes; Ratio = CD4+/CD8+.

Appendix 3. Absolute counts and proportions (%) of granulocyte subsets in HIV positive and HIV negative malaria patients and controls.

NO	Lab_ID	pf	HIV	WBC x 10^3	baso%	easi%	Neut%	baso	eosi	neut
1	MAL2015	1	0	-	-	-	-	-	-	-
2	MAL2036	0	0	8.3	0.8	4.0	52.0	66	332	4316
3	MAL2013	0	0	8.1	-	-	-	-	-	-
4	MAL0002	1	0	-	-	-	-	-	-	-
5	MAL2007	1	0	-	-	-	-	-	-	-
6	MAL1004	1	0	-	-	-	-	-	-	-
7	MAL1005	0	0	-	-	-	-	-	-	-
8	MAL1006	0	0	-	-	-	-	-	-	-
9	MAL1007	1	0	5.6	-	-	-	-	-	-
10	MAL2010	1	0	2.6	-	-	-	-	-	-
11	MAL2009	0	0	3.3	-	-	-	-	-	-
12	MAL2011	0	0	-	-	-	-	-	-	-
13	MAL0003	0	0	14.1	-	-	-	-	-	-
14	MAL0004	0	0	6.0	1.0	15.0	60.0	60	900	3600
15	MAL2041	0	0	4.8	0.4	1.0	80.0	19	48	3840
16	MAL1023	1	0	3.7	0.4	0.4	77.0	14	14	2849
17	MAL0010	1	0	4.9	0.4	0.2	65.0	19	9	3185
18	MAL2016	0	0	5.4	-	-	-	-	-	-
19	MAL0011	1	0	3.6	1.0	0.5	39.0	36	18	2418
20	MAL1029	0	0	6.2	1.0	3.0	56.0	62	186	3472
21	MAL1030	1	0	6.1	0.5	1.0	78.0	30	61	4758
22	MAL2050	0	1	8.6	0.5	7.0	57.0	43	602	4902
23	MAL2051	0	0	6.8	-	-	64.0	-	-	4352
24	MAL2013A	0	0	8.0	1.0	5.0	64.0	81	405	5184
25	MAL1031	1	0	3.4	1.0	1.0	66.0	34	34	2244
26	MAL1032	1	0	6.2	0.4	0.4	83.0	24	24	5146
27	MAL2052	1	0	5.2	0.9	2.0	69.0	46	104	3588
28	MAL2053	1	0	4.7	0.8	0.2	66.0	37	94	3102
29	MAL2054	0	0	7.8	0.6	3.0	81.0	46	234	6318
30	MAL2055	0	0	6.1	1.0	0.5	73.0	61	30	4453
31	MAL2014	0	0	3.9	1.0	10.0	39.0	39	390	1521
32	MAL2056	1	0	5.0	0.7	1.0	77.0	35	50	3850
33	MAL1033	0	0	5.4	1.0	7.0	47.0	54	378	2538
34	MAL2057	0	0	2.7	1.0	0.8	56.0	27	21	1512
35	MAL1034	0	0	4.9	0.8	1.0	65.0	39	49	3185
36	MAL1035	1	0	4.9	0.2	0.3	82.0	9	14	4018
37	MAL1036	1	0	6.2	0.9	0.5	81.0	55	31	5022

38	MAL2018	1	0	3.6	0.3	5.0	46.0	11	180	1656
39	MAL1037	0	0	4.4	1.0	1.0	75.0	44	44	3300

Appendix 3. (Continued)

40	MAL2058	0	0	6.2	1.0	3.0	50.0	62	186	3100
41	MAL2060	1	0	1.5	0.9	0.5	49.0	13	7	735
42	MAL2059	0	0	4.8	-	-	-	-	-	-
43	MAL0014	0	0	5.3	0.5	13.0	62.0	26	689	3286
44	MAL2019	0	0	5.1	0.3	3.0	74.0	15	153	3774
45	MAL1038	0	0	3.5	0.9	1.0	55.0	31	35	1926
46	MAL1039	1	0	2.8	1.0	2.0	20.0	28	56	560
47	MAL1040	1	0	4.1	0.8	0.2	80.0	32	8	3280
48	MAL1041	1	0	5.7	0.6	0.7	69.0	34	39	3933
49	MAL2061	0	0	9.2	0.5	0.2	82.0	46	18	7544
50	MAL1008	1	0	6.0	0.5	1.0	59.0	28	56	3304
51	MAL2062	0	0	4.4	0.6	0.5	67.0	26	22	2948
52	MAL1042	0	0	2.1	1.0	10.0	68.0	21	210	1428
53	MAL1043	1	0	3.5	0.9	2.0	53.0	31	70	1855
54	MAL1044	0	0	2.3	0.3	17.0	61.0	6	391	1403
55	MAL0005	1	0	4.0	0.6	8.0	56.0	26	344	2408
56	MAL2020	0	0	4.8	1.0	3.0	44.0	48	114	2112
57	MAL0015	1	0	5.1	0.4	23.0	62.0	20	1173	3162
58	MAL2065	1	0	3.1	1.0	2.0	47.0	31	62	1457
59	MAL2066	0	0	4.6	0.9	2.0	62.0	41	92	2852
60	MAL2067	0	0	7.1	1.0	2.0	63.0	71	142	4473
61	MAL2068	1	0	5.3	1.0	15.0	37.0	53	795	1961
62	MAL1046	0	0	3.0	0.7	11.0	63.0	21	330	1890
63	MAL0006	1	0	4.0	0.4	3.0	85.0	15	114	3230
64	MAL1047	0	0	0.9	0.4	0.5	72.0	3	4	648
65	MAL2071	0	0	2.3	0.4	0.8	75.0	9	18	1725
66	MAL2072	1	0	5.8	0.9	3.0	48.0	52	174	2784
67	MAL2073	0	0	3.1	0.7	2.0	75.0	21	62	2325
68	MAL1050	0	0	4.2	0.9	0.9	77.0	37	37	3234
69	MAL1010	1	0	6.0	0.6	1.0	50.0	38	64	3200
70	MAL2074	0	0	4.0	0.9	3.0	63.0	31	105	2205
71	MAL0016	1	0	5.8	0.3	0.2	83.0	17	11	4814
72	MAL2075	1	0	4.1	1.0	0.6	55.0	41	24	2255
73	MAL2076	1	0	4.9	0.7	2.0	68.0	37	108	3672
74	MAL0017	0	0	4.9	1.0	4.0	64.0	49	196	3136
75	MAL2021	0	0	4.9	1.0	4.0	53.0	49	196	2597
76	MAL0018	0	0	5.4	0.4	0.1	76.0	19	14	3724
77	MAL2077	0	0	2.7	0.9	7.0	43.0	24	189	1161

78	MAL1051	1	0	3.2	0.4	0.8	68.0	12	25	2176
79	MAL1052	1	0	4.7	1.0	8.0	43.0	47	376	2021
80	MAL2078	0	0	6.6	0.9	0.6	62.0	59	39	4092

Appendix 3. (Continued)

81	MAL1012	0	0	4.0	0.3	1.0	80.0	13	44	3520
82	MAL0019	0	0	8.6	0.2	12.0	75.0	17	1032	6450
83	MAL2079	1	0	5.0	0.6	2.0	57.0	30	100	2850
84	MAL1053	1	0	5.8	0.7	0.8	83.0	40	46	4814
85	MAL2080	1	0	3.9	1.0	2.0	44.0	39	78	1716
86	MAL2081	1	0	6.8	0.8	0.7	71.0	54	47	4828
87	MAL2082	1	0	2.6	0.6	0.7	58.0	15	18	1508
88	MAL1011	0	0	2.0	0.7	3.0	51.0	14	63	1071
89	MAL2084	1	0	3.0	2.0	30.0	10.0	62	930	310
90	MAL0020	1	1	3.0	1.0	1.0	-	31	31	-
91	MAL2085	1	0	5.0	1.0	5.0	47.0	51	255	2397
92	MAL1054	1	0	5.6	0.6	3.0	42.0	33	168	2352
93	MAL2086	1	0	6.3	0.3	0.5	76.0	18	31	4788
94	MAL2023	1	0	4.2	1.0	22.0	39.0	42	924	1638
95	MAL2087	1	0	4.4	-	-	-	-	-	-
96	MAL2024	1	0	8.4	0.6	0.2	80.0	50	16	6720
97	MAL2088	1	0	3.6	-	-	-	-	-	-
98	MAL1013	0	0	8.0	0.8	22.0	49.0	64	1760	3920
99	MAL0020	1	0	5.7	-	-	36.0	-	-	1116
100	MAL1055	1	0	5.8	-	-	-	-	-	-
101	MAL2089	0	0	4.7	0.7	0.3	56.0	32	14	2632
102	MAL1009	0	0	12.0	0.4	8.0	63.0	48	952	7497
103	MAL2090	1	0	4.5	0.8	4.0	74.0	36	180	3330
104	MAL1056	1	0	7.4	0.7	3.0	56.0	51	222	4144
105	MAL1057	0	0	7.0	0.1	0.5	90.0	7	35	6300
106	MAL2091	1	0	5.5	1.0	1.0	59.0	55	55	3246
107	MAL1058	1	0	7.9	0.4	2.0	74.0	31	158	5846
108	MAL2030	0	1	3.1	1.0	9.0	44.0	31	279	1364
109	MAL2092	0	0	7.0	0.1	0.3	89.0	7	21	6230
110	MAL1060	1	0	7.0	0.9	1.0	71.0	63	70	4970
111	MAL0007	0	0	5.3	0.4	6.0	75.0	21	318	3975
112	MAL2094	1	0	5.1	0.7	1.0	69.0	35	51	3519
113	MAL2094-2	1	0	4.8	0.5	1.0	61.0	24	48	2928
114	MAL2095	0	0	5.6	0.3	5.0	72.0	16	280	4032
115	MAL2096	0	0	5.3	0.4	4.0	80.0	21	212	4240
116	MAL2097	0	0	5.4	0.2	5.0	82.0	10	270	4592
117	MAL022	1	0	2.5	1.0	0.4	83.0	25	10	2075

118	MAL2012	0	0	4.0	-	-	-	-	-	-
119	MAL1063	1	0	6.2	0.3	1.0	62.0	18	62	3844
120	MAL2097-2	1	0	5.6	0.1	0.7	76.0	5	39	4104
121	MAL1064	1	0	6.3	0.3	7.0	73.0	18	441	4599

Appendix 3. (Continued)

122	MAL023	1	0	7.7	0.2	0.5	76.0	15	38	5852
123	MAL2022	0	0	4.0	-	-	31.0	-	-	1240
124	MAL1065	1	0	4.1	1.0	1.0	45.0	41	41	1845
125	MAL2098	0	0	7.1	0.5	1.0	63.0	35	71	4473
126	MAL2099	1	0	8.5	0.9	7.0	59.0	76	595	5015
127	MAL1066	0	0	3.7	0.7	1.0	60.0	25	37	2220
128	MAL1067	1	0	6.0	1.0	0.7	62.0	60	42	3720
129	MAL1014	0	0	9.4	0.1	0.7	89.0	9	65	8366
130	MAL1070	0	0	6.9	1.0	3.0	49.0	69	207	3381
131	MAL2100	0	0	5.5	1.0	0.5	58.0	55	27	3190
132	MAL1071	1	0	5.3	0.8	3.0	42.0	42	159	2226
133	MAL1072	0	0	5.0	2.0	0.7	57.0	100	35	2850
134	MAL1073	0	0	1.4	1.0	0.4	61.0	14	7	854
135	MAL2028	0	0	12.4	0.7	34.0	51.0	86	4588	6324
136	MAL1074	1	0	1.8	1.0	1.0	57.0	18	18	1026
137	MAL1076	1	0	2.8	1.0	0.1	71.0	28	2	1988
138	MAL1078	1	0	4.0	0.6	1.0	60.0	24	40	2400
139	MAL1079	1	0	4.9	1.0	1.0	49.0	49	49	2401
140	MAL2103	0	1	4.5	0.5	2.0	67.0	23	90	3015
141	MAL2104	1	0	5.7	1.0	1.0	68.0	57	57	3876
142	MAL2105	0	0	6.0	1.0	5.0	52.0	60	300	3120
143	MAL2106	1	0	5.5	1.0	16.0	33.0	55	880	1815
144	MAL2107	1	0	4.9	0.5	1.0	86.0	25	49	4214
145	MAL2108	1	1	1.9	1.0	4.0	72.0	19	76	1368
146	MAL2109	1	0	7.7	0.6	0.6	78.0	46	46	6006
147	MAL1080	1	0	4.1	0.9	3.0	64.0	36	123	2624
148	MAL2110	1	0	2.5	2.0	2.0	17.0	50	50	425
149	MAL2111	1	0	3.5	1.0	0.8	69.0	35	28	2415
150	MAL2033	0	0	6.7	0.8	4.0	65.0	33	168	2730
151	MAL2025	0	0	6.0	1.0	2.0	64.0	60	120	3840
152	MAL2035	0	0	5.6	1.0	10.0	40.0	56	560	2240
153	MAL2038	0	0	2.6	1.0	2.0	65.0	26	52	1690
154	MAL2042	0	0	6.7	0.5	0.5	83.0	38	38	5561
155	MAL0008	1	1	1.2	1.0	3.0	49.0	12	36	588
156	MAL2039	0	0	4.1	0.8	2.0	44.0	32	82	1804
157	MAL2029	0	1	2.6	0.6	7.0	10.0	15	182	260

158	MAL2017	1	0	5.1	1.0	2.0	70.0	51	102	3570
159	MAL2043	0	0	5.8	2.0	2.0	48.0	116	116	2784
160	MAL1016	1	0	3.1	1.0	7.0	50.0	31	217	1550
161	MAL2046	0	0	8.2	0.3	0.6	86.0	24	49	7052
162	MAL1020	0	1	4.1	0.9	3.0	65.0	36	123	2665

Appendix 3. (Continued)

163	MAL2048	0	0	5.6	0.6	1.0	85.0	33	56	4760
164	MAL1061	1	0	7.7	0.4	1.0	70.0	30	77	5390
165	MAL1017	1	0	8.2	0.2	1.0	92.0	16	82	7544
166	MAL1019	0	0	3.9	1.0	1.0	47.0	39	39	1833
167	W7350-T	0	1	2.8	0.0	9.0	55.0	0	252	1540
168	W7856-S	0	0	2.1	2.0	15.0	37.0	42	315	777
169	WP682-N	0	0	4.0	1.0	6.0	44.0	40	240	1760
170	W7731-A	0	1	5.1	1.0	11.0	41.0	51	561	2091
171	W7630-J	0	1	4.8	1.0	14.0	56.0	48	672	2688
172	W7722-S	0	1	5.4	1.0	26.0	46.0	54	1404	2484
173	W7709-U	0	0	7.1	1.0	13.0	49.0	71	923	3479
174	W7849-P	0	1	6.7	1.0	8.0	34.0	67	536	2278
175	WP349-W	0	1	4.7	0.0	6.0	62.0	0	282	2914
176	W7118-N	0	1	8.6	1.0	18.0	49.0	86	1548	4214
177	W7862-G	0	0	3.9	0.0	4.0	62.0	0	156	2418
178	W7860-K	0	0	7.6	1.0	3.0	55.0	76	228	4180
179	WP651-C	0	0	4.2	1.0	4.0	64.0	42	168	2688
180	W7793-U	0	1	6.7	1.0	15.0	55.0	67	1005	3685
181	W7013-N	0	0	2.3	1.0	2.0	57.0	23	46	1311
182	W7067-J	0	0	9.4	1.0	18.0	37.0	94	1692	3478
183	W7089-G	0	0	7.2	1.0	19.0	31.0	72	1368	2232
184	W7092-J	0	0	4.5	1.0	3.0	56.0	45	135	2520
185	W7102-C	0	1	5.5	1.0	3.0	35.0	55	165	1925
186	W7114-D	0	1	3.4	1.0	9.0	47.0	34	306	1598
187	W7139-H	0	0	8.2	1.0	9.0	60.0	82	738	4920
188	W7140-T	0	1	5.0	1.0	5.0	36.0	49	245	1764
189	W7152-S	0	1	7.2	1.0	6.0	45.0	72	432	3240
190	W7168-U	0	1	6.1	1.0	4.0	48.0	61	244	2928
191	W7173-A	0	1	4.2	2.0	5.0	39.0	84	210	1638
192	W7184-Y	0	0	5.5	1.0	5.0	63.0	55	275	3465
193	W7216-R	0	0	6.5	0.0	11.0	45.0	0	715	2925
194	W7248-X	0	1	6.7	1.0	6.0	55.0	67	402	3685
195	W7264-V	0	1	7.4	0.0	11.0	59.0	0	814	4366
196	W7278-L	0	1	5.1	1.0	4.0	53.0	51	204	2703
197	W7288-V	0	1	6.9	0.0	0.0	71.0	0	0	4899

198	W7294-J	0	1	6.1	1.0	4.0	59.0	61	244	3599
199	W7303-B	0	1	6.0	0.0	1.0	61.0	0	60	3660
200	W7304-D	0	1	5.6	1.0	27.0	28.0	56	1512	1568
201	W7305-Y	0	0	5.1	0.0	6.0	45.0	0	306	2295
202	W7325-G	0	1	5.8	1.0	3.0	68.0	58	174	3944
203	W7346-L	0	0	4.3	1.0	10.0	38.0	43	430	1634

Appendix 3. (Continued)

204	W7373-L	0	1	6.4	1.0	11.0	62.0	64	704	3968
205	W7400-M	0	1	5.4	1.0	3.0	53.0	54	162	2862
206	W7401-C	0	1	4.8	1.0	5.0	33.0	48	240	1584
207	W7410-B	0	1	5.9	0.0	3.0	64.0	0	177	3776
208	W7437-H	0	1	3.9	1.0	8.0	67.0	39	312	2613
209	W7452-G	0	1	5.2	0.0	5.0	49.0	0	260	2548
210	W7489-T	0	0	6.9	1.0	1.0	66.0	69	69	4554
211	W7503-G	0	1	7.0	1.0	7.0	56.0	68	476	3808
212	W7511-D	0	0	6.0	0.0	2.0	68.0	0	120	4080
213	W7557-S	0	1	5.2	1.0	27.0	36.0	52	1404	1872
214	W7620-N	0	0	5.1	1.0	10.0	46.0	51	510	2346
215	W7002-H	0	0	5.9	0.0	2.0	54.0	0	118	3186
216	W7621-P	0	1	3.7	1.0	14.0	30.0	37	518	1110
217	W7649-Y	0	0	4.2	1.0	9.0	42.0	42	378	1764
218	W7669-F	0	0	4.7	1.0	11.0	48.0	47	517	2256
219	W7689-C	0	1	5.3	0.0	18.0	59.0	0	954	3127
220	W7693-U	0	1	7.3	1.0	18.0	53.0	73	1314	3869
221	W7716-S	0	1	7.1	0.0	4.0	66.0	0	284	4686
222	W7727-Q	0	0	4.7	1.0	5.0	35.0	47	235	1645
223	W7742-P	0	0	6.8	0.0	5.0	70.0	0	340	4760
224	W7753-D	0	0	5.2	1.0	10.0	50.0	52	520	2600
225	W7775-G	0	1	4.5	0.0	13.0	54.0	0	585	2430
226	W7003-Y	0	0	1.9	1.0	4.0	30.0	19	76	570
227	W7004-D	0	0	5.0	1.0	12.0	46.0	50	600	2300
228	W7818-S	0	0	4.4	1.0	9.0	44.0	44	396	1936
229	W7823-Q	0	1	3.8	1.0	10.0	54.0	38	380	2052
230	W7831-C	0	0	5.5	1.0	14.0	54.0	55	770	2970
231	W7842-Q	0	0	4.7	1.0	5.0	29.0	47	235	1363
232	W7853-M	0	1	6.2	0.0	9.0	59.0	0	558	3658
233	W7854-P	0	0	8.9	1.0	3.0	61.0	89	267	5429
234	W7861-J	0	0	3.4	2.0	4.0	51.0	68	136	1734
235	W7864-H	0	0	8.0	1.0	13.0	50.0	80	1040	4000
236	W7866-A	0	0	5.6	1.0	4.0	66.0	56	224	3696
237	W7869-J	0	0	9.0	1.0	13.0	56.0	90	1170	5040

238	W7870-G	0	0	7.5	0.0	1.0	67.0	0	75	5025
239	WP188-P	0	0	8.9	1.0	11.0	31.0	89	979	2759
240	W7014-H	0	0	8.5	0.0	13.0	57.0	0	1105	4845
241	WP208-W	0	0	5.0	1.0	4.0	63.0	45	180	2835
242	WP211-W	0	0	4.4	1.0	5.0	51.0	44	220	2244
243	WP275-S	0	0	4.9	1.0	12.0	43.0	49	588	2107
244	WP400-N	0	0	6.1	2.0	13.0	44.0	122	793	2684

Appendix 3. (Continued)

245	WP793-V	0	1	3.8	1.0	8.0	51.0	38	304	1938
246	W7019-P	0	0	5.4	1.0	6.0	65.0	54	324	3510
247	W7027-E	0	0	7.2	0.0	3.0	64.0	0	216	4608
248	W7028-R	0	1	3.6	2.0	14.0	27.0	66	462	891
249	W7048-L	0	0	4.8	1.0	2.0	52.0	48	96	2496
250	W7051-P	0	1	2.8	1.0	2.0	53.0	28	56	1484
251	W7066-R	0	1	3.8	1.0	11.0	44.0	38	418	1672
252	W7090-F	0	0	5.9	1.0	9.0	51.0	59	531	3009
253	W7091-V	0	0	3.5	1.0	14.0	24.0	35	490	840

Note:

Lab-id started with MAL = malaria patients; Lab-id started with W = controls; pf =1 refers falciparum patients; pf = 0 is vivax patients; HIV= 1 is positive, and HIV = 0 is negative for the virus; - = not done; baso = Basophil; eosi = Eosinophil; neut = Neutrophil.

Appendix 4. Patient record form

MALARIA IMMUNOLOGICAL

PATIENT HISTORY RECORD FORM

DATE----------

SITE----------

SUBJECT ID	AGE	SEX	TEMPRETURE ^{0}C	FEVER	REMARK

FEVER = Y= YES

N= NO

PAGE----------