RISK FACTORS AND EFFECTS OF ANTIMALARIAL DRUGS ON *PLASMODIUM FALCIPARUM* GAMETOCYTAEMIA IN NIGERIAN CHILDREN

ADEDEJI, Adebowale Ahmed
Matric No 71919

RISK FACTORS AND EFFECTS OF ANTIMALARIAL DRUGS ON *PLASMODIUM FALCIPARUM* GAMETOCYTAEMIA IN NIGERIAN CHILDREN

BY

ADEDEJI, Adebowale Ahmed
Matric No. 71919

BSc Biochemistry (OOU), M.Sc. Pharmacology (Ibadan), MPhil Pharmaceutical Chemistry (Ibadan)

A dissertation in the Department of Pharmacology and Therapeutics,
Submitted to the Faculty of Basic Medical Sciences
in partial fulfillment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY

of the

UNIVERSITY OF IBADAN

JULY 2007

ABSTRACT

Gametocytes are the sexual forms of Plasmodium species that are essential for the transmission of malaria. However, there is little information on the factors that influence their generation or carriage, a measure of transmission potential of *Plasmodium falciparum*, in Nigerian children. The objectives of the studies were to determine (1) the risk factors for gametocyte carriage and effects of seasons on carriage, in children with acute falciparum malaria, and (2) the effects of selected antimalarial drugs on gametocyte carriage, intensities of carriage, morphological stages and sex ratio changes (GSR).

Overall, 2,317 symptomatic children (M:F= 1024:1293), aged 0.5-14 years, with microscopically-confirmed *P. falciparum* malaria, treated with standard doses of the antimalarial drugs (chloroquine (CQ), pyrimethamine-sulfadoxine (PS), trimethoprimsulfamethoxazole (TS), chloroquine plus chlorpheniramine (CQCP), pyrimethamine-sulfadoxine plus probenecid (PSP) and amodiaquine (AQ) plus pyrimethamine-sulfadoxine, AQPS) were studied. Before, during and following therapy, densities of asexual parasites and of the morphologically distinct developmental stages of gametocytes were quantified in blood over 14 –28 days, using standard methods. Temporal changes in density ratio of Peripheral Young Gametocytes (PYG): Peripheral Mature Gametocyte (PMG) was used as an index of continuing gametocyte generation after treatment. GSR, defined as the proportion of gametocytes that were morphologically males, was also quantified serially over 14 days. CQ blood concentrations were determined by high performance liquid chromatography. Responses of infections to therapy were classified as sensitive or resistant. Data were analyzed using student t- test, analysis of variance, chi square, Kaplan Meier survival test and Multiple logistic regression models.

Gametocyte carriage in the recruited patients was 15% at enrolment. Four factors were found to be independent risk factors for gametocyte carriage at presentation: male gender (adjusted odd ratio, AOR=0.55, 95% Confidence Interval, CI= 0.36-0.83, P= 0.005), absence of fever (AOR= 1.61, 95% CI= 1.05-2.5, P= 0.03), duration of illness >3d (AOR=1.57, 95%, CI= 1.0-2.4, P= 0.047), and asexual parasitaemia <5000/µl (AOR=0.04, 95% CI= 0.02-0.07, P< 0.05). However, 15.6% of all the children developed patent gametocytaemia following treatment. Except for

male gender in the low transmission season, the identified risk factors for gametocyte carriage were little affected by season. A total of 220 children failed to respond to antimalarial drug treatment. Children with CQ-resistant infections (37.1%), and those treated with PS irrespective of outcome, were significantly at risk of gametocyte carriage compared with other antimalarials. CQCP-resistant infection and treatment with PS significantly increased PYG-PMG ratio ($P < 0.05$). In contrast, AQPS significantly reduced the ratio. PSP and TS, like PS, alone, enhanced gametocyte carriage and increased GSR.

Antimalarial drugs modulate gametocyte generation particularly when resistance has developed and may potentially facilitate disease transmission. Presence of peripheral young gametocytes (PYG) was an indicator of resistance to CQCP but not to PS or AQPS. PS may enhance influx of young gametocyte into circulation.

Keywords: *Plasmodium falciparum*, Gametocytaemia, Antimalarial drugs, Transmission, Children (word count=459).

DEDICATION

To

Allah, The Almighty, who makes all things possible

His Rasul

His Dinn-my course of struggle, and

Liberation of the Oppressed all over the world

ACKNOWLEDGMENTS

My sincere appreciation goes to my supervisor, Professor A. Sowunmi, for supporting me at all times and skillfully guiding me during the studies. His financial and moral assistance to a large extent made this project a success.

I also wish to thank Professor A.M.J. Oduola for his care and support in making a career, and Dr. Fehintola F.A. for his continuous concern and support during the studies. I sincerely appreciate the moral support of Dr. G.O. Gbotosho, Dr. O.A.T. Ogundahunsi, Dr. T.C. Happi, Dr. P.I. Azuba, and all staff of Department of Pharmacology both at the University of Ibadan and Obafemi Awolowo University, Ile-Ife and Ikeja Campuses for their assistance throughout the duration of the studies. I specially thank Mr. Babasola A. Fateye, my colleague, for his excellent support and contributions to the studies over the last five years, and Mrs. Moji Amoo, Mrs. Bose Adekoya and Miss Adeola Alabi - for their immense efforts in running the clinics from which the children enrolled in the studies were recruited.

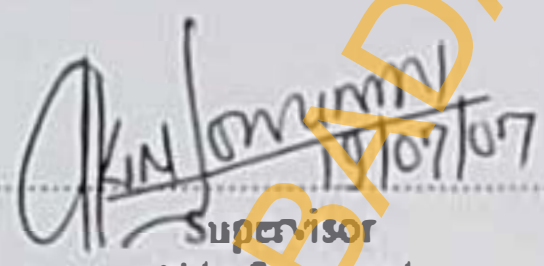
I am very grateful to Mr. Ernest Tembo, Mr Abayomi Sijuade and the entire staff of the Malaria Research laboratories for their encouragement.

I am grateful to my wife for her unquantifiable forbearance, support and patience, and my children who I dream will take over from me (Insha Allah). I thank my numerous friends, beloved ones of the struggle, my clinn and its leadership that has been, by the grace of my Lord, ALLAH, instrumental to making the person I am today, for their support when I needed them and efforts in the realization of my dreams. My love and gratitude go to my special family, my father and mother, the Adedejis, Mrs Gbadebos and others. I pray God continue to support and protect you all. Unto HIM I commit myself and unto HIM I give all thanks and adorations.

The studies received financial support from UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, in particular the Career Development Grant awarded to Prof. A. Sowunmi.

CERTIFICATION

I certify that this work was carried out by Mr. A.A. Adedeji in the Department of Pharmacology and Therapeutics, University of Ibadan



Supervisor
Akin Sowunmi
MB BS, M.Sc., MD, FMCP, FWACP, FRCPath (Lond), FRCPI
Professor of Pharmacology, Department of Pharmacology and Therapeutics
University of Ibadan, Nigeria

TABLE OF CONTENTS

Original papers

This dissertation is supported by the following original papers (see also Appendix):

I Sowunmi, A., Fateye, B.A., Adedeji, A.A., Fehintola, F.A. and Happi, T.C. 2004. Risk factors for gametocyte carriage in uncomplicated falciparum malaria in children. *Parasitology* 129: 255-262.

II Sowunmi, A., Adedeji, A.A., Fateye, B.A., and Babalola, C.P. 2004. *Plasmodium falciparum* hyperparasitaemia in children: risk factors, treatment outcomes and gametocytaemia following treatment. *Parasite* 11: 317-323.

III. Adedeji, A.A., Fehintola, F.A., Fateye, B.A., Happi, T.C., Amoo, A.O.J. and Sowunmi, A. 2005. *Plasmodium falciparum* malaria in Nigerian children during high and low transmission seasons: gametocyte carriage and response to oral chloroquine. *Journal of Tropical Pediatrics* 51: 288-294.

IV. Adedeji, A.A, Fateye, B.A., Amoo, A.O.J. and Sowunmi, A. 2006. Response to chloroquine treatment in children with or without gametocytes during uncomplicated *Plasmodium falciparum* malaria. *European Journal of Clinical Microbiology and Infectious Diseases*, 25: 126-128.

V. Sowunmi, A., Fehintola, F.A., Adedeji, A.A., Gbotosho, G.O., Falade, C.O., Tambo, E., Fateye, B.A., Happi, T.C. and Oduola, A.M.J. 2004. Open randomized study of pyrimethamine-sulfadoxine versus pyrimethamine-sulfadoxine plus probenecid for the treatment of uncomplicated *Plasmodium falciparum* malaria in children. *Tropical Medicine & International Health* 9: 606-614.

VI. Sowunmi, A., Adedeji, A.A., Fateye, B.A. and Fehintola, F.A. 2004. Comparative effects of pyrimethamine-sulfadoxine, with or without probenecid, on *Plasmodium falciparum* gametocytes in children with acute, uncomplicated malaria. *Annals of Tropical Medicine & Parasitology* 98: 873-878.

VII Sowunmi, A., Adedeji, A.A., Gbotosho, G.O., Fateye, B.A. and Happi, T.C. 2006. Effects of pyrimethamine-sulfadoxine, chloroquine plus chlorpheniramine, and amodiaquine plus pyrimethamine-sulfadoxine on gametocytes during and after treatment of acute, uncomplicated malaria in children. *Memorias Instituto Oswaldo Cruz* 101: 887-893.

VIII Sowunmi, A., Fateye, B.A., Adedeji, A.A., Fehintola, F.A., Bamgboye, A.E., Babalola, C.P., Happi, T.C. and Gbotosho, G.O. 2005. Effects of antifolates-cotrimoxazole and pyrimethaminesulfadoxine on gametocytes in children with acute, symptomatic, uncomplicated, *Plasmodium falciparum* malaria. *Memorias Instituto Oswaldo Cruz* 100: 451-455.

LIST OF TABLES

LIST OF FIGURES

GLOSSARY OF ABBREVIATIONS

af	asexual forms
ANOVA	analysis of variance
AQ	amodiaquine
AQPS	amodiaquine plus pyrimethamine-sulphadoxine
AUC_{gm}	area under the curve of gametocytaemia versus time
°C	degree celcius
Ca^{++}	Calcium ion
CI	confidence interval
Cl^-	Chloride ion
CL_{ug}	volume of blood completely cleared of micro- or macro-gametocytes per unit time
cGMP	cyclic guanosine monophosphate
COM	combination antimalarials or pyrimethamine-sulfadoxine combined with chloroquine or amodiaquine
CQ.	chloroquine
CQCP,	chloroquine plus chlorpheniramine
d	day
F	female
FCT	fever clearance time
FGD	fractional gametocyte density
GMGD	geometric mean gametocyte density
GMPMGD	geometric mean peripheral mature gametocyte density
GMPYGD	geometric mean peripheral young gametocyte density
GSR	gametocyte sex ratio
h, hr	hour
HCO_3^-	bicarbonate ion
HCl	hydrochloric acid
HF	halofantrine
HPLC	high performance liquid chromatography
HTS	high transmission season

IQR	interquartile range
K^+	potassium ion
kg	kilogramme
LTS	low transmission season
M	male
M	molar
min	minute
mm	millimeter
n	number
N	normal
NaOH	sodium hydroxide
ns	not significant
OR	odds ratio
P	level of significance
PCR	polymerase chain reaction
PCT	parasite clearance time
PCV	packed cell volume
PMG	peripheral mature gametocyte
PSP, PPS	pyrimethamine-sulfadoxine combined with probenecid
PRR	parasite reduction ratio
PS	pyrimethamine-sulfadoxine
PYG	peripheral young gametocyte
QT-NASBA	quantitative nucleic acid sequence-based amplification
RBC	red blood cells
RI	resistance response type I (mild resistance)
RII	resistance response type II (moderate resistance)
RIII	resistance response type III (severe resistance)
RR	relative risk
S	sensitive response
sd	standard deviation
se	standard error
$t_{1/2}$	apparent half life
$t_{1/2ga}$	apparent half-life of gametocytaemia

Temp	temperature
TS	trimethoprim-sulfamethoxazole
µl	microliter
UV	ultraviolet
vs	versus
WBC	white blood cells
WHO	World Health Organization
χ^2	chi square

Chapter 1

Introduction

CHAPTER 1

Introduction

Plasmodium falciparum, which causes malignant tertian malaria, is the most lethal in man, accounting for majority of malaria attributable morbidity and mortality globally, especially amongst children, pregnant women and non-immune travelers in the tropics (Gilles & Warrell, 1993; Trape, 2001; WHO, 2003). Of the estimated one million deaths reported annually from malaria, approximately 80% occur in young African children. Infants are vulnerable to malaria from approximately 3 months of age, when immunity acquired from the mother starts to wane (WHO, 2003). In southwest Nigeria, *Plasmodium falciparum* is responsible for greater than 95% of the malaria infections (Salako et al., 1990).

The Roll back malaria programme for global malaria control strategies includes early diagnosis and treatment, development of appropriate preventive measures including vector control, and development of local technical capacity (WHO, 2003). However, intense transmission, urbanization, increasing resistance to antimalarial drugs and unusually favourable transmission conditions have hampered the progress in malaria control and have led to unexpected epidemics. In addition, large mosquito populations and ready availability of gametocytes in peripheral blood have made transmission inevitable. It is noteworthy that little information is available on the dynamics of *Plasmodium falciparum* gametocytaemia in Nigerian children.

1.1 Gametocyte development in man

The over 170 species of Plasmodia parasites undergo both sexual and asexual phases (Paul and Bray, 2003). Gametocytes arise from erythrocytic asexual stages. Contrary to production of gametocytes directly from hepatic merozoites as described

in other species (Garnham, 1966). *Plasmodium falciparum* gametocytes (Figure 1.1) arise from mature asexual forms. The mechanism of the switch from asexual to sexual stage and its modulation are complex and incompletely understood (Carter and Miller, 1979; Mons, 1985). These mature asexual stages are absent from peripheral circulation due to cytoadherence of their carrier erythrocytes to microvascular endothelia of organs and tissues, such as heart, lung, liver, skin and brain (MacPherson et al., 1985) to avoid phagocytic clearance from spleen during maturation. The resulting young gametocytes from committed merozoites avoid peripheral blood and sequester preferentially in the bone marrow and spleen (Thomson and Robertson, 1935; Smalley et al., 1980). Bruce and colleagues (1990) showed that merozoites emerging from a single schizont developed further either into asexual stages or into gametocytes. Smith et al. (2000) and Silvestrini et al. (2000) demonstrated that the gametocytes from one schizont are all male or all female. This observation suggests that the trophozoites of the preceding asexual generation were already committed to either sexual development or continuing asexual cycling. Thus, overall, studies on gametocytogenesis consistently support the hypothesis that Plasmodium is committed to sexual development in the preceding asexual generation rather than differentiating following invasion of the erythrocyte by uncommitted merozoites (Carter and Miller, 1979; Inselberg, 1983; Mons, 1986; Bruce et al., 1990).

Field and Shute (1956) were first to describe five different maturation stages of *P. falciparum* gametocytes. The steps were further characterized, using light microscopy by Hawking and co workers (1971) in *P. falciparum*-infected Aotus monkeys. Gametocytes growth process is in stages, I–V, (Table 1.1) and maturation process has been described in rodent parasites and *P. falciparum*. Immature gametocytes of *P. falciparum* (stages I-III) are sequestered in deep tissues of the body, notably the bone narrow (Smalley et al., 1981). One of the most striking features of gametocytes is the presence of pellicular complex, which originates from a small membraneous vesicle beneath the gametocyte plasmalema in late stage I. This structure is absent from asexual stages. It consists of a subpellicular membraneous vacuole subtended by an array of longitudinally- oriented microtubules (Sinden, 1983). This strengthens the parasite and explains the lack of

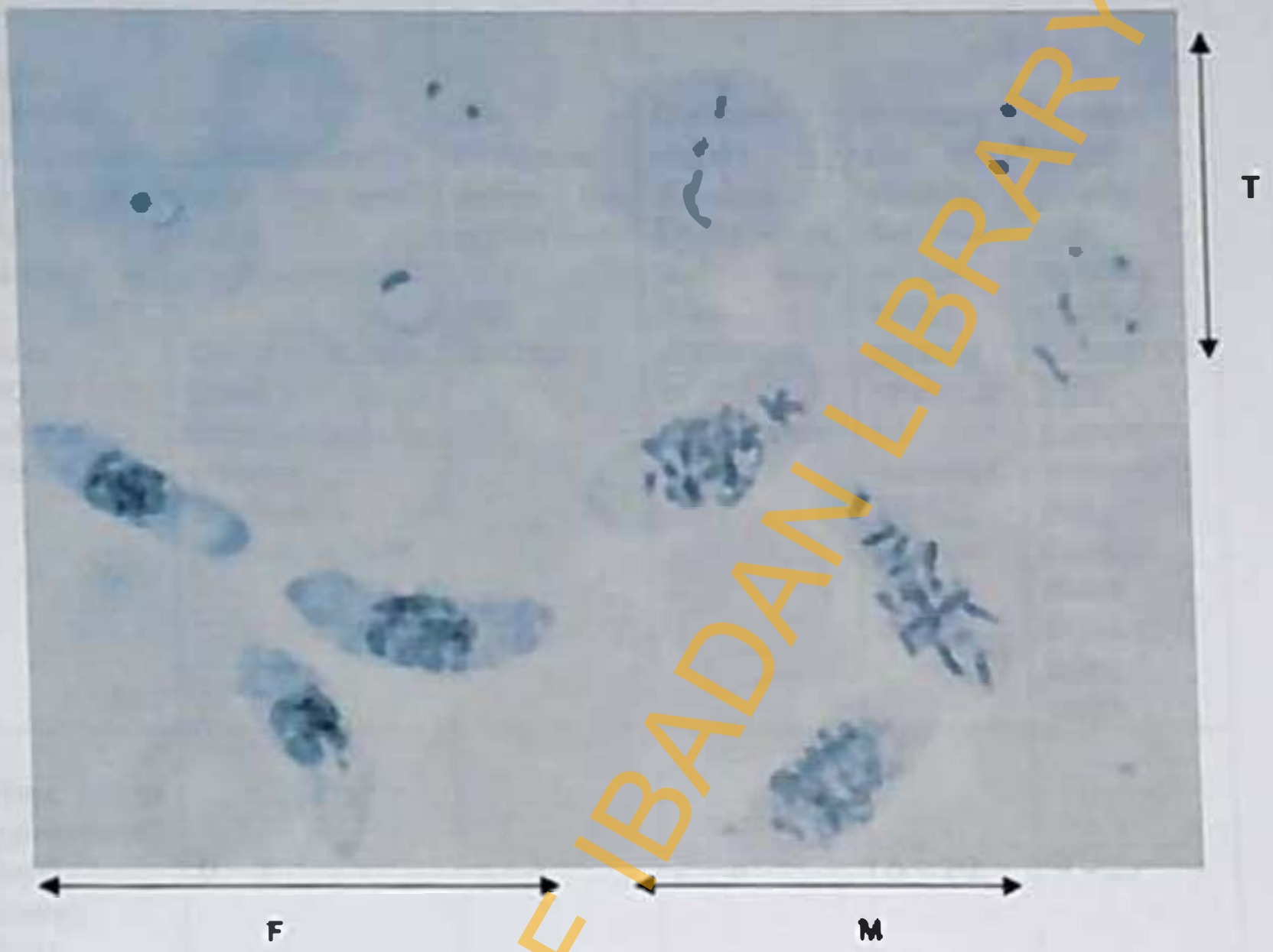


FIGURE 1.1. Mature female (F) and male (M) gametocytes, and trophozoites (T) of *Plasmodium falciparum* in the blood of malaria-infected patient. This picture is a composite of several pictures originating from the same Giemsa-stained thin smear. (From Talman et al., 2004b).

TABLE 1.1. Morphology of gametocytogenesis

	Stage I	Stage II	Stage III	Stage IV	Stage V
Shape (light microscopy) Day et al., 1998; Hawking et al., 1971; Sinden, 1982, Sinden, 1998)	IA: Indistinguishable from the small round trophozoite. IB: Larger round shape distinguished by granular distribution of pigment in food.	IIA: Elongates within the erythrocyte. IIB: D-shape	D-shaped, slightly distorted. Erythrocytes with pink /blue distinction of male/female	Elongate and thin parasite, distorted red cell, Male: pigment tends to be scattered. Female: pigment more dense	Sausage-shaped parasite with rounded/ angular extremities- Male: pigmented scattered pink. Female: dense pigment, light violet.
Time of appearance* (days). (Sinden, 1998)	0 – 2	1 – 4	2 – 8	6 – 10	9 – 23
Point in cell cycle (Sinden and Smalley, 1979)	G1	G1	G0	G0	G0

G0, a temporary resting period or more permanent; G1, cells increase in size, produce RNA and synthesize protein. * time refer to the period after sequestration

amoeboid forms in the asexual parasite (Langreth et al., 1978). The function of this structure is largely unknown.

Gametocyte development takes longer than asexual schizogony, for example, 26 hr as opposed to 22.5 hr in *P. berghei* (Mons et al., 1985) or in the more extreme case of *P. falciparum* 8-12 days as opposed to 48 hr (Sinden, 1983). The proportion of parasites that develop into gametocytes varies greatly during the course of natural infections, even at its peak, but is very low in relation to the total parasitaemia (Smalley et al. 1981) and is reversed during recrudescent infections (Sowunmi and Fateye, 2003 a).

The growth and differentiation of gametocytes of *P. falciparum* spans a period of eight days from merozoite invasion to mature gametocyte, each stage of the gametocyte has been distinguished by successive changes in the organization of the cell (Hawking et al., 1971). The frequency of conversion of asexual parasite to sexual development varies within parasite clones and differs consistently between clones (Carter and Miller, 1979; Graves et al., 1984). These findings suggest that both innate and environmental factors may predispose or trigger the parasite switch from asexual to sexual development (Carter and Miller, 1979; Graves et al., 1984, Bruce et al., 1990).

In a culturing experiment, Suhrbier et al. (1987) showed that sexual commitment is highest in the merozoites derived directly from liver schizont. Day et al. (1993) also showed that continuous asexual passaging was identified as a factor that may lead to sexual commitment. The latter has correlates with accumulated chromosomal breakage and resortment (Frontali, 1994; Bingo et al., 1982) and could be particularly important if the sexual stage genes in Plasmodium are concentrated in certain chromosome, for example, chromosome 12 of *Plasmodium falciparum*.

The investment in securing a mate and the risk of mixing genes with another are noteworthy costs associated with sexual reproduction but at disadvantage compared to asexual reproduction (Dyer and Day, 2000; Otto and Lenormand, 2002). Therefore gametocytogenesis in *P. falciparum* and favourable conditions may

contribute to severity of the burden of the infections as newer strains with considerable biologic difference from original strains may emerge. This will considerably add to the increasing epidemiologic burden of the infection.

Paucity of specific markers that can identify the immature gametocytes or could be used to track the development made full elucidation of the process of gametocytogenesis difficult. Although, there are genes that are expressed later in gametocytogenesis, these were of little usefulness for these studies. The genes, *Pf 11-1* (Scherf et al., 1992; Feng et al., 1993) and *tubulin II* (Rawlings et al., 1992), *Pfg27* (Carter et al., 1989, Pologe, 1994) and the S form of RNA (Thompson and Sinden, 1994, Waters et al., 1989) are expressed significantly after commitment to gametocytogenesis has taken place. Upregulation of the mRNAs for the early gametocyte markers *Pfs16* and *Pfg27* was readily detected in 3D7 parasite line (Silverstrini et al., 2005). One hundred and seventeen genes had expression profiles that correlated to those of *pfs16* and *pfg27*. The northern blot analysis and published proteomic data have been used to identify those proteins whose expression was gametocyte-specific. Immunoflourescence analysis with antibodies against two of these gene products identified two novel parasite membrane proteins associated with sexual stage specific proteins (Silverstrini et al., 2005). One was produced from stage I gametocytes and the second showed peak production in the stage II gametocytes (Silverstrini et al., 2005). The two proteins were named Pfpeg-3 and Pfpeg-4, as *P. falciparum* proteins of early gametocytes (Silverstrini et al., 2005). The search for more molecular markers that will enable improved comprehension of the process of gametocytogenesis is on-going.

1.2 Gametocyte carriage in man

In human, the presence of gametocyte in peripheral blood, gametocytaemia, arises 7-15 days after the initial asexual wave (Eichner et al., 2001; Day et al., 1998) compared to other human species with 1 to 3 days. The ratio of gametocytes to asexual stages in *P. falciparum* is less than 1:10 (Kitchen and Putnam, 1942, Sinden, 1983, Carter and Graves, 1988). Eichner et al (2001) even reported a much lower ratio (1:156). The half-life of the mature gametocyte in blood is reported to be 2.4 days (Smalley and Sinden, 1977) but longer half life and consequent longevity in

blood stream may also occur, for example up to 4 weeks. (Smalley and Sinden, 1977). Eichner et al. (2001) and Sowunmi and Fateye (2003b) also estimated mean circulation time to be 6.4 days or more. Because the mechanism of the switch from asexual to sexual stages is incompletely understood it is necessary to study the dynamics of gametocytes in both host and mosquito vector.

1.3 Studies on gametocyte carriage in man

In sub Saharan Africa, antimalarial drugs used in the treatment of the infection and increasing drug resistance in *P. falciparum* to these drugs have been thought to contribute to gametocyte carriage in man and gametocyte infectivity to mosquitoes (Robert et al., 1996 a, 2000; Hogh et al., 1998). Children, in general, constitute a significant risk group and reservoir of the infection in sub-Saharan Africa (Githeko et al., 1992; Bonnet et al., 2003) with observed pretreatment gametocyte carriage rate during the acute falciparum infections, in West Africa, ranging between 14-17% (von Seidlein et al., 2001).

In Nigeria, the development and increase in antimalarial drug resistance (Falade et al., 1997; Sowunmi et al., 1998 a, b; Sowunmi, 2002) has been associated with increases in gametocyte carriage in children (Sowunmi and Fateye, 2003 a, b). However, there is little or no information on gametocyte carriage rates and factors contributing to gametocyte carriage in Nigerian children. These factors, when identified, may provide support for control of transmission of the infection.

Several factors have been described as risk factors for gametocyte carriage in children in Africa and Asia. In Tanzania, Akim et al. (2000) reported young age, high asexual parasitaemia on presentation and gametocyte positivity on presentation as risk factors for gametocyte carriage in children with acute malaria infections. In children, in The Gambia, hyperparasitaemia, anaemia at enrollment, age, season and location of study site were identified as independent risk factors for gametocyte carriage before treatment (von Seidlein et al., 2001). The development or persistence of gametocytemia during follow up, patent gametocytemia on admission, anaemia, no coincident *P. vivax* malaria infection, presentation with a recrudescent infection and a history of illness longer than two days were risk factors for gametocyte

Chloroquine (CQ) and pyrimethamine-sulfadoxine (PS) are schizonticidal and both have oo effects on mature gametocytes. However, CQ and PS are active against young gametocytes before their appearance in the peripheral circulation (Smalley, 1977; Butcher, 1997). Although it was previously thought that during treatment, CQ or PS does not induce gametocytogenesis, it is now well known that CQ increases gametocytogenesis in *P. chabaudi in-vivo* and *P. falciparum in-vitro* (Buckling et al., 1997, 1999). Pyrimethamine–sulfadoxine, which inhibit dihydrofolate reductase and dihydropteroate synthase, may damage the ookinete and reduce the number of oocyst (Robert et al., 2000), and may consequently affect gametogenesis. Hogh and others observed that CQ enhances *P. falciparum* infectivity to mosquitoes, while PS reduces it (Hogh et al., 1998).

Thus, antimalarials, amongst other factors that trigger and regulate the generation or development of gametocyte, are important and must be continuously evaluated for their impacts on gametocyte carriage in human and infectivity to mosquitoes (Butcher, 1997; Hogh et al., 1998, Chutmongkonkul et al., 1992; Handunnetti et al., 1996, Jones, 1997, Koella et al., 1998, Robert et al., 1996a; Robert and Trape, 1998).

In Nigeria, CQ and PS were antimalarial drugs of choice prior to change of treatment policy to artemisinin based combination therapy (ACTs) in 2004 (Federal Ministry of Health, 2004). Both CQ and PS are still readily available and readily used as monotherapy due to economic reasons and non-affordability of the ACTs, for example, artesunate-PS, by many individuals. It is essential to study the effects of CQ and PS or other antimalarial drugs used for treatment of malaria in endemic areas on the gametocyte generation and carriage during acute infections in children. This will provide useful information that may guide drug combination strategies and deployment with a view to minimizing transmissibility.

1.6 Gametocytes infectiousness to mosquito

Mature and functional gametocytes ingested by an appropriate species of mosquito in a bloodmeal are stimulated to transform into the stages that establish the parasites in their vector (Garnham, 1966). Under the influence of changes in the

mosquito midgut environment, the gametocytes become extracellular within 8-15 min of ingestion. Following emergence from the red blood cell (exflagellation), the male gametes fertilize the female gametes within 60 min of ingestion of blood. The fertilized macrogamete (zygote) differentiates into a single motile ookinete over the next 10-25 hr, and migrates from the bloodmeal through the midgut wall to form an oocyst underneath the basal lamina of the midgut. Each oocyst produces many thousands of invasive sporozoites over a period of 7-12 days. The sporozoites escape from the oocyst and then invade the salivary glands, where they stay for possibly very long periods until injected into another vertebrate host when the next bloodmeal is taken (Sinden, 1984; Carter and Graves, 1988).

1.7 Studies involving gametocytes infectiousness to mosquito

Various triggers are responsible for induction of gametocytes differentiation. Microgametogenesis *in vitro* is, optimally, dependent upon a rise in pH (Nijhout and Carter, 1978), a rise in CO_2 and bicarbonate levels (Carter and Nijhout, 1977; Nijhout and Carter, 1978), a fall in temperature of a few degrees below that of the vertebrate host (Sinden and Croll, 1975) or a very potent factor termed mosquito exflagellation factor (MEF). The latter is a small heat stable molecule from the mosquito head and gut that stimulates gametogenesis via a bicarbonate- and pH-independent mechanism (Nijhout, 1979). Kawamoto et al. (1991) showed *in vitro* that induction of exflagellation of *P. berghei* is triggered by a rise in the intracellular pH (pHi) that is mediated by Ca^{++} and cGMP regulation. pHi can be modulated by alkaline media and is controlled by a complex series of interdependent ion pumps and channels controlling Na^+, K^+, Cl^- and HCO_3^- transport between the parasite and the environment. Other influential factors described include cAMP analogues and inhibitors of phosphodiesterase (Martin et al., 1978). The duration of microgametogenesis is both temperature and species dependent, for example, at 20-22 °C, microgametogenesis may take 7-15 min for *P. falciparum in vitro*, although exflagellation may be detected after shorter periods in the fluid excreted by feeding *Anopheles* (Sinden, 1983). There is no evidence that exflagellation is influenced by factors released by digestion of the blood meal since digestion normally begins several hours later (Graf et al., 1986).

The microgamete formation involves three mitotic divisions with a rapid assembly of eight axonemes on the single microtubule organizing centre that divides and passes to the spindle poles. This division simultaneously segregates the genome and the axoneme so that each of the eight emergent gametes receives a single axoneme and haploid genome, both being connected to a common microtubule-organizing centre. After exflagellation the microgametes, normally bearing a single axoneme, condensed nucleus and kinetosome with its sphere and granule at the distal end, are torn from the microgametocyte surface and rapidly move away into the blood meal (Sinden and Croll, 1975).

Macrogametogenesis at the morphological level involves little more than escape from the host cell (Sinden, 1984). At the cellular level, there is *de novo* synthesis of the proteins that are expressed on the surface of macrogamete (Kumar and Carter, 1984). Scherf and co workers (1992, 1993) identified a gametocyte specific protein of *P. falciparum* called Pfl 1-1 with some evidence that this protein might be involved in the emergence of gametes from the infected erythrocyte. The contributions of these protein factors to gametogenesis are still under studies.

1.8 Studies on gametocyte development in Mosquitoes

Many factors influence the development, survival and infectivity of the parasite during its residence in the midgut lumen of the mosquito. Eyles (1952) has shown that the parasite development ceases at the ookinete stage unless a macromolecular (non- dialyzable) component is present in the blood meal. Studying the influence of red blood cells on the ability of *P. gallinaceum* zygotes fertilized *in vitro* to infect *Aedes aegypti*. Rosenberg et al. (1984) found a linear relationship between erythrocyte density and the number of oocysts up to a 50% hematocrit. Furthermore, they deduced that there are one or more nondialyzable substances (erythrocytic factors) contained in normal erythrocytes, and released by mosquito digestion, that are essential for ookinete invasion of the gut epithelium, where they further develop. In a mosquito feeding experiment with cultured *P. falciparum* (Ponnudurai et al., 1989) and *P. berghei* (Sinden, 1989), gametocytes, dilution with fresh red cells resulted in more oocysts at initial (low) dilutions whereas further dilution reduced oocyst counts.

The involvement of blood factors and/or its digestive products in infectivity has been studied in different parasite-vector models. Using a selected line of *An. stephensi*. Feldmann and Ponnudurai (1989) found mature *P. falciparum* ookinetes in the midgut lumen of refractory mosquitoes but no further penetration of the gut epithelium was observed. The reasons for this limited development in non-compatible mosquitoes could be related to digestive function, since early initiation of hemoglobin degradation and higher aminopeptidase activity have been described in refractory strains of *An. stephensi* (Feldmann et al., 1990). It has also been shown that *P. gallinaceum* develops up to the ookinete stage in the non-compatible mosquito, *An. Stephensi.* at the same time period with those infecting the compatible vector, *Ae. Aegypti.* However, *P. gallinaceum* ookinetes did not escape from the midgut lumen in *An. stephensi* mosquitoes (Rudin et al., 1991).

A possible mechanism causing inhibition of parasite development involves damage of the parasite by digestive enzymes present in the vector. The addition of trypsin inhibitor to blood meal resulted in inhibition of midgut penetration by ookinetes (Rosenberg et al., 1984). Thus trypsin, in particular, and other aminopeptidases are the major proteolytic enzymes involved in blood digestion by female mosquitoes (Briegel and Lea, 1975; Graf and Briegel, 1982; Billingsley, 1990; Billingsley and Hecker, 1991). In animal models, *P. gallinaceum* ookinetes 0-10 hr old (i.e zygote to ookinete transition) were shown to be susceptible to mosquito enzymes in double feeding experiments (Gass, 1977) and *in vitro* damage was observed to cultured ookinetes by proteinases from *An. aegypti* (Gass and Yeates, 1979). However, the finding of Shahabuddin et al (1993) using the same parasite/vector system suggest that the parasite secretes an inactive or partially active chitinase that is activated by a mosquito- produced serine protease. In a recent study, Chege et al (1996) further examined the effect of digestive enzymes on the kinetics of *P. falciparum* ookinete development and oocyst infection rates in *An. albimanus*, *An. freeborni* and *An. gambiae*. Their data indicated that proteolytic enzymes alone do not limit the early stages of sporogonic development in these vector species of *Anopheles*. Studies involving vertebrate host factors are limited.

1.9 Effects of drugs on gametocyte infectiousness to mosquito

Sub-therapeutic doses of antimalarial drugs have been reported to enhance infectivity of *Plasmodium* species to their vectors (Shute and Maryon, 1954). Additionally, numerous compounds including chloroquine (Wilkinson et al., 1976), trimethroprim-sulphamethoxazole (TS), (Wilkinson et al., 1973), pyrimethamine (Shute & Maryon, 1951), pyrimethamine-sulfadoxine (Carter & Graves, 1988) and berenil (Ono et al., 1993) have been suggested to induce gametocyte formation. In some studies, lack of influence of chloroquine (Jeffery et al., 1956, Smalley, 1977, Chutmongkonkul et al., 1992, Hogh et al., 1995) and PS (Hogh et al., 1995) on gametocyte infectivity was observed. It has been demonstrated that pyrimethamine- and halofantrine-treated gametocytes of *P. falciparum* are more infective to *An. stephensi* mosquitoes than untreated controls (Chutmongkonkul et al., 1992). Other studies examined the effects of some schizontocidal agents on the sporogonic cycle of *P. falciparum* and *P. berghei* in anopheline mosquitoes (Coleman et al., 1988, Do Rosario et al., 1988). These studies found that chloroquine, when fed during late sporogony (10-12 days post-infection), may increase the vectorial capacity of some mosquito species.

The effects of chloroquine on the infectivity of chloroquine-sensitive and -resistant populations of *P. yoelii nigeriensis* to *An. stephensi* mosquitoes showed an enhancement of infectivity in sensitive strains but no effect was detected in resistant clones and sublines (Ichimori et al., 1990). Chloroquine use and the subsequent development of resistance over the past years is associated with an increasing human malaria infectiousness (Lines et al., 1991) which may be indirect effects of parasitaemia on the host. Pyrimethamine but not chloroquine or halofantrine showed sporontocidal activity when evaluated by administration with infected blood meal to *An. stephensi* mosquitoes (Chutmongkonkul et al., 1992). Atovaquone (566C80) was noted to have inhibitory activities against ookinete, oocysts and sporozoites of *Plasmodium berghei* in *An. stephensi* (Fowler et al., 1994, 1995).

The reduction of oocyst burden in mosquito and potential to decrease the rate of transmission of resistant parasites have been reported with combination antimalarial drugs, for example, in chloroquine-artesunate (CQ-AS) treated parasite

(Hallett et al., 2004; Drakeley et al., 2004; Sutherland et al., 2003). Thus, combination therapy may prevent the transmission advantage enjoyed by drug-resistant parasites during gametocytes infectiousness and development in mosquitoes.

1.10 Morphological studies on gametocyte sex determination

In order to be transmitted by their mosquito vector, malaria parasites undergo sexual reproduction, which occurs between specialized male and female parasites (gametes) within the blood meal in the mosquito. Until recently, little was known about how Plasmodium determines the sex of its gametocytes (gamete precursors), which are produced in the vertebrate host. Recently, erythropoietin, the vertebrate hormone controlling erythropoiesis in response to anaemia, was implicated in Plasmodium sex determination in animal models of malaria (Paul et al., 2000). The sex ratio of malaria parasites may become progressively more male as conditions that allow motility and subsequent fertilization by the male parasites become adverse in animal models (Paul et al., 2002) and in human host following treatment (Sowunmi and Fateye, 2003 c). Natural and artificial induction of erythropoiesis in vertebrate hosts provokes a shift toward male parasite production. This change in parasite sex ratio often lead to reproductive success in the parasite, suggesting that sex determination is adaptive and could be regulated by the heamatologic state of the host (Paul et al., 2000).

High levels of gametocytaemia (Tchuinkam et al., 1993) and a male biased sex ratio (Robert et al., 1996b) may increase the infectivity of gametocytes to the mosquito feeding on humans. While the effects of some antimalarial drugs on the levels of gametocytaemia following treatment are known (Robert et al., 2000, Sowunmi and Fateye, 2003 a, b), there is little or no information on the temporal changes in sex ratio of *P. falciparum* following treatment with antimalarial drugs in children, the group most at risk for malaria in endemic areas. The effectiveness of an antimalarial drug to contribute to malaria control can be measured, in addition to rapidly clearing parasitaemia and other symptoms of infections, by production of temporal changes in sex ratios that will reduce gametocyte infectivity to mosquitoes.

1.11 Gametocyte sex ratio determination

Carter and Graves (1988) and Robert et al (1996b) described the microscopic determination of gametocytes sexes. Under the microscope, the male gametes (microgametocytes) are smaller than females (macrogametocytes), the nucleus is larger in the males than the females, the ends of the cells are rounded in males and angular in females, with Giemsa the cytoplasm stains pale purple in males and deep blue in females, and the granules of malaria pigment are centrally located in females and more widely scattered in male. The sex ratio is defined as the proportion of peripheral gametocytes that are males (Pickering et al., 2000; West et al., 2001; Sowunmi and Fateye, 2003 c).

Gametocyte sex is not determined by segregation of sex determining genes or chromosomes because malaria parasite is haploid in the vertebrate host and a single clone can produce both male and female gametocyte (Carter and Graves, 1988). Thus an adaptive significance of gametocyte sex ratio exists in parasite transmission. The malaria parasite reproductive success is constrained by the effect of immune system of the host directly on the process of fertilization (Paul et al., 2000). Therefore, parasites maintain their transmission success during course of an infection by either increasing overall gametocytaemia as parasitaemia rises or adjust the gametocyte sex ratio in response to the changing host environment (Paul et al., 2000). This adaptive transmission capacity provides insight to the mechanism of sex determination in malaria parasite.

Several concepts have been developed in the efforts to explain gametocyte sex ratios dynamics in malaria (West et al., 2001). Natural selections often favour genes that maximize their transmission to the next generation- survival of the fittest. A close examination of sex determination in Plasmodium and sex ratios changes are crucial to our understanding of the transmission success, disease epidemiology and evolution, for example, of resistant infections. The occurrence and rate of inbreeding and outcrossing contribute to sex allocation and dynamics of unbeatable sex ratio, the sex ratio that maximize successful transmission (Read et al., 1992, Nee et al., 2002, West et al., 2002). However, little is known of the direct effects or contributions of

host and antimalarial drugs factors to altering the dynamics of sex allocation and transmission success that may result due to gametocyte sex ratio changes.

1.12 Effects of chemotherapy on gametocyte sex ratio

There have been very few studies on the effects of malaria chemotherapy on gametocyte sex ratio in man or *in vivo* in animals. Sowunmi and Fateye (2003 c) showed that pyrimethamine-sulfadoxine treatment of acute falciparum malaria in children enhanced gametocyte maleness. However, these authors (Sowunmi and Fateye, 2003 d) also showed that chloroquine treatment of chloroquine sensitive infections in children produced little or no effect on gametocyte sex ratio. Talman et al., (2004 a) demonstrated that following treatment of *P. falciparum* malaria in human and treatment of *P. vinckei petteri* in experimental animals with chloroquine and pyrimethamine-sulfadoxine, the sex ratio of the gametocytes did not become male biased. These differences in the findings by Sowunmi and Fateye (2003 c, d) and Talman et al. (2004 a), suggest that more studies are urgently needed to evaluate the effects of antimalarial drugs on gametocyte sex ratio.

1.13 Recent advances in estimation of gametocytaemia

For many years, presence and estimation or quantification of gametocytes in peripheral blood have been done by microscopy. Gametocyte detection by microscopy is laborious. Recently, it has been shown that submicroscopic gametocytaemia is common in children in areas of high and low transmission in Africa using molecular gametocyte detection technique, the real-time nucleic acid sequence-based amplification, (QT-NASBA) (Bousema et al., 2006; Shekalaghe et al., 2007). The technique is based on the amplification of gametocyte specific messenger ribonucleic acid (Pfs25 mRNA). The lower limit of sensitivity of this method for the quantification of *P. falciparum* gametocytaemia is 20- 100 per milliliter of blood. Using this method, it has been shown that following treatment of acute infections with mono and combination therapies, submicroscopic gametocytaemia is common and can be infectious to mosquitoes (Bousema et al., 2006).

1.14 Areas where studies are lacking and additional knowledge is required

Although gametocyte carriage has long been documented in Nigerian children with acute falciparum infections, (Bruce-Chwatt, 1951) and contribution of drug resistance of *P. falciparum* to increasing the prevalence and intensities of gametocytaemia have been documented (Sowunmi and Fateye, 2003 a, b, c.), there is dearth of information on gametocyte carriage in Nigerian children, the host factors that support gametocyte carriage and transmission, and role of seasonality of infection on gametocyte generation and carriage. In addition, the effects, following treatment, of mono and combination drug therapies on carriage, intensity and sex ratio dynamics that contribute to continuing transmission are unknown. Certain compounds have been used to reverse chloroquine resistance *in vivo* in children with acute malaria infections in Nigeria (Sowunmi et al., 1997) and to chemosensitize *P. falciparum* to PS *in vitro* (Nzila et al., 2003), little is known of the potential effects of these compounds on gametocyte generation and contribution to transmission success of *P. falciparum*, should field and clinical evaluation of their use be favoured by health policy in the near future.

Aims of the present study

From the foregoing, it is clear that an essential component for successful control of malaria is understanding of the dynamics of gametocytaemia, a measure of transmissibility of *P. falciparum*, and the influence of currently available antimalarials including potentially used combination adjuncts on malaria transmissibility. In addition, there is a need for systematic study of the epidemiology of gametocyte carriage and the influence of antimalarial drugs on gametocyte dynamics and gametocyte sex ratio.

It is for these reasons that the studies reported in this dissertation were carried out.

The objectives of the studies reported in this dissertation were:

1. To determine the risk factors for gametocyte carriage in Nigerian children with acute uncomplicated *Plasmodium falciparum* malaria.
2. To evaluate the effects of season on gametocyte carriage and response to therapy in children during acute *Plasmodium falciparum* malaria.

3. To evaluate the relationship between hyperparasitaemia and gametocyte carriage in children.
4. To compare the response to treatment with chloroquine in children who had with those who did not have gametocytes and assess the relationship between chloroquine blood levels and gametocyte carriage.
5. To assess the safety, treatment efficacy, and effects on gametocyte carriage of adding probenecid, a chemosensitizer of *P. falciparum* to pyrimethamine-sulphadoxine, to pyrimethamine sulfadoxine.
6. To compare the effects of probenecid when added to PS and PS alone on gametocyte carriage, gametocytaemia and gametocyte sex ratios in children with acute uncomplicated falciparum malaria.
7. To evaluate the effects of pyrimethamine-sulfadoxine, chloroquine plus chlorpheniramine and amodiaquine plus pyrimethamine-sulfadoxine on gametocyte production in children with acute, symptomatic, uncomplicated falciparum malaria.
8. To compare the effects of trimethoprim-sulfamethoxazole and pyrimethamine-sulfadoxine on prevalence and intensity of gametocytaemia and gametocyte sex ratios in children with acute, symptomatic, uncomplicated falciparum malaria.

Chapter 2

General Methodology

CHAPTER 2

General Methodology

Patients

Patients included in the study of risk factors for gametocyte carriage were children with acute, uncomplicated, falciparum malaria and were aged less than 12 years. Those enrolled in the study of the relationship between hyperparasitaemia and gametocyte carriage were aged 12 years or below. Children included in the study of effects of season on *P. falciparum* malaria and gametocyte carriage were children with febrile illness suspected to be malaria who were aged 15 years or less. Children aged 0.5-12 years suffering from acute falciparum malaria were included in studies of effects of drugs on *P. falciparum* gametocytaemia in children; and in the safety, treatment efficacy, and effects on gametocyte carriage and gametocyte sex ratios of adding probenecid, a chemosensitizer of *P. falciparum* to pyrimethamine-sulfadoxine. Under-twelve-year olds with acute, symptomatic, uncomplicated, falciparum malaria were included in the studies of the effects of pyrimethamine-sulfadoxine, chloroquine plus chlorpheniramine, amodiaquine plus pyrimethamine-sulfadoxine, on gametocyte production and the effects of trimethoprim-sulfamethoxazole (TS) and pyrimethamine – sulfadoxine on prevalence and intensity of gametocytaemia and gametocyte sex ratios. Patients included in the evaluation of the relationship between chloroquine levels and gametocyte carriage were children aged 3-15 years with acute falciparum malaria.

Methods of study

The methods of study, including the selection criteria are stated in the individual study. The sampling times, appropriate biochemical tests and follow up of patients are also indicated in the individual study.

Drug treatment

Children enrolled in the studies were treated with chloroquine (CQ), amodiaquine (AQ), pyrimethamine sulfadoxine (PS), chloroquine plus chlorpheniramine (CQCP), chloroquine plus pyrimethamine sulfadoxine (CQPS), amodiaquine plus pyrimethamine sulfadoxine (AQPS), Probenecid plus pyrimethamine sulfadoxine (PPS). The dosing regimens are indicated in the individual study as appropriate.

Quantification of asexual and sexual parasitaemia and determination of gametocyte sex and sex ratio

All blood films for examination of malaria parasite densities were stained with Giemsa. Parasites were counted against white cells in thick films under an oil immersion objective. Five hundred asexual forms of *P. falciparum* or the number of such parasites corresponding to 1000 white cells were counted, whichever occurred first. From this figure, parasite density was calculated from the known white blood cell count or by assuming a white cell count of 6000 /µl if the actual white blood cell count was unknown.

Gametocytaemia was quantified using the Giemsa stained thick blood smears. Levels of gametocytaemia (sexual forms/ µl) were estimated by counting gametocytes against 1000 leucocytes and assuming each patient had 6000 leucocytes/µl blood. If the level of gametocytaemia was at least 10 sexual forms/µl, the gametocytes were sexed on the basis that males (microgametocytes) are smaller than females (macrogametocytes), the nucleus is larger in the males than the females, the ends of the cells are rounded in males and angular in females, with Giemsa the cytoplasm stains pale purple in males and deep blue in females, and the granules of malaria pigment are centrally located in females and more widely scattered in males (Carter and Graves, 1988, Robert et al., 1996b). Gametocyte sex ratio was defined as the proportion of gametocytes in peripheral blood that were microgametocytes (Pickering et al., 2000). A minimum of 200 fields was counted before declaring any slide negative.

Disposition kinetics of gametocytaemia

Gametocyte kinetic parameters were estimated from the levels of micro- and macro-gametocytaemia by a non-compartmental method, using the computer programme *Turbo Ken* (Clinical Pharmacology Group, University of Southampton, U.K., through the courtesy of Professor A.G. Renwick), generally as previously described (Sowunmi and Fateye, 2003 b). The time taken to attain a sex ratio of 1 (SR1) was defined as the time elapsing from drug treatment until this ratio was achieved and was calculated for each patient, from a plot of sex ratio *vs* time, by computer extrapolation. The data from the patients who did not have at least three estimates of gametocyte sex ratios were excluded from the estimation of SR1 and the exploration of the disposition kinetics of gametocytaemia. After determining SR1, the absolute counts of micro- and macro-gametocytaemia were log-transformed for each patient and plotted against time. The following parameters were noted or determined: (1) time to attain SR1 (ts_{R1}), (2) area under the curve of the plot of micro- or macro-gametocytaemia *vs* time, from ts_{R1} to day 14 (AUC_{SR1-14}), (3) the half-lives ($t_{1/2}$) of the micro- and macro-gametocytaemia, calculated from t_{SR1}, and (4) the volume of blood completely cleared of micro- and macro-gametocytaemia from t_{SR1}, defined as (the level of micro- and macro-gametocytaemia at ts_{R1}/AUC_{SR1-14}. Since it was difficult to determine the time that gametocyte recruitment stopped in the patient, the levels of micro- and macro-gametocytaemia at ts_{R1} were assumed to be the levels when recruitment stopped.

Handling of samples

Blood for haematological and biochemical tests were collected in appropriate sample bottles and were processed according to individual specifications. In general, blood was collected in heparinized bottles and immediately processed as required for the tests. Venous blood (5 ml) was obtained for drug level determination before treatment (day 0), on the eighth day following initiation of treatment (day 7) and on the day of failure or recrudescence of parasitaemia. Blood samples for drug level estimation in plasma and red blood cells were collected into heparinized bottles and

centrifuged immediately at 2000 x g. The plasma sample was separated and both samples were stored frozen at -20°C until analysis. Blood was also collected into heparinized bottles from healthy volunteers for the chloroquine calibration curves and were processed in the same ways as samples collected from patients

Chloroquine assay

Red blood cell and plasma chloroquine concentration levels were estimated in 12 and 10 children with resistance and sensitive response, respectively. Chloroquine was assayed in plasma and red blood cell by high perfomance liquid chromatography (HPLC) with ultraviolet (UV) detection, using a modified method previously used for the estimation of quinine (Babalola et al., 1993). Plasma (1ml) and the internal standard 10µl of papaverine (5µg/ml) were alkalinized with 1ml of 2 *M* NaOH and whirl mixed for 1 minute. The mixture was extracted with 2ml diethyl ether and vortexed for 1 minute. The organic layer was separated following centrifugation at 1200 revolution per minute (rpm) for ten minutes. 100µl of 0.1 *N* HCl was added to 2ml of the organic layer and the mixture vortexed for 1 minute and centrifuged at 1200 rpm. The ether upper layer was removed and 20 µl of chloroquine extract injected into the HPLC (Beckman Gold, Model 127, Switzerland with computerized recorder). The mobile phase was a buffer consisting of 0.2 *M* sodium dihydrogen phosphate, methanol and acetonitrile at a ratio of 65 : 30 : 5, with 1 ml perchloric acid /100 ml of solution at pH of 3.7. The mobile phase was degassed in a sonicator just before use and pumped through the column at a flow rate of 1ml/min. The column contained a Bondapak C_{18} (3.9 x 300mm). The UV detector was set at 254nm. The thawed red blood cell, already lysed by the storage condition, was processed in the same way as the plasma. A typical chromatogram is shown in Figure 2.1

Calibration curve of chloroquine in plasma and red blood cell by HPLC method

Calibration curves based on peak area ratios (Drug/ Internal standard) were obtained by spiking drug-free samples with standard concentrations of chloroquine. One millilitre of red blood cell or plasma each taken in extraction tubes were spiked with chloroquine to give a concentration range of 50 - 1000ng/ml and with 10µl of 5µg/ml papaverine solution as the internal standard. 1ml of 2*M* NaOH solution was

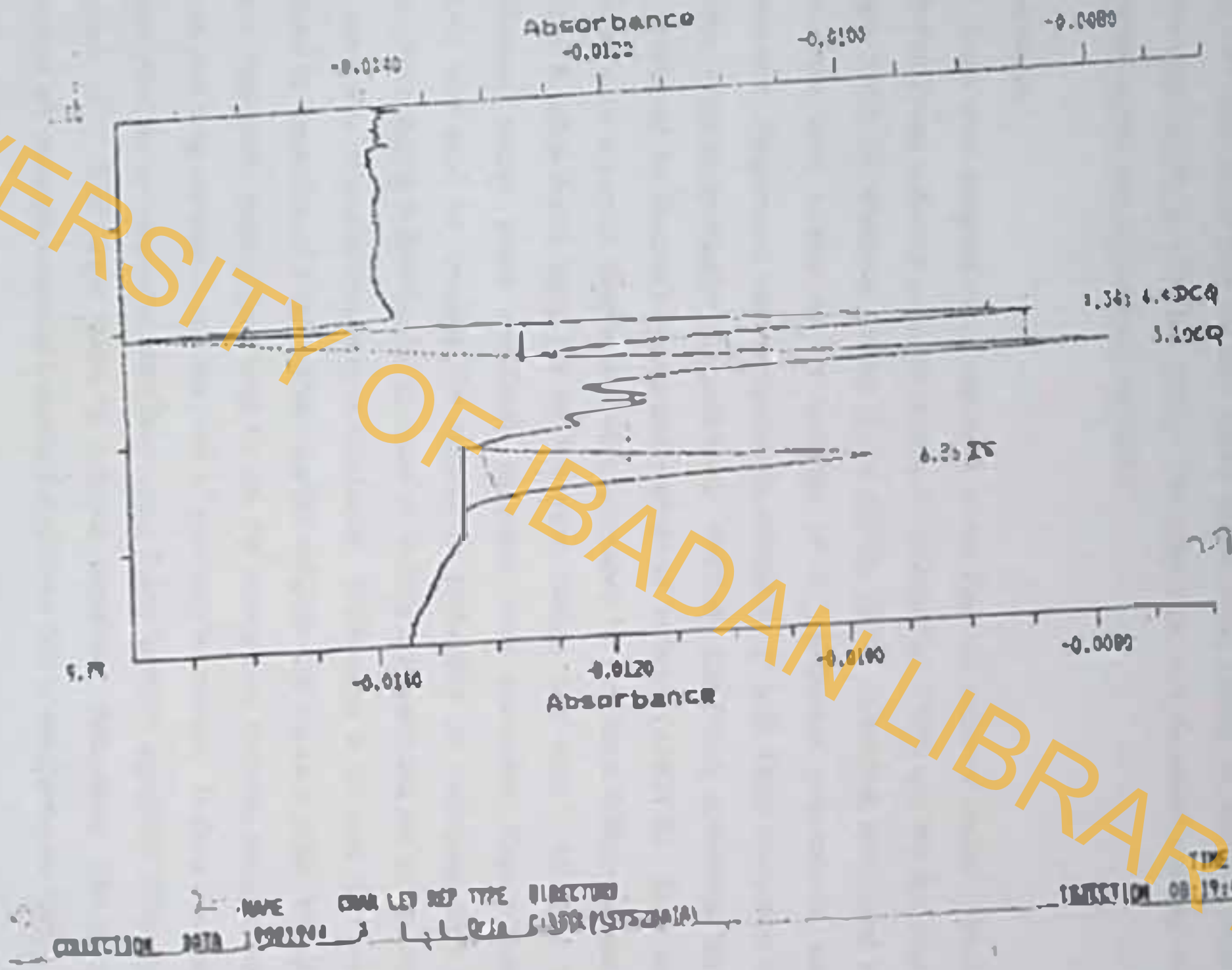


Figure 21 Chromatogram of chloroquine in red blood cell
(DCQ- desethylchloroquine, CQ- chloroquine, IS- internal standard)

added followed by 2ml of diethyl ether. The tube was whirl-mixed for 1min and then centrifuged for 10min at 2000 rpm. The ether layer was transferred into a tapered tube and 100µl of 0.1*N* HCl added before whirl mixing for another 2min. This was centrifuged for a further 10min. The ether layer was discarded and 10µl of the aqueous phase was injected into the HPLC.

Data analysis

Data were analysed using version 6 of the Epi-Info software (Anon., 1994), Graph Pad Prizm software package version 3.0 (GrapbPad, 1999) and the statistical program SPSS for Windows version 10.01 (SPSS, 1999). Variables considered in the analysis were related to the densities of *P. falciparum* gametocytes and trophozoites. Proportions were compared by calculating χ^2 with Yates' correction or by Fisher exact or by Mantel Haenszel tests. Normally distributed, continuous data were compared by Student's t-tests and analysis of variance (ANOVA). Data not conforming to a normal distribution were compared by the Mann-Whitney U-tests and the Kruskal-Wallis tests (or by Wilcoxon ranked sum test). All tests of significance, except where specifically indicated, were two-tailed. Kaplan-Meier analysis was used to estimate the cumulative probability of remaining free of gametocytes during follow-up for all cases of malaria combined and for those cases that were free of gametocytaemia at enrolment. Differences in survival time were assessed by inspection of Kaplan-Meier curves and pair wise log-rank tests. Multiple logistic regression models were used to test the associations between parasite, host factors and drug treatment, and the risks of gametocyte carriage. In drug treatment groups, *post hoc* comparisons were done using Tukey honestly significant difference. P-values of ≤ 0.05 were taken to indicate significant differences. The values presented are generally means and standard deviations (sd) or standard error (se).

Ethical clearance

Ethical clearance for all the studies was obtained from the Joint University of Ibadan/ University College Hospital, Ibadan ethics review committee, and the Ethics Committee of the Ministry of Health, Ibadan.

Section I

Epidemiology of P. falciparum gametocytaemia

Chapter 3

Risk factors for gametocyte carriage in uncomplicated falciparum malaria in children

CHAPTER 3

Risk factors for gametocyte carriage in uncomplicated falciparum malaria in children

Introduction

The transmission of *Plasmodium falciparum* from humans to mosquitoes can only occur through the gametocyte, its sexual stage that develops from proliferating asexual parasite. Gametocytes, in turn, are essential for the infection of new hosts by the mosquito (Sinden et al., 1978; Carter and Graves, 1988). Although the mechanisms of the switch from asexual to sexual stage and its modulation are complex and incompletely understood (Carter and Miller, 1979; Mons, 1985), the process, and the infectivity of the gametocytes arising from the switch may be influenced by antimalarial drugs (Wilkinson et al., 1976; Butcher, 1997; Buckling et al., 1999).

In sub Saharan Africa, increasing drug resistance in *P. falciparum* has led to increases in malaria related morbidity and mortality (Trape et al., 1998; Trape, 2001) and is thought to be associated with increases in gametocyte carriage and gametocyte infectivity to mosquitoes (Robert et al., 1996a, 2000; Hogh et al., 1998). In West African children, pretreatment gametocyte carriage in those with acute falciparum infections may reach 14-17% (von Seidlein et al., 2001; Sowunmi and Fateye 2003 b); and children, in general, are thought to constitute a significant reservoir of infection in sub-Saharan Africa (Githeko et al., 1992; Bonnet et al., 2003). A recent study from The Gambia (von Seidlein et al., 2001), an area of lesser intensity of malaria transmission than Nigeria (Salako et al., 1990), has shown that anaemia, absence of fever and parasitaemia less than 100,000 asexual forms per µL were independent risk factors for gametocyte carriage at presentation in Gambian children

In addition, treatment with pyrimethamine-sulfadoxine (PS) alone was associated with increased risk of gametocyte carriage seven days after treatment compared to chloroquine (CQ) or artemisinin-based combination therapy. It is unclear whether these factors, alone or in addition to others, are associated with gametocyte carriage in Nigerian children.

Although with increasing antimalarial drug resistance (Falade et al., 1997; Sowunmi et al., 1998 a, b; Sowunmi, 2002) there has been associated increases in gametocyte carriage in Nigerian children (Sowunmi and Fateye, 2003 b), there is little information on the risk factors associated with gametocyte carriage pre- or post-treatment in Nigerian children. Such information is necessary as it may potentially harness the efforts aimed at the management and control of drug resistance in the community. In the present study the factors that influence the production of gametocytes were evaluated, in children presenting with acute, symptomatic, uncomplicated, *P. falciparum* malaria in a hyperendemic area of malaria in southwest Nigeria. The main aims were to define the host, parasite and drug factors that contribute to gametocyte production and carriage.

Patients and methods

Patients

The study took place between July 1996 and December 2002 in patients presenting at the University College Hospital in Ibadan, a hyperendemic area for malaria in southwestern Nigeria (Salako *et al.* 1990). Ethical clearance was provided by the local ethics committee. During the period, a series of antimalarial drug studies were conducted to evaluate the efficacy and safety of different treatment regimens. Studies on CQ were done during the entire six years period, those of chloroquine plus chlorpheniramine (CQ-CP) in the first three years, those of PS in the first two years and the last 2 years, those of amodiaquine (AQ) alone in the last three years, and those of combination antimalarials in the last two years. However, there was considerable degree of overlap in the study periods. Details of the studies have been described before (Sowunmi et al., 1998 a, b, c; Sowunmi 2002, 2003; Sowunmi and Fateye 2003 b). Briefly, children with symptoms compatible with acute falciparum malaria who fulfilled the following criteria were enlisted in the study: age below 120 months, pure *P. falciparum* parasitaemia greater than 2000 asexual forms/μl blood, negative urine tests for antimalarial drugs (Dill-Glazko and lignin tests), absence of concomitant illness, no evidence of severe malaria (WHO, 2000) and written informed consent given by parents or guardians. After enrolment and start of treatment (day 0), follow-up with clinical and parasitological evaluation was at days 1-7, and then on days 14, and when necessary, on days 21 and 28, for example, in patients who received PS combined with CQ (CQPS) or AQ (AQPS). Clinical evaluation consisted of a general clinical examination including measurement of weight, core temperature and physical examination.

Assessment of parasitaemia

Thick and thin blood films prepared from a finger prick were Giemsa-stained and were examined by light microscopy under an oil-immersion objective, at x 1000 magnification, by two independent assessors. Parasitaemia in thick films was estimated by counting asexual parasites relative to 1000 leukocytes, or 500 asexual forms, whichever occurred first. From this figure, the parasite density was calculated assuming a leukocyte count of 6000/μL of blood. Gametocytes were also counted in thick blood films against 1000 leukocytes assuming an average leukocyte count of

6000/μL of blood (Shaper and Lewis, 1971; Ezeilo, 1971; Sowunmi et al., 1995). Haematocrit was done at enrolment in 124 of the patients treated with CQPS or AQPS in order to evaluate the safety of combination antimalarial therapy.

Evaluation of response to drug treatment

Response to drug treatment was assessed using World Health Organization (WHO) criteria (WHO, 1973) as follows: S = sensitive, clearance of parasitaemia without recurrence; RI (mild resistance) = parasitaemia disappears but reappears within 7 to 14 days; RII (moderate resistance) = decrease of parasitaemia but no complete clearance from peripheral blood; RIII (severe resistance) = no pronounced decrease or increase in parasitaemia at 48 hours after treatment. In those with sensitive or RI response, parasite clearance time (PCT) was defined as the time elapsing from drug administration until there was no patent parasitaemia for at least 72 h. Asexual parasite reduction ratio [PRR] (White, 1997) was defined as the ratio of day 0/day 2 parasitaemia.

Statistical analysis

Data were analysed using version 6 of the Epi-Info software (Anon., 1994), and the statistical program SPSS for Windows version 10.01 (SPSS, 1999). Proportions were compared by calculating χ^2 with Yates' correction. Normally distributed, continuous data were compared by Student's t-tests and analysis of variance (ANOVA). In the drug treatment groups posthoc comparisons were done using Tukey honestly significant difference (Tukey HSD). Data not conforming to a normal distribution were compared by the Mann-Whitney U-test and the Kruskal-Wallis test (or by Wilcoxon rank sum test). A multiple logistic regression model was used to test the association between gametocytaemia (yes or no at presentation or during follow up) and factors that were significant at univariate analysis: male gender, presence of fever, duration of illness before presentation, asexual parasitaemia at presentation, drug treatment, and recrudescence of asexual parasites within fourteen days of initiating treatment. Because the study was conducted over a

period of 6 years, time in years since the commencement of trials was included as a covariate in the model for pretreatment gametocytaemia. The values presented below are generally means and standard deviations (sd) or standard error (se) or median with interquartile range [IQR]. P-values of < 0.05 were taken to indicate significant differences.

Results

Patent gametocytaemia (geometric mean 26, range 6 – 1344/μL) was present in 115 (15%) of the 767 children at enrolment.

Risk factors for gametocyte carriage at enrolment.

The responses of the asexual parasitaemia to drug treatment and gametocyte carriage during and/ or after treatment are shown in Table 3.1. PRR in children treated with AQPS or CQPS was significantly higher than all other treatment groups ($P < 0.001$) with the exception of the AQ and PS groups, which, compared to CQPS, did not differ significantly ($P = 0.099$ and 0.30 respectively, Tukey HSD). PCT was significantly shorter in those treated with AQPS and CQPS compared to other treatment groups ($P < 0.001$) except AQ ($P = 0.052$ and 0.25, respectively, Tukey HSD). PCT was also significantly shorter in those treated with AQ compared to CQ ($P = 0.019$, Tukey HSD). Factors associated with gametocytaemia at enrolment are presented in Table 3.2. Male gender, absence of fever, duration of illness > 3 d, and asexual parasite densities less than 5000/μL were related to the presence of gametocytaemia at enrolment. Neither age nor packed cell volume at presentation was an independent risk factor for gametocyte carriage (Table 3.2).

Risk factors for gametocyte carriage during follow up

During follow-up, 15.6% of all patients (i.e. 120 patients) developed patent gametocytaemia, which in 85% (102 patients) had developed by day 7 following treatment. Gametocyte densities at enrolment were similar in all treatment groups, were significantly higher on day 14 in those treated with PS, and a significantly higher proportion of children treated with PS carried gametocytes throughout the duration of the study (Table 3.3). In the cohort of children in whom gametocytes were not detected at enrolment, 36 of 259 (13.9%) children treated with CQ, 9 of 82 (11.%) treated with CQCP, 3 of 93 (3.2%) treated with AQ, 1 of 64 (1.6%) treated with CQPS, 3 of 64 (4.7%) children treated with AQPS, and 50 of 90 (55.6%)

TABLE 3.1 Responses of asexual parasitaemia and gametocyte carriage following treatment with antimalarial drugs (Standard doses of drugs were given at presentation (day 0) and asexual parasitaemia quantification was done daily for 8 d (days 0-7) and then on day 14. Gametocyte carriage was assessed on days 0, 7 and 14)

	CQ (n = 315)	CQCP (n = 104)	AQ (n = 104)	PS (n = 109)	CQPS (n = 65)	AQPS (n = 70)	P value
% of children with gametocytes							
at enrolment	17.8 (n = 56)	21.1 (n = 22)	10.6 (n = 11)	17.4 (n = 19)	1.5 (n = 1)	8.6 (n = 6)	0.001
on day 7	24.8 (n = 78)	23.1 (n = 24)	10.6 (n = 11)	61.5 (n = 67)	3.1 (n = 2)	10.0 (n = 7)	0.001
on day 14	17.1 (n = 54)	10.6 (n = 11)	7.7 (n = 8)	48.6 (n = 53)	-	4.3 (n = 3)	0.001
PRR							
Median	2.27	2.30	2.75	3.18	3.94	3.70	<0.001*
Interquartile range	1.42 – 3.97	1.73 – 3.93	1.85 – 4.11	1.91 – 4.13			
Range	-0.7 – 5.77	-0.10 – 5.32	-0.40 – 5.10	-0.21 – 5.6	-1.0 – 5.66	0.72 – 5.65	
PCT (days)	2.9 ± 0.9	2.8 ± 0.8	2.6 ± 0.8	2.9 ± 1.1	2.3 ± 0.8	2.2 ± 0.8	0.001**
	1-6	1-5	1-5	1-6	1-4	1-4	
S (no. of patients)	198	97	102	78	65	70	0.001
RI	87	6	2	18	-	-	
RII	15	-	-	9	-	-	
RIII	15	1	-	4	-	-	
Cure rate (%)	62.9	93.2	98.1	71.5	100	100	0.001

PRR, parasite reduction ratio; PCT, parasite clearance time; PS, Pyrimethamine-sulfadoxine; CQ, chloroquine; CQCP, chloroquine plus chlorpheniramine; AQ, amodiaquine; CQPS, pyrimethamine-sulfadoxine combined with chloroquine or amodiaquine; RI = parasitaemia disappears but reappears within 7 to 14 days; RII = decrease of parasitaemia but no complete clearance from peripheral blood; RIII = no pronounced decrease or increase in parasitaemia at 48 hours after treatment; S = sensitive response. *PRR of AQPS- and CQPS-treated children were significantly higher than in other treatment groups except those treated with AQ or PS (compared with CQPS, P = 0.09 and 0.30).

** PCT was significantly shorter in those treated with AQPS and CQPS compared to other treatment groups ($P \leq 0.001$) except AQ (P = 0.052 and 0.25, respectively). PCT was also significantly shorter in those treated with AQ compared to CQ (P = 0.019, Tukey HSD).

TABLE 3.2. Risk factors for *P. falciparum* gametocytaemia at enrolment

		No. of children with gametocytes	Crude OR (95% CI)	P. value	Adjusted OR (95% CI)	P. value
Age (y)						
< 5	420	69	1	-	-	-
≥ 5	347	46	0.78 (0.52 – 1.2)	0.26		
Gender						
male	354	66	1		1	
female	413	49	0.6 (0.4–0.9)	0.01 [a]	0.55 (0.36 – 0.83)	0.005
Parasitaemia (/μL)						
< 5000	82	21	1		1	
≥ 5000	685	94	0.46 (0.26 – 0.83)	0.007	0.42 (0.24-0.73)	0.002
Fever *						
Febrile	533	73	1		1	
Afebrile	208	42	1.6 (1.06–2.13)	0.04 [a]	1.61 (1.05 – 2.5)	0.03
Duration of illness						
≤ 3 d	575	76	1		1	
> 3 d	162	39	1.7 (1.1 – 2.7)	0.001 [a]	1.57 (1.0 – 2.4)	0.047
PCV (%)						
≤ 25%	24	3	1		-	-
> 25%	100	10	0.78 (0.52 – 1.2)	0.71		

PCV, packed cell volume
*Fever, axillary temperature ≥ 37.5°C
+ Time was included as a covariate in the analysis

OR, odds ratio
CI, confidence interval

[a] χ^2 with Yate's correction

TABLE 3.3. Gametocyte densities at enrolment and following treatment with various antimalarial drugs

	CQ (n = 315)	CQCP (n = 104)	AQ (n = 104)	PS (n = 109)	CQPS* (n = 65)	AQPS (n = 70)	P. value
Gametocytaemia							
At enrolment							
GMGD (/μL)	25 (n = 56)	24 (n = 22)	29 (n = 11)	24 (n = 19)	132 (n = 1)	40 (n = 6)	0.55
Range	6 – 1344	12 – 576	12 – 740	6 - 444	132	12 – 288	
On day 7							
GMGD (/μL)	34 (n = 78)	43 (n = 24)	34 (n = 11)	75 (n = 67)	54 (n = 2)	31 (n = 7)	0.054
Range	6 – 1476	12 – 696	12 – 636	6 – 3520	24 – 120	12 – 468	
On day 14							
GMGD (/μL)	21 (n = 54)	41 (n = 11)	16 (n = 8)	50 (n = 53)	-	19 (n = 3)	0.003
Range	6 – 144	12 – 168	12 - 36	6 – 480		12 - 48	
Proportion (%) of children with gametocytaemia on days 0, 7 & 14	6.7 (n = 29)	7.7 (n = 8)	3.8 (n = 4)	12.8 (n = 14)	-	1.4 (n = 1)	0.030

GMGD, Geometric mean gametocyte density; PS, pyrimethamine-sulfadoxine; CQ, chloroquine; CQCP, chloroquine plus chlorpheniramine; AQ, amodiaquine; COM, pyrimethamine-sulfadoxine combined with chloroquine or amodiaquine
*CQPS not included in the comparison due to small number

children treated with PS developed patent gametocytaemia within 7 days of enrolment. Thus, the proportion of children who developed gametocytaemia following treatment were significantly higher in those treated with PS compared with other treatment regimens ($\chi^2 = 136.9$, P = < 0.001)

Presence of patent gametocytaemia at enrolment, and recrudescence of asexual parasites within 14 d were associated with presence of gamctocytaemia 7 or 14 days after enrolment (Table 3.4). Delay in the time taken to clear the initial parasitaemia was associated with increased risk of subsequent gametocyte carriage, but this association was not significant following multivariate analysis (Table 3.4). Children treated with AQ, AQPS or CQPS were significantly less likely to have delayed (> 2 d) parasite clearance compared with those treated with CQ or PS alone ($\chi^2 = 41.7$, degree of freedom (df) = 5, P < 0.001)

Presence of gametocytes on day 7 or 14 was significantly associated with treatment outcome by day 14 in children treated with CQ ($\chi^2 = 18.3$, df = 1, P = < 0.001) and CQCP ($\chi^2 = 10.1$, df = 1, P = 0.001), but not PS ($\chi^2 = 0.21$, df = 1, P = 0.64), and AQ ($\chi^2 = 0.24$, df = 1, P = 0.62) and AQPS or CQPS in which all children were clinically cured.

TABLE 3.4. Risk factors for *P. falciparum* gametocytaemia 7 days after treatment

Status at enrolment	Total no.	No. of children with gametocytes on day 7	Crude OR (95% CI)	P. value	Adjusted OR (95% CI)	P. value
Gametocytes						
Present	115	86	1		1	
Absent	652	102	0.06 (0.04-0.09)	<0.001[a]	0.01 (0.02 – 0.07)	<0.001
PCT						
≤ 2 d	298	61	1		1	
> 2 d	469	127	1.4 (1.00-2.07)	0.047[a]	1.4 (0.9 – 2.1)	0.20
Patent asexual parasitaemia within 14days						
Present	157	68	1		1	
Absent	610	121	0.32 (0.22 – 0.47)	<0.001	0.50 (0.3 – 0.8)	0.007
Drug treatment*						
PS	109	67	1	1	1	1
CQ	315	78	4.8 (3.0 – 7.9)	<0.001	8.5 (4.9 – 14.6)	<0.001
CQCP	104	24	5.3 (2.8 – 10.1)	<0.001	9.4 (4.5 – 197)	<0.001
AQ	104	11	13.5 (6.2 – 30.2)	<0.001	17.4 (7.3 – 41.0)	<0.001
AQPS	70	6	14.4 (5.7 – 38.0)	<0.001	14.9 (5.5 –10.2)	<0001
CQPS	65	1	50.2 (112 – 313.7)	<0.001	35.6 (7.8 – 163.5)	<0.001

OR odds ratio. CI Confidence interval. [a] χ^2 with Yate's correction. PCT, parasite clearance time
PS, pyrimethamine-sulfadoxine; CQ, chloroquine; CQCP, chloroquine plus chlorpheniramine; AQ, amodiaquine; COM, pyrimethamine-sulfadoxine combined with chloroquine or amodiaquine
CI, confidence interval
*Values of OR represent chances of being gametocyte free

Discussion

Gametocytes are often detectable in peripheral blood for a variable period after acute falciparum infection, with morphologically mature gametocytes being detectable in the blood 10-14 days after originating from merozoites (Thomson, 1911, Smalley, 1976). Carriage rates may vary widely and are dependent on several factors. In the current study, gametocyte prevalence was much higher than those reported from western Thailand (2%, Price et al., 1999) and Tanzania (8%, Akim et al., 2000) but similar to that from The Gambia (17%, von Seidlein et al., 2001) in the same region of Africa. However, despite regional differences in prevalence rates, the risk factors associated with gametocyte carriage were remarkably similar.

Gametocyte prevalence in the study area before the 1990s, a period of full sensitivity to CQ, was less than 2% (L.A. Salako, unpublished observation). Presently, in the area, CQ treatment of CQ-resistant infections is associated with significant gametocyte carriage and gametocytaemia, and slower clearance of gametocytaemia (Sowunmi and Fateye, 2003 a, b). Therefore, it would appear that the present relatively high prevalence rate, in part, may be due to increasing CQ resistance. Seventy percent of all cases of acute malaria infections in our area of study occur in children aged less than ten years (Salako et al., 1990), the similar gametocyte carriage in all age groups (Table 3.2) suggests children aged below 10 years were uniformly susceptible to gametocyte carriage. In other studies involving a broader age range than we evaluated, younger age was associated with increased gametocyte prevalence, for example, in Tanzania (Akim et al., 2000).

It is unclear why male gender is a risk factor for gametocyte carriage at presentation despite similar duration of illness and other characteristics in both gender groups enrolled in the present study. So far in records, this is the first report of the association between male gender and gametocyte carriage in African children with falciparum malaria. Could this simply be a chance finding? Plasma testosterone is often significantly raised in pre-pubertal- male than -female children (Griffin and Wilson,

1991), and testosterone and other corticosteroids may stimulate *P. falciparum* gametocytogenesis *in vitro* (Maswoswe et al., 1985; Lingnau et al., 1993). It seems possible that differences in sex hormone levels may be contributory, but hormone concentrations were not measured in the children. Gender-related differences as risk factors for gametocyte carriage require further evaluation in African children.

As was expected, duration of illness longer than 3 days was associated with increased risk of gametocyte carriage on presentation. In areas of low transmission, duration of illness longer than 2 days has also been associated with gametocyte carriage (Price et al., 1999). As longer established *P. falciparum* infections are more likely to produce gametocytes (Smalley et al., 1981), it is likely that longer duration of illness before presentation allowed sufficient time for the progression of committed asexual parasites to gametocytes. Since absence of fever is associated with increased risk of gametocyte carriage, afebrile children may have harboured the infection for longer periods. Alternatively, children with longer duration of illness may have had a relatively shorter duration of fever resulting in reduced noxious effects of fever on gametocyte development. Low parasitaemia (as in the present study) and anaemia are also significantly associated with gametocyte carriage (Price et al., 1999; von Seidlein et al., 2001) but haematocrit values less than 25% was not associated with gametocyte carriage in our cohort of children. There is no clear explanation for this observation. Anaemia in uncomplicated falciparum malaria may be enhanced by pre-existing helminth infections (Nacher et al., 2002), and both conditions may enhance gametocyte carriage (von Seidlein et al., 2001; Nacher et al., 2002), frequently co-exist and, are common in tropical endemic regions.

Despite lower efficacy (Table 3.1), CQ treatment resulted in lower gametocyte carriage than PS. A similar observation has been made in Senegal and The Gambia (Robert et al., 2000; von Seidlein et al., 2001). The ability to release more gametocytes into the circulation following PS treatment may, in part, be independent of parasite sensitivity to PS (Sowunmi and Fateye, 2003 c) and may partly explain this observation.

Irrespective of treatment regimen given, children with patent gametocytaemia at presentation were significantly more likely to be gametocytaemic 7 days later than children without patent gametocytaemia. This suggests the drugs evaluated had little or no effect on mature circulating gametocytes.

As was expected, recrudescent infections were associated with higher gametocyte prevalence, as was delay in peripheral parasite clearance as parasites develop resistance to drugs. The increase in gametocyte carriage and density as resistance develops to antimalarial drugs may confer survival and propagations advantages on the parasite in the population (Handunnetti et al., 1996; Robert et al., 1996 a; Sowunmi and Fateye, 2003 a, b). In the current study, delayed clearance of peripheral parasitaemia and increased recrudescence rates were most frequently seen in those treated with CQ or PS and least frequent in those treated with AQ, AQPS or CQPS. Similar observations have been made elsewhere (Price et al., 1999; Robert et al., 2000; Akim et al., 2000; von Seidlein et al., 2001). The significantly higher gametocyte density in those treated with PS than CQ at recrudescence of asexual parasitaemia would suggest that the former may increase the propensity for the transmission of drug-resistant infections than the latter since gametocyte infectivity to mosquitoes may correlate with level of gametocytaemia (Tchuinkam et al., 1993; Robert et al., 2000). Since leukocyte counts may vary widely, one of the possible sources of errors in the estimation of gametocyte density is assuming an average leukocyte count of 6000/μL of blood.

The findings of the present study may have potential implications for the management of acute infections in this endemic area: prompt treatment of falciparum infections with effective drugs is often associated with low gametocyte carriage (and may invariably reduce transmission of gametocytes to mosquitoes); treatment of acute infections should, preferably, employ rapidly acting schizontocide to reduce the development of gametocytes. The artemisinin derivatives may reduce transmissibility by this mode of action (Price et al., 1996). Finally, the findings may have important

implications with respect to malarial control in sub-Saharan Africa, where combination antimalarial therapy (WHO, 2001 a, b) is presently being proposed for the treatment of malaria in the region.

Low parasitaemia, amongst other factors in this study, is associated with gametocyte carriage. However, *P. falciparum* infections in children in an endemic area could present with hyperparasitaemia- a feature of severity of infection. This condition can be managed using oral treatment with antimalarial drugs and may provide a favourable condition for asexual parasite committed to gametocytogenesis. Little is known of the effects of drug treatment of hyperparasitaemia on gametocyte dynamics-release, carriage and intensity in children. It is important to determine the contributions of hyperparasitaemia in acute malaria and effects of its management on gametocyte carriage in children.

Chapter 4

Plasmodium falciparum hyperparasitaemia in children: risk factors, treatment outcomes, and gametocytaemia following treatment

CHAPTER 4

Plasmodium falciparum hyperparasitaemia in children: risk factors, treatment outcomes, and gametocytaemia following treatment

Introduction

Plasmodium falciparum infections may result in rapid multiplication of asexual parasites and astronomical increases in circulating peripheral parasites particularly in the relatively non-immune or, less frequently, in the semi-immune. These astronomic increases may reach or surpass a threshold referred to as hyperparasitaemia. Hyperparasitaemia, defined as 5% or more parasitized erythrocytes or a parasitaemia greater than 250,000/μL blood, is considered one of the several features of severe malaria (Warrell et al., 1990).

Hyperparasitaemia not accompanied by other features of severe malaria (uncomplicated hyperparasitaemia) often poses management problems in patients resident in endemic areas. Apart from a general recommendation of parenteral antimalarials (WHO, 2000), there are no other clear-cut guidelines for the management of uncomplicated hyperparasitaemia in children resident in such areas. However, it has been suggested that uncomplicated hyperparasitaemia in children in these endemic areas be treated with oral antimalarial drugs providing the drug is rapidly absorbed and the parasites are fully sensitive to the antimalarial drug(s) chosen (Sowunmi et al., 1992, 1996, 2000 a). Such a suggestion needs review in view of the increasing resistance in *P. falciparum* to many antimalarials and the lack of facilities to monitor drug sensitivity of *P. falciparum in vitro* and *in vivo* in many endemic areas.

There is little information on, for example, the risk factors associated with uncomplicated hyperparasitaemia or the time-course of gametocytaemias following oral antimalaria treatment of uncomplicated hyperparasitaemias in African children. Such information is necessary in view of the increasing resistance in *P. falciparum* to chloroquine (CQ) and other commonly available antimalarials and the increasing morbidity and mortality associated with drug resistance (Trape et al., 1998; Trape, 2001). In addition, it may improve the overall management of these cases. The present study was designed to address these issues. The main aims of the study were to evaluate the risk factors associated with hyperparasitaemia in a group of children presenting with acute, symptomatic, apparently uncomplicated, *P. falciparum* malaria in an endemic area; to assess the outcomes of oral antimalarial treatment of uncomplicated hyperparasitaemia, and to follow the course of changes in gamctocytaemias in children with hyperparasitaemias who were treated with oral antimalarial drugs.

Patients and methods

Patients

The study took place between July 1996 and September 2003 in patients presenting at the University College Hospital in Ibadan, a hyperendemic area for malaria in southwestern Nigeria (Salako et al., 1990). Ethical clearance was provided by the local ethics committee. During the period, a series of antimalarial drug studies were conducted to evaluate the efficacy and safety of different treatment regimens. All antimalarial drugs were given orally. The details of the studies have been described before (Sowunmi et al., 1998 a, b, c, 2000 a; Sowunmi, 2002, 2003; Sowunmi and Fateye, 2003 a). Briefly, children with symptoms compatible with acute falciparum malaria who fulfilled the following criteria were enlisted in the study: age below 12 years, pure *P. falciparum* parasitaemia greater than 2000 asexual forms/μL blood, negative urine tests for antimalarial drugs (Dill-Glazko and lignin tests), absence of concomitant illness, no evidence of severe malaria (WHO, 2000) and written informed consent given by parents or guardians. After enrolment and start of treatment (day 0), follow-up with clinical and parasitological evaluation was at days 1-7, and then on days 14, and when necessary, on days 21 and 28, for example, in patients who received pyrimethamine-sulfadoxine (PS) combined with chloroquine or amodiaquine (COM). Clinical evaluation consisted of a general clinical examination including measurement of weight, core temperature and physical examination.

Assessment of parasitaemia

Thick and thin blood films prepared from a finger prick were Giemsa-stained and were examined by light microscopy under an oil-immersion objective, at x 1000 magnification, by two independent assessors. Parasitaemia in thick films was estimated by counting asexual parasites relative to 1000 leukocytes, or 500 asexual forms, whichever occurred first. From this figure, the parasite density was calculated assuming an average leukocyte count of 6000/μL of blood (Shaper & Lewis, 1971; Ezeifo, 1971; Sowunmi et al., 1995). Gametocytes were also counted in thick films against 1000 leukocytes assuming an average leukocyte count of 6000/μL of blood at enrolment (day 0) and on days 7 and 14. Fractional gametocyte density (FGD) at enrolment was defined

as gametocyte count divided by total asexual and sexual count (Price et al., 1999). Haematocrit was done at enrolment in 124 of the patients treated with PS or CQPS, AQPS or PPS.

Evaluation of response to drug treatment

Response to drug treatment was assessed using World Health Organization (WHO) criteria (WHO, 1973) as follows: S = sensitive, clearance of parasitaemia without recurrence; RI (mild resistance) = parasitaemia disappears but reappears within 7 to 14 days; RII (moderate resistance) = decrease of parasitaemia but no complete clearance from peripheral blood; RIII (severe resistance) = no pronounced decrease or increase in parasitaemia at 48 hours after treatment. In those with sensitive or RI response, parasite clearance time (PCT) was defined as the time elapsing from drug administration until there was no patent parasitaemia for at least 72 h. Asexual parasite reduction ratio [PRR] (White, 1997) was defined as the ratio of day 0/day 2 parasitaemia.

Statistical analysis

Data were analysed using version 6 of the Epi-Info software (Anon., 1994), and the statistical program SPSS for Windows version 10.01 (SPSS, 1999). Proportions were compared by calculating χ^2 with Yates' correction or Fisher exact test. Normally distributed, continuous data were compared by Student's t-tests and analysis of variance (ANOVA). Data not conforming to a normal distribution were compared by the Mann-Whitney U-test and the Kruskal-Wallis test (or by Wilcoxon rank sum test). A multiple logistic regression model was used to test the association between hyperparasitaemia (yes or no at presentation or during follow up) and factors that were significant at univariate analysis: age $\leq$ 5 years, and presence of fever $\geq$ 39.5°C. Because the study was conducted over a period of 7 years, time was included as a covariate in the analysis. P-values of $\leq$ 0.05 were taken to indicate significant differences.

Results

The demographic characteristics of the children enrolled in the study are summarized in Table 4.1. At enrolment, 303, 173, 104, 203, 143, 78 and 44 of the 1048 children were allotted to, and were subsequently treated with chloroquine (CQ) only; pyrimethamine-sulfadoxine (PS) only; amodiaquine (AQ) only; CQ plus chlorpheniramine (CQCP); PS combined with CQ or AQ (CQPS or AQPS); PS combined with probenecid (PPS); and halofantrine (HF), respectively. Hyperparasitaemia was found in 100 (9.5%) of the 1048 children at enrolment.

Risk factors for hyperparasitaemia at enrolment.

Factors associated with hyperparasitaemia at enrolment are presented in Table 4.2. An age ≤ 5 years, and a core temperature ≥ 39.5°C were independent risk factors for uncomplicated hyperparasitaemia at enrolment.

Hyperparasitaemia during follow up

Following oral therapy, 1.2% of all patients (i.e. 13 of the 1048 patients) became hyperparasitaemic, which developed in all the 13 patients by day 1 of follow-up. The 13 patients who developed hyperparasitaemia following treatment were treated with CQ (10 patients), PS (1 patient) or COM (2 patients), and following treatment, all but two had sensitive response. The two children in the COM group who became hyperparasitaemic on day 1 specifically received PS combined with CQ. The two children with resistance response (1 RI, 1 RII) were treated with CQ. Compared with other treatment groups, there was a significant difference in the proportion of children treated with CQ who became hyperparasitaemic on day 1 following treatment (P = 0.01).

Treatment outcomes of hyperparasitaemia

The clinical and parasitological characteristics of the 100 children who had hyperparasitaemia at enrolment and were treated with oral antimalarial drugs are

TABLE 4.1. Summary of demographic and other characteristics of the 1048 children enrolled in the study

Variables	Value [mean ± sd (range)]
Age (years)	5.5 ± 2.5 (0.5 – 11.9)
M : F	493 : 555
Weight (kg)	15.1 ± 4.8 (6.6 - 27)
Presenting body temperature (°C)	38.6 ± 1.2 (36.4 – 40.8)
Duration of illness (d)	3.0 ± 1.5 (1 - 14)
Asexual parasite density (per μL)	
Geometric mean	30129
Range	2090-2341000
No. > 250000	100

TABLE 4.2. Risk factors for *P. falciparum* hyperparasitaemia in children at enrolment

	Total no.	No. of children with hyperparasitaemia	Crude OR (95% CI)	P. value	Adjusted OR (95% CI)	P. value
Age (years)						
≤ 5	533	62	1.65		1.61	
> 5	515	38	1 (1.06 – 2.6)	0.025	1 (1.05-2.47)	0.026
Gender						
male	493	45	1.1			
female	555	55	1 (0.58–1.4)	0.7[a]	-	-
Duration of Illness (d)						
≥ 3	696	66	0.98			
< 3	352	34	1. (0.62 – 1.56)	0.9	-	-
Fever *					1.84	
≥ 39.5°C	214	31	1.88		1 (1.17 – 2.89)	0.009
< 39.5°C	834	69	1 (1.15–3.01)	0.008 [a]		
Gametocytes						
Yes	124	11	0.91			
No	924	89	1 (0.43-1.78)	0.9[a]	-	

OR. odds ratio
* χ^2 with Yate's correction
CI. confidence interval

summarized in Table 4.3. Despite enrolment at different periods, these characteristics were similar (primarily because the criteria for enrolment into all studies were similar). No child with hyperparasitaemia was treated with AQ alone.

The responses of the asexual hyperparasitaemia to drug treatment are shown in Table 4.4. The cure rate following treatment with CQ was significantly lower than the other treatment groups (P = 0.001).

Comparison of outcomes of treatment of non hyperparasitaemia and hyperparasitaemia

Sixteen of 948 children without hyperparasitaemia had RIII response to treatment compared to 6 of 100 children with hyperparasitaemia. The difference between these proportions was significant (χ^2 = 6.22, P = 0.001). Four children (3 treated with CQ and 1 with PS) aged ≤ 3 years who had hyperparasitaemia progressed to cerebral malaria, while 2 of the 948 children without hyperparasitaemia had the same outcome. The difference between these two proportions was significant (P = 0.001, by Fisher exact test). The 2 children without hyperparasitaemia who progressed to cerebral malaria were treated with CQ. Adverse reactions reported following drug treatment were similar in children with hyperparasitaemia and in age- and gender-matched children without hyperparasitaemia who were treated with the same drugs (data not shown). For example, in those treated with CQ, pruritus occurred in 5 (of 33) and 4 (of 33) children with and without hyperparasitaemia, respectively.

TABLE 4.3 Clinical and parasitological characteristics of 100 children with *P. falciparum* hyperparasitaemia who were treated with oral antimalarial drugs

	CQ (n = 33)	CQCP (n = 25)	PS (n = 25)	COM (n = 11)	PPS* (n = 5)	HF* (n = 1)	P value
Age (years)							
Mean ± sd	4.3 ± 2.3	5.1 ± 2.3	4.6 ± 2.8	4.9 ± 2.2	7.5 ± 1.5	5.0	0.6
Range	1.5 – 9	0.7 – 10.5	0.5-10.5	3-8.1	6.3-10	-	
M:F	12 : 21	13 : 12	12 : 13	6 : 5	3 : 2	1 : 0	-
Duration of illness (d)							
Mean	2.8 ± 1.2	3.2 ± 1.3	3.5 ± 2.5	3.0 ± 0.7	3.0 ± 0.0	3.0	0.5
Range	1-6	1-6	1-14	2-4	3-3	-	
Parasitaemia (/μL)							
Geometric mean	438649	467090	398318	387090	750304	-**	0.4
Range	253091 - 1500000	253600 – 2341000	253091 – 1254000	250145 – 716000	414750 – 1388000		

PS, pyrimethamine-sulfadoxine; CQ, chloroquine; CQCP, chloroquine plus chlorpheniramine; COM, pyrimethamine-sulfadoxine combined with chloroquine or amodiaquine; PPS pyrimethamine-sulfadoxine combined with probenecid; HF, halofantrine
*Excluded from multiple comparisons because of relatively small number of patients
**Parasitaemia at enrolment was 438649 per μL

TABLE 4.4 Therapeutic responses of 100 children with acute *P. falciparum* malaria who had hyperparasitaemia at enrolment

	CQ (n = 33)	CQCP (n = 25)	PS (n = 25)	COM (n = 11)	PPS* (n = 5)	HF* (n = 1)	P value
FCT (d)							
Mean ± sd	2.0 ± 0.9	2.3 ± 1.0	2.2 ± 1.2	1.6 ± 0.5	2.2 ± 1.3	1.0	0.5
Range	1 – 4	1 – 4	1-4	1-2	1-4	-	
PRR							
Median ($\times 10^4$)	3.6	1.0	37.1	13.1	30.2		-
Interquartile range ($\times 10^4$)	0.006-72.2	0.03-44.6	27.3-77.6	0.09-45.1		-	
PCT (d)							
Mean	2.8 ± 1.1	3.2 ± 0.8	2.8 ± 1.3	2.6 ± 0.5	2.6 ± 0.9	3.0	0.4
Range	2-6	2-5	2-6	2-3	2-4	-	
S (no. of patients)	18	22	22	11	5	1	
RI	8	1	3	0	0	0	
RII	2	1	0	0	0	0	
RIII	5	1	0	0	0	0	
Cure rate (%)	54.5	88	88	100	100	-	0.001

PRR, parasite reduction ratio. FCT, fever clearance time, PCT, parasite clearance time; PS, pyrimethamine-sulfadoxine; CQ, chloroquine; CQCP, chloroquine plus chlorpheniramine, COM, pyrimethamine-sulfadoxine combined with chloroquine or amodiaquine; PPS pyrimethamine-sulfadoxine combined with probenecid. HF, halofantrine, RI = parasitaemia disappears but reappears within 7 to 14 days. RII = decrease of parasitaemia but no complete clearance from peripheral blood. RIII = no pronounced decrease or increase in parasitaemia at 48 hours after treatment. S = sensitive response. *Excluded from multiple comparison because of relatively small number of patients

Gametocyte carriage and gametocytaemia in children with hyperparasitaemia

In order to evaluate gametocyte carriage and gametocytaemia in those who were hyperparasitaemic at presentation, children with hyperparasitaemia were matched with those without hyperparasitaemia for time of presentation, age, gender, and drug treatment.

At enrolment gametocyte carriage was similar in children with hyperparasitaemia and in age- and gender- matched children without hyperparasitaemia who received the same drug treatment (6 of 100 v 11 of 100 children, $\chi^2 = 1.03$, P = 0.3). Similarly following treatment, gametocyte carriage was similar on day 7 (16 of 100 v 27 of 100 children, $\chi^2 = 2.9$, P = 0.08) and on day 14 (9 of 100 v 17 of 100 children, $\chi^2 = 2.2$, P = 0.14).

At enrolment gametocytaemia was similar in children with hyperparasitaemia and in age- and gender- matched children without hyperparasitaemia who received the same drug treatment (geometric mean 12, range 6-24/uL v 14 range 6-72, P = 0.5). Similarly following treatment, gametocytaemia was similar on day 7 (geometric mean 71, range 6-1320/µL v 66, range 6-828, P = 0.4) and on day 14 (geometric mean 57, range 12-480/µL v 70 range 12-360, P = 0.7).

Fractional gametocyte density was insignificantly lower in children with hyperparasitaemia compared with those without hyperparasitaemia (median 0.003, range 0.001- 0.005 v 0.048, range 0.0015-2.3%, P = 0.24).

Discussion

Uncomplicated hyperparasitaemia is not uncommon in African children presenting with acute, symptomatic, *P. falciparum* malaria (Salako et al., 1990; Sowunmi et al., 1992, 1996, 2000 a). Prevalence rates in endemic and non endemic areas in Africa probably vary widely; in southwest Nigeria, the rate is approximately 10-12% (Sowunmi, unpublished data). The 10% prevalence recorded in the present study was similar to that previously reported from the same area in the early 1990's (Salako et al., 1990).

The risk factors associated with uncomplicated hyperparasitaemia at presentation are not frequently documented. In falciparum infections, younger age (≤ 3 years) has been associated with hyperparasitaemia and increased risk of progression to cerebral malaria (Sowunmi et al., 2000 a). In the present study, age ≤ 5 years and a core temperature ≥ 39.5°C were independent risk factors associated with hyperparasitaemia at presentation. In falciparum infections in young children, the general trend is for parasitaemia to increase with time, and more specifically, to be accompanied by increases in body temperature. However, in severe infections there may be hypothermia. In practice many children with lower core temperatures than this model found may be hyperparasitemic. This would be so because many parents or guardians have ready access to over the counter remedies including antipyretics before presentation. This 'blunting' of presenting core temperature may mislead the attending health care provider and distract attention from the possible presence of hyperparasitaemia.

The responses of apparently uncomplicated hyperparasitaemia to oral therapy are less frequently reported, probably because of the dangers associated with oral therapy in a condition that may rapidly progress to a fatal outcome, and probably also because of increasing resistance in *P. falciparum* to antimalarial drugs leading to reluctance to try oral therapy. Providing the parasites are fully sensitive to the oral drugs chosen, responses to drug therapy appears to be independent of parasite load. Thus in a comparative study, therapeutic responses of those with and without hyperparasitaemia were similar in children from an endemic area in West Africa (Sowunmi et al., 2000 a). In addition, in drug sensitive infections, the disposition of parasitaemia appears to follow

a first order kinetics (Sowunmi et al , 2000 a, b). In this cohort of children, CQ was the least effective drug in children with hyperparsitaemia and clearly represented a significant decline in the sensitivity of *P. falciparum* to this drug. Thus with prevailing degree of CQ resistance, this drug may not be ideal for the treatment of malaria irrespective of parasite load. The significantly higher proportions of children without hyperparasitaemia who subsequently developed it following treatment with CQ or PS compared with the other treatment groups suggest slow onset of antimalarial action or reduced sensitivity to these drugs and a risk for development of post-treatment hyperparasitaemia.

The similar frequencies of pruritus (and other adverse drug reactions following treatment in those with and in those without hyperparasitaemia who were treated with the same drugs [data not shown]) suggest that hyperparasitaemia does not predispose to undue adverse drug reactions following treatment (Sowunmi et al , 2000 a).

Hyperparasitaemia is a potentially life threatening condition, and with or without other features of severe malaria requires close clinical and parasitological monitoring. Its occurrence in children from this endemic area without other overt features of severe falciparum malaria suggests the presence of some degree of immunity, although these children are, in general, considered relatively non-immune compared with adults from the same endemic area, and are prone to multiple infections (Happi et al , 2003). Should oral CQ or PS continued to be used for a potentially life threatening situation in view of increasing resistance of *P. falciparum* to these drugs in Africa? This should not be so. A recent study suggests that AQ, a drug more effective than CQ in both CQ- sensitive and resistant- *P. falciparum* infections, rapidly clears hyperparasitaemia (Ndounga & Basco, 2003). In the small number of children treated with a combination of PS plus AQ in the study population, neither clearance nor parasite reduction ratio was significantly faster or higher, respectively than those of other treatments. In view of the fact that artemisinin and its derivatives clear parasitaemia more rapidly than most of the currently available antimalarials (Hien & White, 1993), these drugs combined with, for example, AQ may be tried for the management of uncomplicated hyperparasitaemia in children from Africa. This suggestion is predicated on the fact that AQ is a relatively safe drug (Olliaro et al ,

1996), and may be a suitable partner combination drug with the artemisinin derivatives, for example, artesunate for use in Africa (Adjuik et al., 2002). Studies to assess the efficacy of such combinations in uncomplicated and complicated hyperparasitaemias are under way in the study area

As was expected, gametocyte carriage and FGD were lower in children with than in those without hyperparasitaemia. The median FGD was 16 folds higher in those without than in those with hyperparasitaemia. Relatively low asexual parasitaemia and absence of fever are some of the risk factors associated with gametocyte carriage in falciparum infections (Price et al., 1999; Akim et al., 2000, von Seidlein et al., 2001). The lower gametocyte carriage and gametocytaemia following treatment indicate that oral therapy of this condition is not associated with undue generation and/or release of gametocytes into the peripheral circulation. However, it is not known whether gametocytes arising from patients who had hyperparasitaemia are more infectious to the mosquito than those arising from patients without hyperparasitaemia who were treated with the same drugs

Chapter 5

Plasmodium falciparum malaria in Nigerian children during high and low transmission seasons: gametocyte carriage and response to oral chloroquine

CHAPTER 5

Plasmodium falciparum malaria in Nigerian children during high and low transmission seasons: gametocyte carriage and response to oral chloroquine

Introduction

The incidence of *Plasmodium falciparum* malaria often has a seasonal pattern. Gametocyte generation, carriage and infectivity to mosquitoes are crucial to successful transmission of falciparum malaria infection, particularly in endemic areas. Carter and Miller (1979) demonstrated that the rate at which sexual differentiation occur in *Plasmodium falciparum* erythrocytic stages depends on certain environmental factors. Several other studies have reported immunological stress (Smalley and Brown, 1981; Ono et al., 1986), impact of host response to parasite (Mons 1986, Schneweis et al., 1991; Sinden, 1998) and chemotherapy (Buckling et al., 1997; Roberts et al., 1996 a, 2000, Sutherland et al., 2002; Hogh et al., 1998; Sowunmi and Fateye 2003 a, b) as important factors involved in the induction of gametocytogenesis.

Although some studies have reported seasonal influence on vectorial capacity, gametocyte carriage and trophozoite densities at the onset of dry or during rainy season in endemic area in Africa and Thailand (Rosenberg et al., 1990, McElroy et al., 1994, Molineaux 1980, Nacher et al., 2004), little is known about the effects of seasonal variations on gametocyte carriage and response to chloroquine treatment in endemic area of southwest Nigeria. Such information is crucial to our understanding of the potential contribution of seasonal changes to malaria transmission. Thus, in the present study, an evaluation of the effects of low and high transmission seasons on gametocyte carriage and response of children to chloroquine during *P. falciparum* malaria infection in hyperendemic southwest Nigeria has been made.

Patients and methods

Patients

The study took place between July 1996 and December 2002 in patients presenting at the University College Hospital in Ibadan, a hyperendemic area for malaria in southwestern Nigeria (Salako et al., 1990). Ethical clearance was provided by the local ethics committee. During the period, a series of antimalarial drug studies were conducted to evaluate the efficacy and safety of different treatment regimens spanning the two periods of high (April to October) and low (November to March) transmission seasons known in the area (HTS and LTS, respectively). The details of the studies have been described before (Sowunmi et al., 1998 a, b, c; Sowunmi 2002, 2003). Briefly, children with symptoms compatible with acute falciparum malaria who fulfilled the following criteria were enlisted in the study: age 13 years or below, pure *P. falciparum* parasitaemia greater than 2000 asexual forms/μl blood, negative urine tests for antimalarial drugs (Dill-Glazko and lignin tests), absence of concomitant illness, no evidence of severe malaria (WHO, 2000) and written informed consent given by parents or guardians. After enrolment and start of treatment (day 0), follow-up with clinical and parasitological evaluation was at days 1-7, and then on days 14, and when necessary, on days 21 and 28. Clinical evaluation consisted of a general clinical examination including measurement of weight, core temperature and physical examination.

Assessment of parasitaemia and gametocytaemia

Thick and thin blood films prepared from a finger prick were Giemsa-stained and were examined by light microscopy under an oil-immersion objective, at x 1000 magnification, by two independent assessors. Parasitaemia in thick films was estimated by counting asexual parasites relative to 1000 leukocytes, or 500 asexual forms, whichever occurred first. From this figure, the parasite density was calculated assuming a leukocyte count of 6000/μL of blood. Gametocytes were also counted in thick blood films against 1000 leukocytes assuming an average leukocyte count of 6000/μl of blood (Shaper & Lewis, 1971; Ezeilo, 1971; Sowunmi et al., 1995).

Evaluation of response to drug treatment

In order to evaluate the response of children to chloroquine treatment during the HTS and LTS, 25mg/kg body weight of the drug over three days (10 mg/kg on day 1, 10mg/kg on day 2 and 5mg/kg on day 3) was administered to children. Response to drug treatment was assessed using World Health Organization (WHO) criteria (WHO, 1973) as follows: S = sensitive, clearance of parasitaemia without recurrence, RI (mild resistance) = parasitaemia disappears but reappears within 7 to 14 days; RII (moderate resistance) = decrease of parasitaemia but no complete clearance from peripheral blood, RIII (severe resistance) = no pronounced decrease or increase in parasitaemia at 48 hours after treatment. In those with sensitive or RI response, parasite clearance time (PCT) was defined as the time elapsing from drug administration until there was no patent parasitaemia for at least 72 h.

Statistical analysis

Data were analysed using version 6 of the Epi-Info software (Anon., 1994), and the statistical program SPSS for Windows version 10.01 (SPSS, 1999). Proportions were compared by calculating χ^2 with Yates' correction or by Fisher exact or by Mantel Haenszel tests. Normally distributed, continuous data were compared by Student's t-tests and analysis of variance (ANOVA). Data not conforming to a normal distribution were compared by the Mann-Whitney U-test and the Kruskal-Wallis test (or by Wilcoxon rank sum test). A multiple logistic regression model was used to test the association between gametocytaemia (yes or no at presentation) and factors that were significant at univariate analysis: male gender, presence of fever, duration of illness before presentation and asexual parasitaemia at presentation. The values presented below are generally means and standard deviations (sd) or standard error (se). P-values of <0.05 were taken to indicate significant differences.

Results

Clinical and parasitological features at enrolment

The summary of demographic and other characteristics of the children enrolled in the study is presented in Table 5.1. Of 1031 children enrolled into the studies, 693 and 338 children were recruited during the high and low transmission seasons, respectively between 1996-2003. Patent gametocytaemia (geometric mean 27, range 6 – 1344/μL) was present in 73 (10.5%) of 693 and 40 (11.8%) of 338 children at enrolment in both high and low transmission seasons respectively. These proportions were not significantly different (χ^2 = 0.27, P= 0.6). The parasite densities at enrolment in these children were 36748 (Geometric mean, range 209-150000) per μL and 27961 (Geometric mean, range 1116-565333) per μL in both high and low transmission seasons, respectively (P= 0.001)

The responses of the asexual parasitaemia to drug treatments have been reported elsewhere. Factors associated with gametocytaemia at enrolment during the high transmission seasons (HTS) are presented in Table 5.2. Duration of illness > 3 d, and asexual parasite densities less than 10000/μL were related to the presence of gametocytaemia at enrolment. None of age, gender or fever at presentation was independent risk factor for gametocyte carriage (Table 5.2). However, during low transmission seasons, gender, duration of illness >4 d, and asexual parasite densities less than 5000/μL were the independent factors associated with gametocytaemia at enrolment (Table 5.3)

Clinical features and response to chloroquine

Of 333 children that were treated with chloroquine during the study, 168 were placed in the HTS and 165 in the LTS. The clinical features at presentation and parasitological parameters of these children are summarized in Table 5.4. The clinical features were similar, although those enrolled in the LTS were significantly younger (P= 0.03), had significantly lower presenting temperature (P=0.03) and lower geometric mean parasite density (P= 0.001). Although, the fever clearance times were similar in the HTS and LTS, the parasite clearance times were significantly different (P=0.003). The other therapeutic responses (Table 5.4) were similar in the two

TABLE 5.1. Summary of demographic and other characteristics of the 1031 children enrolled in the study

Variables	Value [mean ± sd (range)]
Age (years)	5.6 ± 2.9 (0.5 – 12.0)
M: F	493 : 538
Weight (kg)	16.4 ± 4.8 (5.0 - 27)
Presenting body temperature (°C)	38.6 ± 1.2 (35.7 – 42.0)
Duration of illness (d)	3.2 ± 1.7 (1 – 21)
Asexual parasite density (per µL)	
Geometric mean	34063
Range	2090-2341000
No. > 250000	100

TABLE 5.2 Risk factors for *P. falciparum* gametocytaemia at enrolment during the high transmission seasons

		No. of children with gametocytes	Crude OR (95% CI)	P. value	Adjusted OR (95% CI)	P. value
Age (y)						
≥ 5	346	35	1		-	-
< 5	347	38	1.09 (0.6 – 1.8)	0.8		
Gender						
male	320	36	1		-	-
female	373	37	1.2 (0.7-2.0)	0.4		
Parasitaemia (/μL)						
≥ 10000	561	51	1		1	
< 10000	132	21	1.89 (1.04 – 3.35)	0.03	1.85 (1.11-3.33)	0.03
Fever *						
Febrile	437	46	1		-	-
Afebrile	258	27	0.99 (0.5–1.68)	0.9		
Duration of illness (day)						
>3 d			1		1	
≤ 3 d	156	25	0.51 (0.31 – 0.9)	0.01	0.55 (0.33 – 1.0)	0.03
	537	48				

OR odds ratio

Fever, axillary temperature ≥ 37.5°C CI confidence interval

TABLE 5.3. Risk factors for *P. falciparum* gametocytaemia at enrolment during the low transmission seasons

		No. of children with gametocytes	Crude OR (95% CI)	P. value	Adjusted OR (95% CI)	P. value
Age (y)					-	-
≥ 5	159	13	1			
< 5	179	27	0.5 (0.23 – 1.05)	0.7		
Gender						
male	159	28	1		1	
female	179	12	0.34 (0.15-0.72)	0.003	0.3 (0.1 – 0.6)	0.002
Parasitaemia (/µL)						
< 5000	23	8	1		1	
> 5000	315	32	0.21 (0.08 – 0.63)	0.001	0.22 (0.08-0.64)	0.005
Fever *					-	-
Febrile	98	9	1			
Afebrile	240	31	0.68 (0.3–1.54)	0.4		
Duration of illness (day)						
≤ 4 d	299	30	1		1	
> 4 d	39	10	3.1 (1.2 – 7.3)	0.01	3.1 (1.2 – 7.3)	0.014

OR odds ratio
CI confidence interval

* Fever: axillary temperature ≥ 37.5°C

TABLE 5.4: Comparison of clinical parameters of 333 children with acute falciparum malaria at presentation and their therapeutic response following treatment with chloroquine during high and low transmission seasons

	HTS	LTS	P values
Number of patients	168	165	
Age (years)			
mean ± sd	5.6 ± 2.8	4.9 ± 3.0	0.03
range	0.7 - 13.0	0.6 - 12.0	
Weight (kg)			
mean ± sd	16.0 ± 5.6	15.1 ± 5.6	0.16
range	6.5 -33.0	6.5 - 31.0	
Duration of symptoms (days)			
mean ± sd	3.3 ± 1.8	3.1 ± 1.5	0.14
range	1.0 -14.0	1.0 - 8.0	
Body Temperature (°C)			
mean ± sd	38.5 ± 1.2	38.1 ± 1.1	0.03
range	36.1 - 42.0	36.5 - 40.6	
Parasitaemia (/µL)			
GMPD (actual)	36748	27961	0.001
Range	2090 - 1500000	2116 - 565333	
FCT (d)			
mean ± sd	1.5 ± 0.8	1.5± 0.8	0.99
range	1 – 4	1 – 5	
PCT (d)			
mean ± sd	2.7 + 0.9	3.0 + 0.9	0.003
range	1 – 6	1 – 5	
Response			
No Cured	97	92	0.7
No with RI	60	52	0.4
No with RII	6	10	0.4
No with RIII	5	11	0.1

GMPD, geometric mean parasite density; PRR, parasite reduction ratio; FCT, fever clearance time; PCT, parasite clearance time; RI = parasitaemia disappears but reappears within 7 to 14 days; RII = decrease of parasitaemia but no complete clearance from peripheral blood; RIII = no pronounced decrease or increase in parasitaemia at 48 hours after treatment; d= day

seasons. Analysis of the treatment failures showed that of the 71 that had resistance response in the HTS, 60, 6 and 5 children had RI, RII, and RIII respectively; similarly in the LTS, 52 had RI, 10 had RII and 11 had RIII responses. RIII response occur more in the LTS than HTS but the difference was not significant (P= 0.1).

Gametocytaemia during treatment with chloroquine and follow up

Gametocytaemia was found in twenty seven of 168 and twenty eight of 165 during the HTS and LTS, respectively at enrolment. There was no difference in the geometric mean gametocyte densities (24, range 12-1344/µl, *vs.* 26, range 6-150/µl; P= 0.3). Gametocytaemia increased significantly in densities by day 7 and 14 in children treated in the HTS when compared to the gametocyte densities obtained on these days in those treated during LTS following chloroquine treatment (Table 5.5). However, the cumulative gametocyte carriage by day 7 and 14 were significantly higher in the children treated with chloroquine during the LTS (P= 0.015 and P=0.03) than those treated during the HTS.

TABLE 5.5. Comparison of gametocyte intensities at presentation and following treatment in 333 children with acute falciparum malaria during high and low transmission seasons

	HTS	LTS	P values
Number of patients	168	165	
Parasitaemia (per µl) on day 0			
GMPD (gametocytes)	24 (n=27)	26 (n=28)	0.29
Range	12 - 134	6 - 150	
Parasitaemia (per µl) on day 7			
GMPD (gametocytes)	48 (n=29)*	27 (n=48)	0.04
Range	12 - 1476	6 – 264	
Parasitaemia (per µl) on day 14			
GMPD (gametocytes)	29 (n=20)*	18 (n=35)	0.02
Range	12 - 144	6 – 102	

GMPD, geometric mean parasite density
* number of children carrying gametocyte, Gametocyte carriage was significantly higher in LTS by day 7 and 14 (P= 0.015 and 0.03)

Discussion

The primary purpose of the present study was to evaluate the effect of seasons in the low and high transmission period characteristic of malaria infection in Nigerian children, on gametocyte carriage, response to oral chloroquine and gametocyte carriage following treatment. Gametocyte carriage rates may vary widely and depend on several factors. In this study, the observed prevalence of malaria infection was significantly higher in the high transmission season (1354 of 1986) than in the LTS (253 of 789), but the gametocyte carriage rate was slightly higher in the latter. Such seasonal effect has been observed earlier in the same area (Molineaux, 1980). Prompt visit to clinic and early treatment of the infection during HTS compared to slow response of infected individuals during LTS may be contributory. People in this setting appear to suspect malaria infection more in the rainy season once symptomatic or pyrexic. It is noteworthy that asexual parasitaemia at enrolment was markedly higher in the HTS than in the LTS. The reason(s) for this is not clear from the present study. A similar observation of low parasite rate during the low transmission period has been earlier reported for the area (Sowunmi 1995; Salako et al., 1990). It may be that the features of asexual *Plasmodium falciparum* infectivity or clinical presentation vary with season or respond to changes in the environment in such a way to favour its propagation.

A critical evaluation of the risk factors for carriage of the sexual forms may provide some clues in respect of the above observation. In the present study, two and three independent factors were associated with gametocyte carriage in the HTS and LTS, respectively. Why male gender should be a risk factor for gametocyte carriage in LTS and not in the HTS remains unclear. Testosterone and corticosteroids has been reported to stimulate *P. falciparum* gametocytogenesis *in vitro* (Naswoswe et al., 1985; Lingnau et al., 1993). Could there be seasonal variation in the levels of sex hormones in the prepubertal male and female? This finding would require further investigation in African children.

The duration of illness longer than 4 days and reduced parasitaemia found as risk factors for gametocyte carriage in the LTS contrast sharply to the shorter duration of

illness and two fold parasite density in the HTS and may suggest that there is delayed presentation of symptoms or possibly low degree of virulence in the circulating asexual parasites during the LTS. Smalley et al. (1981) observed that longer established *P. falciparum* infections are likely to produce gametocytes. It is likely therefore that longer duration of illness before presentation in the LTS may allow sufficient time for the progression of committed asexual parasites to gametocytes.

The effects of antimalarial drugs on sexual differentiation in *P. falciparum* is still not fully understood. Certain antimalarial drugs, for example, chloroquine and pyrimethamine –sulphadoxine, have been reported to contribute to gametocytogenesis *in vitro* (Buckling et al., 1999) or gametocyte generation or release *in vivo* (Butcher, 1997, Sowunmi and Fateye, 2003a, b). It is remarkable to note that the children in the cohorts treated with chloroquine in this study during LTS were significantly younger, had lower presenting temperature and low parasite density compared to those treated with chloroquine during the HTS. Although fever clearance times were similar, the parasite clearance times were significantly different in the two transmission seasons. The children treated during the LTS had delayed clearance of their asexual forms suggesting differing parasite behaviour and dynamics during transmission seasons. Thus the use of chloroquine in children in the study area in the HTS appeared more favourable and important to reduce circulating parasite load. Despite similar therapeutic outcome and resistance rates in the two transmission periods, early resistance of RII and RIII occur in more children during the LTS.

Surprisingly, the post treatment gametocytaemia and gametocyte carriage differ significantly in the two seasons compared to pretreatment gametocytaemia and gametocyte carriage that were similar. In the HTS, post treatment gametocyte intensity was high but significantly fewer children were carriers compared with low gametocyte intensity and high carriage rate in the LTS. Thus antimalarial drug chemotherapy may impose stress on the parasite, response to which could result in increased gametocyte production (Buckling et al., 1999, Smalley, 1977). The higher sexual parasite density in the HTS may in addition support increased parasite burden on mosquito and probability of

mosquito infection (Carter and Graves, 1988; Taylor and Read, 1997; Buckling et al., 1999). Thus creating heavy burden of malaria and high transmission in the area.

The increased resistance to chloroquine, which still remained the most common, readily available, cheap and first line antimalarial drug in the study area, may be contributory to differences in the post treatment gametocyte generation or release and carriage in children. Patients with slow response to treatment are likely to carry gametocytes than those that responded rapidly (Price et al., 1999). This may find relevance in our understanding of how the parasite ensures transmission despite chemotherapy of the infection. More studies would be needed to elucidate parasite response and behaviour to other antimalarial drugs during low and high transmission seasons.

Overall, a strategy that avoids the identified risk factors for gametocyte carriage in the two transmission seasons and controlled use of antimalarial drugs may reduce gametocyte prevalence and contribute to a reduction in malaria transmission.

Section II

Effects of antimalarials on P. falciparum gametocytaemia and gametocyte sex ratio changes

CHAPTER 6

Response to chloroquine treatment of children who had or did not have gametocytes during uncomplicated *Plasmodium falciparum* malaria and influence of host chloroquine blood concentrations on gametocyte carriage

Introduction

Chemotherapy still remains the most widely used approach to combat malaria infections. Continuing use of chloroquine in the treatment of malaria infections has been associated with increasing rise in failure to clear parasites in patients. In endemic areas, as high as 40% and 80% in West African and East African patients, respectively may fail chloroquine treatment (Sowunmi and Fateye, 2003 a; Woklay et al., 1995). This has necessitated alternative antimalarial drug therapy. While awaiting the emergence of alternative to chloroquine in these areas, efforts need be geared towards minimizing the morbidity and mortality that may result from continuing use of chloroquine. One strategy, to consider to extend the period in which the antimalarial is useful in a patient is the parasite host-related characteristics, for instance gametocyte carriage, and clinical response to chloroquine

Gametocytes generation, carriage in host, and infectivity to mosquitoes are crucial to successful transmission of malaria infection and may contribute to sustenance and spread of chloroquine resistance in endemic areas (Sowunmi and Fateye, 2003 a, b; Sinden, 1983). In addition, little is known of the effects of chloroquine concentration levels on gametocyte carriage and contribution to spread of chloroquine resistant infections in children. Thus the aims of the present study is to evaluate the effect of gametocytes at point time of treatment with chloroquine and during follow up period on clinical outcome and resistance pattern in children during acute, uncomplicated, falciparum malaria, and assess the effects of CQ blood levels on gametocyte dynamics

Patients and Methods

The study is a part of a large study on efficacy of antimalarial drugs carried out in Ibadan, Nigeria from July 1996 till March 2003 (Sowunmi et al., 1998a, b, c; Sowunmi, 2002; Sowunmi, 2003). One hundred and forty two children with acute uncomplicated *P. falciparum* malaria were enrolled consecutively into two groups of those who had gametocytes at enrolment or and during follow-up period and those who did not have gametocytes at enrolment or any point during treatment. They were treated with chloroquine (25 mg/kg of body weight given over a 3-day period: 10 mg/kg on days 0 and 1 and 5 mg/kg on day 2). The study was approved by local ethics committee. To be enrolled into the study, a child had to be aged ≤ 13 years, have pure *P. falciparum* parasitaemia of > 1000 asexual forms/µl, give negative results in (Dill-Glazko and lignin) urine test for antimalarial drugs, have no concomitant illness or evidence of severe malaria and have the written informed consent of his or her parents or guardians. After enrolment and start of treatment (day 0), follow-up with clinical and parasitological evaluation was on days 1-7, 14, and when necessary, on days 21 and 28. Clinical evaluation consisted of a general clinical examination including measurement of weight, core temperature and physical examination.

Assessment of parasitaemia and gametocytaemia

Thick and thin blood films prepared from a finger prick were Giemsa-stained and were examined by light microscopy under an oil-immersion objective, at x 1000 magnification, by two independent assessors. Parasitaemia in thick films was estimated by counting asexual parasites relative to 1000 leukocytes, or 500 asexual forms, whichever occurred first. From this figure, the parasite density was calculated assuming a leukocyte count of 6000/µL of blood. Gametocytes were also counted in thick blood films against 1000 leukocytes assuming an average leukocyte count of 6000/µl of blood (Shaper and Lewis, 1971, Ezeilo, 1971, Sowunmi et al., 1995). Gametocytaemia was quantified on days 0, 3, 5, 7 and 14 using the thick blood smears prepared on those days (Sowunmi and Fateye, 2003a, b).

Evaluation of response to drug treatment

Response to drug treatment was assessed using World Health Organization (WHO) criteria (WHO, 1973) as follows: S = sensitive, clearance of parasitaemia without recurrence; RI (mild resistance) = parasitaemia disappears but reappears within 7 to 14 days, RII (moderate resistance) = decrease of parasitaemia but no complete clearance from peripheral blood; RIII (severe resistance) = no pronounced decrease or increase in parasitaemia at 48 hours after treatment. In those with sensitive or RI response, parasite clearance time (PCT) was defined as the time elapsing from drug administration until there was no patent parasitaemia for at least 72 h.

Assessment of effects of CQ blood levels on gametocyte dynamics

Venous blood (5ml) was obtained from 22 children enrolled in the study at presentation and on day 3 and 7 for chloroquine concentrations in plasma and red blood cells. In addition, blood was obtained from all cases of treatment failure before retreatment with pyrimethamine sulfadoxine or amodiaquine at standard doses. Blood was immediately centrifuge at 1200 x g, plasma separated from red blood cell and both plasma and red blood cells stored at −20°C until analysis. Chloroquine was determined in plasma and red blood cells by high performance chromatography (HPLC), using a modified method previously used for the estimation of quinine (Babalola et al., 1993). Briefly, Plasma (1ml) and the internal standard 10µl of papaverine (5µg/ml) were alkalinized with 1ml of 2 *M* NaOH and whirl mixed for 1 minute. The mixture was extracted with 2ml diethyl ether and vortexed for 1 minute. The organic layer was separated following centrifugation at 1200 revolution per minute (rpm) for ten minutes. 100µl of 0.1 *N* HCl was added to 2ml of the organic layer and the mixture vortexed for 1 minute and centrifuged at 1200 rpm. The ether upper layer was removed and 20 µl of chloroquine extract injected into the HPLC.

The mobile phase was a buffer consisting of 0.2 *M* sodium dihydrogen phosphate, methanol and acetonitrile at a ratio of 65: 30: 5, with 1 ml perchloric acid /100 ml of solution at pH of 3.7. The mobile phase was degassed in a sonicator just before use and pumped through the column at a flow rate of 1 ml/min. The column contained a Bondapak

C_{18} (3.9 x 300mm). The fluorescence detector was set at 254nm. The compound eluted from the column in the following order chloroquine and papaverine. The retention times were 4.6 and 6.8 min for chloroquine and internal standard, respectively.

The thawed red blood cell, already lysed by the storage condition, were centrifuged at 1200 rpm for 10 min and the upper layer 1ml processed in the same way as the plasma. The lower limit of detection was 5ng/ml. Recoveries over the concentration range 50 -1000ng/ml were 90% in plasma and 85% in red blood cell. The intra and inter samples coefficient of variation was 4.5%. Calibration plots were linear ($r^2 = 0.98$) up to 1000ng/ml. The peak area ratios were calculated and concentrations determined by extrapolation from the standard plots using a Graph Pad Prism software package (GraphPad, 1999).

Statistical analysis

Data were analysed using version 6 of the Epi-Info software (Anon., 1994), and the statistical program SPSS for Windows version 10.01 (SPSS, 1999). Proportions were compared by calculating χ^2 with Yates' correction or by Fisher exact or by Mantel Haenszel tests. Normally distributed, continuous data were compared by Student's t-tests and analysis of variance (ANOVA). Data not conforming to a normal distribution were compared by the Mann-Whitney U-test and the Kruskal-Wallis test (or by Wilcoxon rank sum test). The values presented below are generally means and standard deviations (sd) or standard error (se). P-values of < 0.05 were taken to indicate significant differences.

Results

Clinical characteristics and response to chloroquine treatment in children who had or did not have gametocytes

The seventy one children each in the two groups of those who had gametocytes at presentation or during treatment with CQ and those who did not have at presentation had similar clinical characteristics, parasite clearance times (2.9 ± 1.1 vs 3.0 ± 0.8, P= 0.9) and fever clearance times 1.6 ± 0.9 vs 1.4 ± 0.7, P= 0.6). 43 of 71 gametocytaemic children during the treatment of their acute malaria with chloroquine had gametocyte at presentation. These children were younger and had low presenting parasiteamia when compared with 43 children on consecutive enrolment who did not have gametocytes (Table 6.1). The therapeutic response (Table 6.2) to chloroquine treatment differs in these two groups. Children who had gametocytes at presentation had significantly shorter parasite clearance time and fever clearance time when compared with those who did not have gametocyte at presentation or during treatment of the infection (2.7 ± 0.9 vs 3.1 ± 0.9 d, P= 0.03; 1.2 ± 0.5 vs 1.6 ± 0.9 d, P= 0.01 respectively).

Gametocyte carriage, gametocyte intensity and chloroquine concentrations in plasma and red blood cells in 22 children

Gametocytes were carried in peripheral blood in three of the 22 children in whom chloroquine concentrations were determined, at presentation, and additional 3 children during treatment, with mean (geometric) gametocyte densities of 19 /µl blood (day 0) and 46 /µl blood (day 7), respectively. Four of the six children who had gametocyte at presentation or developed during treatment were aged less than 5 years. Table 6.3 shows the clinical parameters and therapeutic response of these children. All the six children who had gametocyte at presentation or developed during treatment had resistance response.

TABLE 6.1: Comparison of clinical parameters of 86 children with acute falciparum malaria who at presentation had or did not have gametocyte

Parameters	Gametocytaemic	Agametocytaemic	P values
Number of patients	43	43	
Age (years)			
mean ± sd	5.6 ± 3.0	6.9 ± 2.8	0.04
range	0.7-12.0	0.6-13.0	
Weight (kg)			
mean ± sd	16.5 ± 6.4	18.8 ± 6.2	0.09
range	7.0-30.0	8.5-28.0	
Duration of symptoms (days)			
mean ± sd	3.5 ± 2.3	3.2 ± 1.4	0.46
range	1.0-14.0	1.0-7.0	
Body Temperature (°C)			
mean ± sd	38.4 ± 1.2	38.5 ± 1.2	0.69
range	36.5-40.6	36.5-40.6	
Parasitaemia (per ul)			
GMPD (asexual)	13588	21716	0.05
Range	209-262426	681-236866	

GMPD, geometric mean parasite density

TABLE 6.2. Therapeutic response following treatment with chloroquine of 86 children with acute falciparum malaria who at presentation had or did not have gametocyte

Parameters	Gametocytaemic	Agametocytaemic	P values
Number of patients	43	43	
FCT (d)			
mean + sd	1.2 + 0.5	1.6 + 0.9	0.01
range	1 – 3	1 – 4	
PCT (d)			
mean ± sd	2.7 + 0.9	3.1 + 0.9	0.03
range	1 – 6	1 – 5	
Day 14 responses			
Cured (%)	20 (46.5)	33 (76.7)	0.001
RI	20	7	
RII	3	0	
RIII	0	3	

FCT, fever clearance time; PCT, parasite clearance time; RI = parasitaemia disappears but reappears within 7 to 14 days, RII = decrease of parasitaemia but no complete clearance from peripheral blood, RIII = no pronounced decrease or increase in parasitaemia at 48 hours after treatment. S = sensitive response.

TABLE 6.3. Clinical parameters and therapeutic response of 22 children with acute falciparum malaria in whom chloroquine blood concentrations were assessed

Parameters	mean ± sd	range
Age (years)	6.2 ± 3.1	1.5 -12.0
Age < 5 years	9	
Sex (M:F)	13:9	
Weight (kg)	17.6 ± 5.5	9.0 - 26.0
Duration of symptoms (days)	3.0 ± 0.6	2.0 - 6.0
Body Temperature (°C)	38.4 ± 1.2	35.7 - 40.2
Parasitaemia (per ul) GMPD (asexual)	26451	6240 – 262426
Fever Clearance time (d)	1.1 ± 0.6	1.0 - 2.0
Parasite clearance time (d)	3.0 ± 0.8	2.0 - 5.0
Response		
Sensitive (S)	9	
Resistant (R)	13	

GMPD, geometric mean parasite density

Plasma and red cell chloroquine profiles were available for all the 22 children. The gametocyte carriage and intensity, and chloroquine concentrations in plasma and red blood cells the 22 children are shown in Table 6.4. Following therapy, overall, 13 of the 22 children had resistance response and had significantly higher chloroquine concentrations in red blood cell compared to those (9) with sensitive response (P = 0.0001). The plasma chloroquine concentrations and the red blood cells- plasma ratio were similar in these two treatment outcomes.

There was a significant negative correlation between gametocytaemia on day 7 and red blood cells- plasma chloroquine concentration ratio obtained on day 3 in the children (Spearmans rho = - 0.92, P = 0.008), but not with plasma (Spearmans rho = - 0.70, P = 0.11) or red blood cell concentrations (Spearmans rho = - 0.5, P = 0.9).

TABLE 6.4. Comparison of gametocytaemia and chloroquine concentrations in plasma and red blood cells of 22 children with resistant or sensitive response following treatment with chloroquine

Parameters	CQ resistant group	CQ sensitive group	P values
Number of patients	13	9	
Gametocyte density (/µl blood)			
Day 0 (n = 3)	19*	-	
Day 3 (n = 6)	32	-	
Day 5 (n = 6)	42	-	
Day 7 (n= 6)	46	-	
Day 14 (n= 4)	21	-	
Plasma chloroquine concentration on day 7 or day of failure (ng/ml)			
mean ± sem	303.8 ± 48.3	167.3 ± 54.3	0.08
range	82.3 - 683.2	22.4 - 538.7	
RBC chloroquine concentration on day 7 or day of failure (ng/ml)			
mean ± sem	1096.1 ± 143.4	437.6 ± 74.9	0.02
range	379.6 - 1875.4	158.6 - 876.8	
RBC-plasma chloroquine concentration ratio on day 7 or day of failure (ng/ml)			
mean ± sem	5.6 ± 1.5	3.8 ± 0.5	0.38
range	0.7 – 19.2	1.1 - 7.0	

*GMPD, geometric mean (sexual) parasite density

Discussion

An interesting feature of the study was the significant difference in fever and parasite clearance times in children who had gametocytes compared to those who did not have at presentation following treatment with chloroquine. The reason for this finding is not clear from the present study. Children who presented with gametocytes are supposedly carrying trophozoites probably committed to gametocyte production (Bruce et al., 1990). The behaviour of the trophozoites committed to gametocyte production in the presence of antimalarial drug is little known. Also why the asexual forms of the parasites in the cohort of children with gametocytes at presentation appeared less virulent and cleared from the peripheral circulation earlier in the present study is unknown. However, the treatment of the infection with chloroquine may impose considerable stress and greatly reduce parasite number (Buckling et al., 1997).

The presence or absence of gametocytes at presentation in the children studied modulates significantly the therapeutic response of these children to chloroquine. As children without gametocytes at presentation responded to chloroquine treatment with significantly higher cure rates, there was comparatively significant resistance response to the drug in those with gametocytes at enrolment. It is clear that chloroquine therapy may favour malarious children who had no gametocytes at point of drug administration. In such children a combination of gametocidal drugs plus chloroquine or chloroquine in combination with all stage acting antimalarial, like artemether, may be of advantage. Once a child present with gametocytes in the peripheral blood, alternative antimalarial superior to chloroquine may be administered. However more studies are needed to evaluate the effect of presence or absence of gametocytes on clinical response of infected children to available antimalarial drugs in an area and potential contribution to controlling the infections.

It is interesting to note that chloroquine concentrations in red blood cells in children were significantly higher in children with resistance response. The reason(s) for this is not clear from the present study. Although, about 50% of these children carried gametocyte or asexual forms already committed to gametocyte formation, it is possible

that the handling of chloroquine by these parasites allows concentration of the drug in the red blood cell milieu. It is thus hypothesized that a 'conventional circulating exchange' of CQ may occur between the parasite cytoplasm and red blood cell environment, via a balance between concentration gradient and efflux mechanism (Krogstad et al., 1987; Ginsburg and Krugliak, 1992; Sanchez et al., 1997), with a consideration that relatively less exchange occur between the red cell and plasma compartment. The increasing density of gametocytes over time in this cohort and its correlation with low red blood cell-plasma CQ concentration ratio is not clear. A possible explanation is that switch to gametocyte formation of the committed asexual form and the increased gametocyte carriage and intensity is triggered following increased drug pressure and stress on the parasite, an observation earlier reported in some studies (Taylor and Read, 1997, Buckling et al., 1997). More studies would be required to investigate this observation. Whether or not this hypothesis explains the observations, it is clear from this finding that host handling of CQ creates adaptive support for the parasite in a way that contributes partly to spread of resistant parasites.

Chapter 7

Open randomized study of pyrimethamine-sulfadoxine versus pyrimethamine-sulfadoxine plus probenecid for the treatment of uncomplicated Plasmodium falciparum malaria in children

CHAPTER 7

Open randomized study of pyrimethamine-sulfadoxine versus pyrimethamine-sulfadoxine plus brobenecid for the treatment of uncomplicated *Plasmodium falciparum* malaria in children

Introduction

Drug resistance in *P. falciparum* to chloroquine is a major public health problem in much of sub-Saharan Africa, accounting for recent increases in malaria-related morbidity and mortality (Trape et al., 1998, Trape, 2001), gametocyte carriage, and enhanced transmission of drug-resistant infections in Africa (Robert et al., 1996 a, 2000, Sutherland et al., 2002, Drakeley et al., 2004, Happi et al., 2003; Sowunmi and Fateye 2003 a, b).

As an alternative to chloroquine, pyrimethamine-sulfadoxine is widely used in sub-Saharan Africa, but resistance is rapidly emerging (Sibley et al., 2001), is associated with point mutations in dihydrofolate reductase and dihydropteroate synthetase genes of the parasite (Plowe et al., 1997, Wang et al., 1997, Diourté et al., 1999), and confers survival and propagation advantages on the parasite in the population (Sowunmi and Fateye, 2003 b).

These developments have led to renewed search for effective alternatives to both chloroquine and pyrimethamine-sulfadoxine, and to the use of both drugs in combination with each other, or in combination with other antimalarials with modes of action different from those of chloroquine and pyrimethamine-sulfadoxine, with the aims of slowing the progression of resistance to these drugs and prolonging their lifespan (von Seidlein et al., 2000, Sowunmi, 2002, Basco et al., 2002, Gasasira et al., 2003, Drakeley et al., 2004).

It has also led to the use of chloroquine in combination with resistance modulators, for example, chlorpheniramine (Sowunmi et al., 1997).

Experience with chloroquine plus chlorpheniramine for treating chloroquine-resistant infections comes from southwest Nigeria where the prevalence of chloroquine-resistant infection is 35-40% (Sowunmi et al., 1998 a, b, c; Sowunmi, 2003). A recent study has shown that probenecid, an inhibitor of organic anion transporters and multiresistance-associated proteins can chemosensitize *P. falciparum* to pyrimethamine, sulfadoxine or chloroquine *in vitro* (Nzila et al., 2003), but the clinical significance is unclear. To date no study has examined, clinically, the usefulness of probenecid in combination with pyrimethamine-sulfadoxine for the treatment of malaria in African children. Such a study is essential for a number of reasons: 1. It is possible that the combination, given in appropriate doses, may improve treatment efficacy. 2. Malaria transmission may be reduced if it modulates the gametocytogenesis-enhancing effect of pyrimethamine-sulfadoxine. 3. It may potentially modify the management of paediatric cases of malaria.

The present study reports the safety, antimalarial treatment efficacy, and effect on gametocyte carriage of pyrimethamine-sulfadoxine-probenecid and pyrimethamine-sulfadoxine alone in children aged 12 years or below with acute, symptomatic, uncomplicated, *P. falciparum* malaria.

Materials and methods

Study area

The study was carried out in Ibadan, southwest Nigeria from July to September 2003. In this area of hyperendemic malaria, transmission occurs all year round but is more intense during the rainy season from April to October. In the area, it is difficult, clinically, to distinguish recrudescence from re-infection 14 days after commencing antimalarial treatment, and traditionally antimalarial efficacy tests have usually been conducted for 14 rather than the customary 28 days (Ekanem et al., 1990; Salako et al., 1990). Chloroquine resistance was reported in the area in the 1980s (Ekanem, 1985; Salako and Aderounmu, 1987) and pyrimethamine-sulfadoxine resistance in the 1990s (Sowunmi et al., 1993, 1998 a; Falade et al., 1997). Presently, chloroquine resistance reaches approximately 35-40% (Sowunmi, 2003) and, pyrimethamine-sulfadoxine resistance approximately 25% in the under-five-year-olds (Sowunmi and Fateye, unpublished).

Patients, treatment and follow-up

Patients were eligible to join the study if they were aged 12 years or below, had symptoms compatible with acute uncomplicated malaria, with pure *P. falciparum* parasitaemia > 2000 asexual forms/μL, a temperature > 37.4 °C or recent pyrexial antecedents, absence of other concomitant illness, no history of antimalarial use in the 2 weeks preceding presentation, negative urine tests for antimalarial drugs (Dill-Glazko and lignin), and written informed consent given by parents or guardians. Patients with severe malaria (WHO, 2000), severe malnutrition, serious underlying diseases (renal, cardiac, or hepatic), and known allergy to study drugs were excluded from the study. The protocol was approved by the local ethics committee. The disease history was recorded by asking patients or their parents when the present symptomatic period had started, and was followed by a full physical examination.

Enrolled patients were randomly assigned pyrimethamine-sulfadoxine 25 mg/kg of body weight of the sulfadoxine component at presentation (days 0) or pyrimethamine-sulfadoxine as above plus probenecid (Batch 2D13, Industria Farmaceutica Nova

Argentina Milano, Italy) 20-25 mg/kg of bodyweight in two divided doses daily for 3 days (days 0, 1 and 2). All drugs were given orally; except the second daily doses of probenecid, all drugs were administered in the clinic, and all patients waited for at least 3 h after drug administration to ensure the drug was not vomited. If it was, the patient was excluded form the study. If necessary, patients were provided with antipyretic (paracetamol tablets, 10-15 mg/kg 8 hourly for 24-48 h). Drug administration was controlled by a physician.

Follow-up with clinical and parasitological evaluation was done daily for seven days (days 1-7) and then on days 14, 21 and 28. Thick and thin blood films prepared from a finger prick were Giemsa-stained and were examined by light microscopy under an oil-immersion objective, at x 1000 magnification, by two independent assessors who did not know the drug treatment of the patient. Parasitaemia (asexual or sexual) in thick films was estimated by counting asexual or sexual parasites relative to 1000 leukocytes, or 500 asexual or sexual forms, whichever occurred first. From this figure, the parasite density was calculated assuming a leukocyte count of 6000/µl of blood.

Routine haematological (haematocrit) and biochemical tests (concentrations of alanine aminotransferase, aspartate aminotransferase, bilirubin, and creatinine) were done on a proportion of patients, pre-treatment and on day 14. Blood was spotted on filter papers on days 0, 3, 7, 14, 21 and 28, and at the time of treatment failures for parasite genotyping.

Classification of responses to drug treatment was according to WHO criteria (WHO, 1973). Parasite clearance time was defined as the time elapsing between drug administration and absence of detectable parasitaemia for at least 48 h. Fever clearance time was defined as the time from drug administration until the core temperature fell to or below 37.4°C and remained so for 48 h.

Cure rates were defined as the percentages of patients who remained free of parasitaemia on days 14, 21 and 28 of follow-up. [This step was necessary because of

intense transmission in the study area, making it difficult to distinguish, clinically, between re-infection and recrudescence after day 14 (Ekanem et al., 1990; Salako et al., 1990) and the relatively long half life of pyrimethamine-sulfadoxine).

Retreatment of drug treatment failures

Patients who failed treatment (within 14 days) with pyrimethamine-sulfadoxine were retreated with pyrimethamine-sulfadoxine-probenecid and were followed for another 14-28 days. Those failing pyrimethamine-sulfadoxine-probenecid were retreated with oral amodiaquine 30 mg/kg over 3 d and were followed for another 14-28 days. Patients were retreated whenever they became symptomatic (usually between 14-21 days after initial enrolment). Patients with profound clinical (hyperpyrexia, oral fluid intolerance) and parasitological deterioration during follow-up were treated with artemether (9.6 mg/kg, over 5 days and were regarded as treatment failures.

Data analysis

Sample size was calculated on the basis of recent cure rate (75% on day 14) for pyrimethamine-sulfadoxine in the under five-year olds. Data were analysed using version 6 of the Epi-Info software (Anon., 1994). Variables considered in the analysis were related to the densities of *P. falciparum* gametocytes and trophozoites. Proportions were compared by calculating χ^2 with Yates' correction or by Fisher exact or by Mantel Haenszel tests. Normally distributed, continuous data were compared by Student's t-tests and analysis of variance (ANOVA). Data not conforming to a normal distribution were compared by the Mann-Whitney U-tests and the Kruskal-Wallis tests (or by Wilcoxon ranked sum test). All tests of significance, except where specifically indicated, were two-tailed. P-values of < 0.05 were taken to indicate significant differences. The values presented are generally means and standard deviations (sd) or standard error (se).

Results

Patients' characteristics

A total of 529 children aged 12 years or below with symptoms compatible with acute, uncomplicated falciparum malaria was screened during the period. Parasitaemia was present in 313 children, 256 children were eligible for participation but only 153 children were enrolled. Of the 153 children enrolled, 79 were treated with pyrimethamine-sulfadoxine-probenecid and 74 with pyrimethamine-sulfadoxine. Two children, one from each of the treatment arms, were lost to follow up after day 7 because of parental relocation. These children were excluded from the data analysis. Figure 7.1 shows the trial profile. Overall results are for 151 children. The demographic and clinical characteristics of patients at enrolment are shown in Table 7.1. These characteristics were similar in the two treatment arms, but the duration of illness at presentation was significantly longer in those treated with pyrimethamine-sulfadoxine-probenecid.

Fever and parasite clearance, and gametocyte carriage

One hundred and seven children were febrile at enrolment, 57 in pyrimethamine-sulfadoxine-probenecid and 50 in pyrimethamine-sulfadoxine groups. By day 2, fever cleared in 42 and 26 children, respectively. There was a significant difference in the proportion of patients in whom fever cleared by day 2 ($\chi^2 = 4.5$, $P = 0.03$). Overall, fever clearance was significantly shorter in those treated with pyrimethamine-sulfadoxine-probenecid (1.9 ± 1.1 vs 2.4 ± 1.2 d, $P = 0.02$) (Table 7.2).

Compared with pyrimethamine-sulfadoxine, pyrimethamine-sulfadoxine-probenecid substantially accelerated the clearance of parasitaemia. By day 2, 53 and 37 children in the pyrimethamine-sulfadoxine-probenecid and pyrimethamine-sulfadoxine treatment arms, respectively had cleared their parasitaemias. The difference in this proportion was significant ($\chi^2 = 3.98$, $P = 0.04$). Overall, parasite clearance was significantly shorter in those treated with pyrimethamine-sulfadoxine-probenecid (2.3 ± 0.9 vs 2.7 ± 1.1 d, $P = 0.04$) (Table 7.2). The cure rate on day 14 (96.2 vs 83.5%, $\chi^2 = 5.3$, $P = 0.02$) but not day

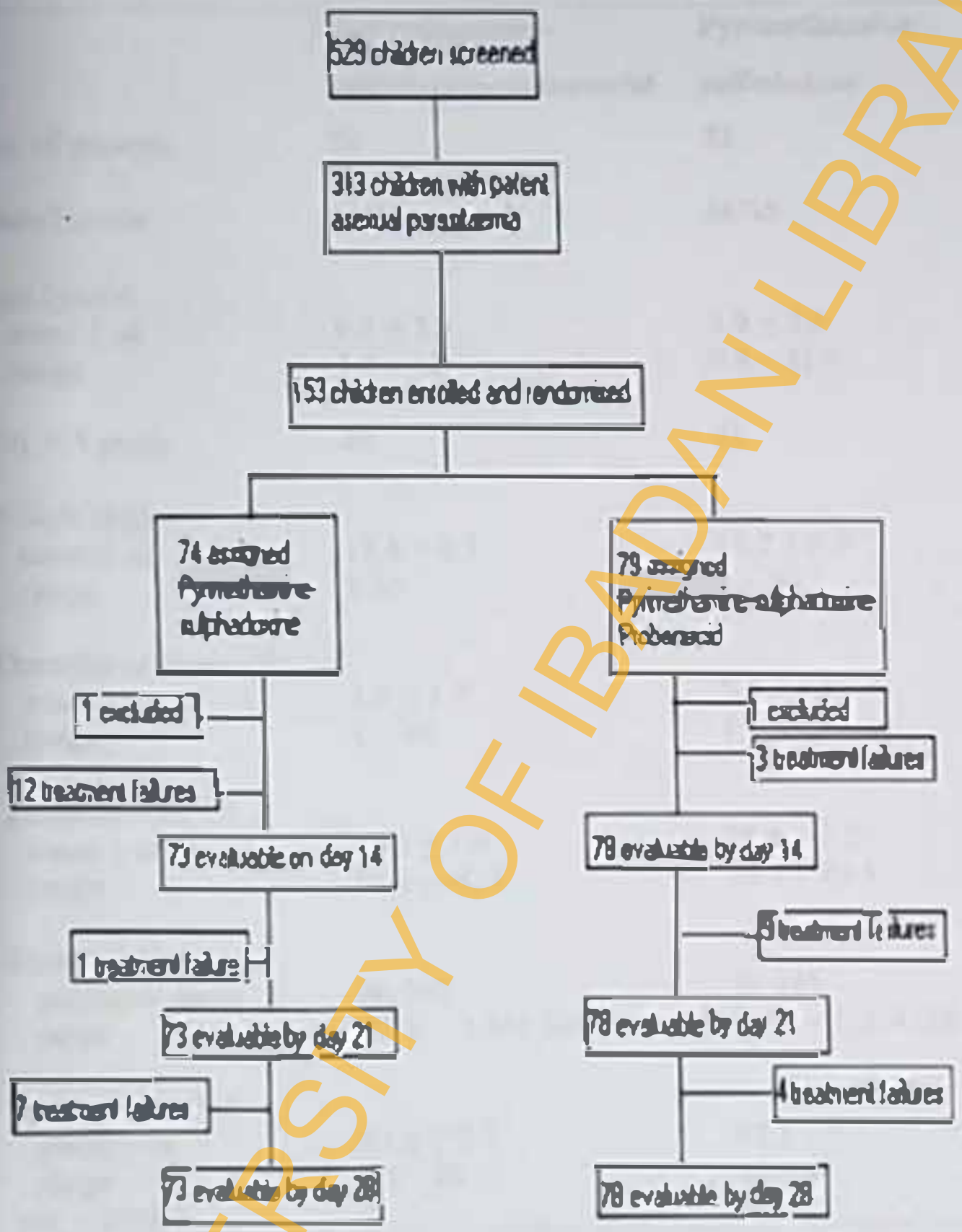


FIGURE. 7.1. Trial profile of patients enrolled in the pyrimethamine sulfadoxine vs pyrimethamine-sulfadoxine-probenecid study

TABLE 7.1. Demographic and clinical characteristics at enrolment of the 151 children with acute falciparum malaria who were treated with pyrimethamine-sulfadoxine probenecid or pyrimethamine-sulfadoxine

	Pyrimethamine-sulfadoxine-probenecid	Pyrimethamine-sulfadoxine	P. value
No. of patients	78	73	-
Male/Female	41/37	38/35	
Age (years)			
mean ± sd	6.3 ± 2.9	5.9 ± 2.9	0.3
range	1.5 – 12	0.8 – 11.5	
No. < 5 years	29	25	0.8
Weight (kg)			
mean ± sd	17.8 ± 6.1	17.2 ± 5.6	0.5
range	7-35	5 – 30	
Duration of illness (d)			
mean ± sd	3.5 ± 1.7	3.0 ± 1.3	0.04
range	1 – 10	1 – 9	
Temperature (°C)			
mean ± sd	38.1 ± 1.0	38.4 ± 1.2	0.2
range	35.9 – 40.3	36.1 – 40.5	
Parasite count (/µL)			
geometric mean	46,792	57,745	0.5
range	2010 – 1,388,000	2020 – 1,254,000	
Haematocrit (%)			
mean ± sd	31.6 ± 5.5	33.1 ± 5.1	0.1
range	18 – 43	22 – 46	
No. < 25%	8	2	0.1

TABLE 7.2. Therapeutic responses to pyrimethamine-sulfadoxine-probenecid or pyrimethamine sulfadoxine of children with acute falciparum malaria

	Pyrimethamine-sulfadoxine-probenecid	Pyrimethamine-sulfadoxine	P value
No. of patients	78	73	-
Fever clearance time (d)			
mean ± sd	1.9 ± 1.1	2.4 ± 1.2	0.02
range	1-5	1-7	
Parasite clearance time (d)			
mean ± sd	2.3 ± 0.9	2.7 ± 1.1	0.04
range	1-5	1-6	
Day 14 responses			
No. cured	75	61	
No. RI	1	10	
No. RII	2	1	
No. RIII	0	1	
Cure rate (%)	96.2	83.5	0.02
Day 21 responses			
No. cured	66	60	
No. RI	10	11	
No. RII	2	1	
No. RIII	0	1	
Cure rate (%)	84.6	82.2	0.8
Day 28 responses			
No. cured	62	53	
No. RI	14	18	
No. RII	2	1	
No. RIII	0	1	
Cure rate (%)	79.4	72.6	0.4

28 (79.4 vs 72.6%, $\chi^2 = 0.6$, P = 0.4), was significantly higher in children treated with pyrimethamine-sulfadoxine-probenecid than in those treated with pyrimethamine-sulfadoxine. Response to both treatment regimens was not related to age: one child and 2 children from the 29 and 49 < 5 and ≥ 5 year-olds, respectively treated with pyrimethamine-sulfadoxine-probenecid failed treatment by day 14 (P = 1.0, by Fisher exact test). Similarly, 4 and 8 children from the 25 and 48 < 5 and ≥ 5 year-olds, respectively treated with pyrimethamine-sulfadoxine failed treatment by day 14 (P = 1.0, by Fisher exact test).

Gametocyte carriage in those who did not have gametocytaemia at enrolment (n = 73 and 72, respectively in the pyrimethamine-sulfadoxine-probenecid and pyrimethamine-sulfadoxine) was similar on days 7 (32 of 73 (43.8%) vs 31 of 72 (43%), $\chi^2 = 0.01$, P = 0.9) and 14 (16 of 73 (21.9%) vs 21 of 72 (29.1%), $\chi^2 = 0.66$, P = 0.4) with both regimens.

Response to pyrimethamine-sulfadoxine-probenecid of children with pyrimethamine-sulfadoxine-treatment failures

Seven of 12 children who failed initial treatment with pyrimethamine-sulfadoxine, were retreated with pyrimethamine-sulfadoxine-probenecid. The therapeutic responses of these children are summarized in Table 7.3. Parasitaemia and fever cleared within 2-4 days of treatment with pyrimethamine-sulfadoxine-probenecid. The child with RII response to pyrimethamine-sulfadoxine during initial treatment had a RI response following retreatment with pyrimethamine-sulfadoxine-probenecid. The cure rates on days 14 and 28 were 86% and 72%, respectively. None of the three children who failed treatment with pyrimethamine-sulfadoxine-probenecid on or before day 14 (see Table 7.2) and were subsequently retreated with amodiaquine failed treatment during a 28-day follow-up period. In these children fever and parasitaemia cleared within 2-3 days of initiating amodiaquine therapy.

TABLE 7.3. Clinical and parasitological parameters of the 7 children with *Plasmodium falciparum* malaria who had resistance response to pyrimethamine-sulphadoxine during initial treatment and subsequently treated with pyrimethamine-sulphadoxine-probenecid

	Pyrimethamine-sulfadoxine	Pyrimethamine-sulfadoxine-probenecid	P. value
No. of patients	7	7	-
Age (years)			
mean ± sd	7.8 ± 2.6		
range	3.3 – 11.5		
Weight (kg)			
mean ± sd	19.9 ± 3.4	20.1 ± 3.8	0.5
range	15 - 26	15 – 27.5	
Temperature (°C)			
mean + sd	38.8 ± 1.1	37.8 ± 1.4	0.1
range	37.0 – 40.3	36.0 – 39.5	
Parasite count (/µL)			
geometric mean	47,835	8,156	0.01
range	2,020 – 115,500	716 – 27,622	
Fever clearance time (d)			
mean ± sd	2.1 ± 1.3	1.2 ± 0.4	0.35
range	1 - 4	1 – 2	
Parasite clearance time (d)			
mean ± sd	3.6 ± 1.0	2.8 ± 0.9	0.26
range	2 - 5	2 – 4	
Day 14 responses			
No. cured	0	6	0.004
No. RI	6	1	
No. RII	1	0	
No. RIII	0	0	
Cure rate (%)	0	86	
Day 28 responses			
No. cured	0	5	0.001
No. RI	6	2	
No. RII	1	0	
No. RIII	0	0	
Cure rate (%)	0	72	

Adverse events

Pyrimethamine-sulfadoxine-probenecid and pyrimethamine-sulfadoxine were well tolerated; no child was withdrawn because of drug intolerance Symptoms reported within the first week and during followup were similar (Table 7.4). However, vomiting was more frequently reported by those treated with pyrimethamine-sulfadoxine. None of the 7 children who failed initial treatment with pyrimethamine-sulfadoxine and were retreated with pyrimethamine-sulfadoxine-probenecid reported adverse symptoms.

Haematological and biochemical parameters

Except for haematocrit values below 25% at enrolment in 8 and 2 children in pyrimethamine-sulfadoxine-probenecid and pyrimethamine-sulfadoxine groups, respectively, and at day 7 in 4 and 4 children, respectively, haematological, biochemical and other parameters remained normal before and after treatment in all subjects. Thrombocytopenia was present in 10 and 12 children in pyrimethamine-sulfadoxine-probenecid and pyrimethamine-sulfadoxine groups, respectively, at enrolment, but was not seen on day 14 in any child.

TABLE 7.4. Adverse drug reactions reported during the study

	Pyrimethamine sulfadoxine-probenecid	Pyrimethamine-sulfadoxine
No. of children investigated	78	73
Reporting		
Pruritus	0	0
Vomiting	2	2
Abdominal pain	5	2
Diarrhoea	0	2
Anorexia	0	4*
Drowsiness	0	0
Cough	4	7
Headache	3	3
Weight gain ≥ 1.0kg	38 (n = 66)	32 (n = 60)

* Significant statistical difference, P = 0.05

TABLE 7.4. Adverse drug reactions reported by children with acute falciparum malaria treated with pyrimethamine-sulphadoxine-probenecid or pyrimethamine-sulphadoxine

	Pyrimethamine sulfadoxine-probenecid	Pyrimethamine-sulfadoxine
No. of children investigated	78	73
Reporting		
Pruritus	0	0
Vomiting	2	2
Abdominal pain	5	2
Diarrhoea	0	2
Anorexia	0	4*
Drowsiness	0	0
Cough	4	7
Headache	3	3
Weight gain ≥ 1.0kg	38 (n = 66)*	32 (n = 60)

* Significant statistical difference P = 0.05. • Number with increase in weight

may also be due to additional increases in drug levels arising from repeated administration of pyrimethamine-sulfadoxine. Although no untoward effect was observed following re-treatment, caution is required with this step, since it may increase the chances of adverse drug reactions to pyrimethamine-sulfadoxine.

The drugs used were well tolerated. The most frequently reported adverse reactions were of gastrointestinal origin, and most were indistinguishable from the symptoms of malaria. Malaria, and the drugs evaluated, can cause anorexia. It is possible the significantly reduced reporting of anorexia by those treated with pyrimethamine-sulfadoxine-probenecid was related to the accelerated clearance of fever and parasitaemia. Both probenecid and sulfadoxine can also induce haemolysis in Glucose-6 Phosphate Dehydrogenase (G6PD)-deficient subjects, but no child, following treatment, reported features suggestive of drug-induced haemolytic anaemia.

It remains unclear exactly how probenecid enhanced the antimalarial effect of pyrimethamine-sulfadoxine in the cohort of children studied. Probenecid can reduce folate uptake by *P. falciparum in vitro* (Nzila et al., 2003), in addition to increasing plasma sulfonamides concentrations by reducing renal tubular secretion of the latter. Both of these actions are independent of parasite sensitivity status to pyrimethamine-sulfadoxine. It is possible that following treatment with pyrimethamine-sulfadoxine-probenecid, sulfadoxine concentrations were significantly higher than in those treated with pyrimethamine-sulfadoxine alone, but drug levels were not measured. Probenecid can also reverse resistance in cancer cells to methotrexate (Hooijberg et al., 1999) and resistance in *P. falciparum* to chloroquine *in vitro* (Nzila et al., 2003), by inhibiting the multi-drug resistance associated proteins. Inhibition of the multi-drug resistance associated proteins is an unlikely mechanism of the enhancement of the antimalarial effect of pyrimethamine-sulfadoxine by probenecid since resistance to pyrimethamine-sulfadoxine is associated with mutations in the dihydropteroate synthetase and dihydrofolate reductase, and not mutations in the pfmdr1 gene of the parasite (Wang et al., 1997, Diourté et al., 1999, Duraisingh et al., 1997).

There are justifications for the dosing regimen; the relatively moderate dose was based on the dose used to retard tubular secretion of penicillin in children- a convenient starting point since the drug has not been previously co-administered with pyrimethamine-sulfadoxine for the treatment of malaria in children; the three-day dosing regimen is practicable, and compliance is more likely than if it were for longer periods. Certainly pharmacokinetic and pharmacodynamic studies are required before optimal dosing regimens can be achieved. There are potential clinical applications of these findings. If at moderate doses probenecid enhances the antimalarial efficacy of pyrimethamine-sulfadoxine, it follows that as resistance increases to pyrimethamine-sulfadoxine, higher doses of probenecid may be effective, since it is possible that enhancement may be dose related.

Chapter 8

Comparative effects of pyrimethamine-sulfadoxine with or without probenecid on gametocytaemia and gametocyte sex ratios in children with acute, symptomatic, uncomplicated, falciparum malaria

CHAPTER 8

Comparative effects of pyrimethamine-sulfadoxine with or without probenecid on gametocytaemia and gametocyte sex ratios in children with acute, symptomatic, uncomplicated, falciparum malaria

Introduction

The increasing spread of *Plasmodium falciparum* resistant to pyrimethamine-sulfadoxine (PS), the first or second line treatment of malaria in most endemic countries in Africa (Falade et al., 1997; Plowe et al., 1997; Wang et al., 1997; Diourte et al., 1999; Omar et al., 2001 a, b; Sibley et al., 2001), has led to renewed search for cheap, effective alternatives to PS, and to renewed efforts to prolong the clinical utility of the drug in Africa (Nzila et al., 2003). When used as part of combination therapy, particularly with the 4-aminoquinoline, amodiaquine, or the artemisisin derivatives, not only is there a rapid clearance of asexual parasitaemia, the frequency of gametocyte carriage and level of gametocytaemia during treatment with PS may also be significantly reduced (Sowunmi, 2002; Sowunmi and Fateye, 2003 b)). However, it is not clear whether non-antimalarial drugs that can potentially enhance the activity of pyrimethamine and sulfadoxine *in vitro* and *in vivo* will influence the PS-induced increases in frequency of gametocyte carriage, level of gametocytaemia and male-biased sex ratio (Sowunmi and Fateye, 2003 c).

Probenecid, an inhibitor of organic anion transporters and multiresistance-associated proteins, can chemosensitise *Plasmodium falciparum* to pyrimethamine and sulfadoxine *in vitro* (Nzila et al., 2003), and at least, *in vivo* in Nigerian children treated with pyrimethamine-sulfadoxine (Sowunmi et al., unpublished data). However, its effects, if any, when added to PS for the treatment of acute, symptomatic, uncomplicated, falciparum malaria in children, on the frequency of gametocyte carriage, level of gametocytaemia, and temporal changes in gametocyte sex ratios are unknown. Such information is essential as increases in both the level of

ietocytaemia and proportion of gametocytes that are microgametocytes may ificantly increase the infectivity of the human population to the mosquitoes ding on it (Boyd et al., 1935; Robert et al., 1996 b). If the addition of probenecid to attenuates the potentials of PS to enhance malaria transmission, and without oducing undue toxicity, it may be the ideal chemosensitizer of *P. falciparum in vivo*.

ie aims of the present study were (1) to determine the effects, on the frequency of metocyte carriage and the level of gametocytaemia, of the addition of probenecid to S (PSP) and (2) to follow the temporal changes in gametocyte sex ratios in children eated with PSP.

Patients and methods

Patients

The study took place at the University College Hospital in Ibadan, a hyperendemic area for malaria in southwestern Nigeria (Salako et al., 1990) from July to September 2003. The subjects were 151 children presenting with acute, symptomatic, uncomplicated *Plasmodium falciparum* malaria that were randomized to the following treatment regimens: PS given orally at presentation (day 0) as 25 mg/kg of the sulfadoxine component, or PS given as above plus probenecid 20-25 mg/kg given orally in two divided doses daily for 3 d (days 0-2). The study protocol was approved by the local ethics committee.

The clinical aspects of the study were as reported in the previous chapter (Chapter 7). Briefly, to be enrolled, a child had to have acute, symptomatic, uncomplicated *P. falciparum* malaria, to be aged 12 years or below, to have a pure *P. falciparum* parasitaemia of > 2000 asexual forms/μl blood, to give negative results in (Dill-Glazko and lignin) urine tests for antimalarial drugs, to have no concomitant illness or evidence of severe malaria, and to have the written informed consent of his or her parents or guardians.

After detailed clinical and parasitological assessment and drug administration at presentation, each child, as follow-up, was checked clinically and parasitologically on each of days 1-7 and 14. Fingerprick samples of blood, collected on days 0-7 and 14 were used to make thin and thick smears so that the levels of parasitaemia could be estimated (Sowunmi and Fateye, 2003 b).

Quantification of gametocytaemia

Gametocytaemia was quantified on days 0, 3, 5, 7 and 14, using the thick blood smears prepared on those days (Sowunmi and Fateye, 2003 b). Levels of gametocytaemia (sexual forms/ μl) were estimated by counting gametocytes against 1000 leucocytes and assuming each patient had 6000 leucocytes/μl blood. If the level of gametocytaemia was at least 10 sexual forms/μl, the gametocytes were sexed on the basis that males (microgametocytes) are smaller than females (macrogametocytes), the nucleus is larger in the males than the females, the ends of the cells are rounded in

males and angular in females, with Giemsa the cytoplasm stains pale purple in males and deep blue in females, and the granules of malaria pigment are centrally located in females and more widely scattered in males (Carter and Graves, 1988; Robert et al., 1996 b). The time taken to attain a sex ratio of 1 (SR1) was defined as the time elapsing from drug treatment until this ratio was achieved and was calculated for each patient, from a plot of sex ratio *v.* time, by computer extrapolation. The data from the patients who did not have at least three estimates of gametocyte sex ratios were excluded from the estimation of SR1 and the exploration of the disposition kinetics of gametocytaemia.

Disposition kinetics of micro- and macro-gametocytaemia

Gametocyte kinetic parameters were estimated from the levels of micro- and macro-gametocytaemia by a non-compartmental method, using the computer programme *Turbo Ken* (Clinical Pharmacology Group, University of Southampton, U.K., through the courtesy of Professor A.G. Renwick), generally as previously described (Sowunmi & Fateye, 2003 d). After determining SR1, the absolute counts of micro- and macro-gametocytaemia were log-transformed for each patient and plotted against time. The following parameters were noted or determined: (1) time to attain SR1 (ts_{R1}), (2) area under the curve of the plot of micro- or macro-gametocytaemia *v.* time, from ts_{R1} to day 14 (AUC_{SR1-14}), (3) the half-lives ($t_{1/2}$) of the micro- and macro-gametocytaemia, calculated from ts_{R1}, and (4) the volume of blood completely cleared of micro- and macro-gametocytaemia from ts_{R1}, defined as (the level of micro- and macro-gametocytaemia at ts_{R1}/AUC_{SR1-14}. Since it was difficult to determine the time that gametocyte recruitment stopped in the patient, the levels of micro- and macro-gametocytaemia at ts_{R1} were assumed to be the levels when recruitment stopped.

Statistical analysis

Data were analysed using version 6 of the Epi-Info software (Anon., 1994). Proportions were compared by calculating χ^2 with Yates' correction or by Fisher exact or by Mantel Haenszel tests. Normally distributed, continuous data were compared by Student's t-tests and analysis of variance (ANOVA). Data not conforming to a normal distribution were compared by the Mann-Whitney U-tests and the Kruskal-Wallis tests

(or by Wilcoxon rank sum test). Correlations were assessed by linear regression. All tests of significance were two-tailed. P-values of ≤ 0.05 were taken to indicate significant differences. The values presented below are generally means and standard deviations (sd) or standard error (se).

Results

Clinical features at enrolment and responses to therapy

Seventy eight children were treated with PSP and 73 with PS. The details of the clinical and parasitological responses to the two treatment regimens are presented in the previous chapter (Chapter 7). Briefly, the clinical and parasitological parameters at enrolment were similar in the two treatment groups. Mean age at enrolment was 6.3 ± 2.9 and 5.9 ± 2.9 years in the PSP and PS groups, respectively, (P = 0.3). Fever clearance was significantly faster in those treated with PSP (1.9 ± 1.1 d), than in those treated with PS (2.4 ± 1.2 d). The difference between these values was significant (P = 0.02). Similarly, parasite clearance was significantly faster, and the cure rate on day 14 was significantly higher in those treated with PSP than in those treated with PS. For example, the cure rate on day 14 was 96.2% in those treated with PSP and 83.5% in those treated with PS (P = 0.02).

Frequency of gametocyte carriage and level of gametocytaemia

During the entire study period, gametocytaemia was found in 39 patients treated with PSP and in 34 treated with PS. The frequency of gametocyte carriage was significantly higher on each of days 7 and 14 (P < 0.01) than on day 0, both in the PSP- and PS-treated patients (Table 8.1). Similarly, the levels of gametocytaemia were significantly higher on each of days 3, 5, 7 and 14 than on day 0, both in the PSP- and PS-treated patients. The level of gametocytaemia was, however, significantly higher on day 5 in PS- than in PSP- treated patients (P = 0.004, Table 8.1). Two children and one child treated with PSP and PS, respectively were gametocyte carriers at all times during the study period.

Gametocyte sex ratios

In the 39 children treated with PSP who had gametocytaemia during the study period, 42, 32, 132, 460 and 138, gametocytes were counted on days 0, 3, 5, 7 and 14, respectively, and most of these gametocytes (39, 32, 130, 453, and 138 on days 0, 3, 5, 7 and 14, respectively) could be sexed. In the 34 children treated with PS who had gametocytaemia during the study period, 12, 38, 122, 578, and 143 gametocytes were counted on days 0, 3, 5, 7 and 14, respectively, and most of these gametocytes (12, 37,

TABLE 8.1 Prevalence and intensities of *Plasmodium falciparum* gametocytaemia at presentation and during follow-up of malarious children treated with pyrimethamine sulfadoxine-probenecid (PSP) or pyrimethamine sulfadoxine(PS)

Parameter	Day					
	0	3	5	7	14	P
PSP (n = 78)						
No. with gametocytaemia	5	3	9	32	16	0.00001*
GMGD (gametocyte/μl)	17	32	33	63	44	0.002*
Range	12 - 36	24 – 36	24 - 48	12 - 960	12 - 216	
PS (n = 73)						
No. with gametocytaemia	1	3	5	31	22	0.00001*
GMGD (gametocyte/μl)	12	50	67	41	30	00001*
Range	-	36 – 72	48 - 84	12 - 687	12 – 84	

GMGD, geometric mean gametocyte density. *Kruskal-Wallis test, *χ^2 test with Yates correction; All comparisons were two-tail

121, 576, and 142 on days 0, 3, 5, 7 and 14, respectively) could be sexed. The data on the sex ratios for both PSP and PS were pooled because of the small number of gametocyte carriers observed pre-treatment (five among the PSP children and one among the PS children). Overall the sex ratio was male-biased, a mean (se) of 59 (12%), range 30-100% (95% confidence interval 26-92%). At presentation there was no significant correlation between the proportion of gametocytes that were male and asexual parasitaemia ($r = 0.7$, $P = 0.17$), core temperature ($r = 0.2$, $P = 0.8$) or gametocytaemia ($r = 0.02$, $P = 0.98$).

The temporal changes observed in the gametocyte sex ratios were similar for the PSP and PS treated children (Figure 8.1). There was a progressive increase in the proportion of gametocytes that were male such that by day 7, over 80% of the gametocytes were male in both treatment groups. In 3 children (one in PSP and 2 in PS) with pre-treatment female biased sex ratio, SR1 was reached by day 5. On day 7, 5 of 32 children with gametocytaemia who were treated with PSP had female biased sex ratio, while on day 14, one of 16 with gametocytaemia had a female-biased ratio. In the children treated with PS, 1 of 31 children with gametocytaemia on day 7 had female-biased ratio, while none had such ratio on day 14. In those treated with PSP, the proportions of gametocytes that were male on days 7 and 14 were significantly higher than the proportion on day 0 ($\chi^2 = 17.1$, $P = 0.00003$, and $\chi^2 = 27.1$, $P = 0.00001$, respectively). Similarly, in those treated with PS, the proportions of gametocytes that were male on days 7 and 14 were significantly higher than the proportion on day 0 ($\chi^2 = 38.5$, $P = 0.000001$ and $\chi^2 = 51.4$, $P = 0.000001$, respectively).

The levels of micro- and macro-gametocytaemia before and after treatment with PSP and PS for all the 73 children with gametocytaemia during the study period are shown in Table 8.2. In PSP treated children, the levels of micro- and macro-gametocytaemia were similar between days 0-5. However, by day 7 the level of microgametocytaemia was significantly higher than that of macrogametocytaemia. In those treated with PS, macrogametocyatemia mildly predominated between days 0-5. However, by day 7, microgametocytaemia predominated, the level of microgametocytaemia being significantly higher than those of macrogametocytaemia.

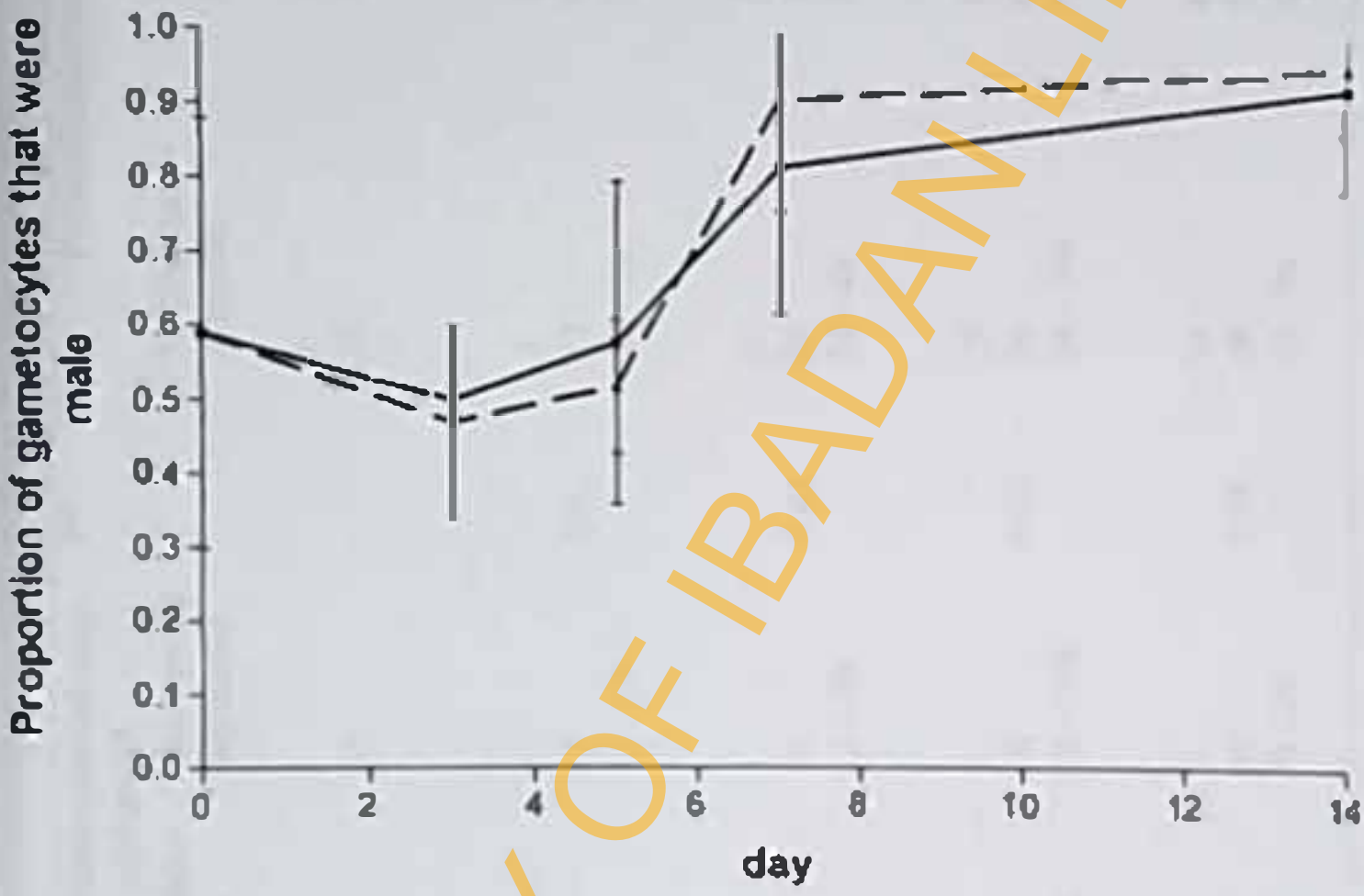


FIGURE 8.1 Changes in sex ratio of gametocytes before and after pyrimethamine-sulfadoxine-probenecid (-■-) or pyrimethamine-sulfadoxine (-▲-) treatment of acute *Plasmodium falciparum* malaria in children

TABLE 8.2 Prevalence and intensities of micro- and macro-gametocytaemia densities in 73 malarious children treated with pyrimethamine-sulfadoxine-probenecid or pyrimethamine-sulfadoxine

	Pyrimethamine-sulfadoxine-probenecid		P*	Pyrimethamine-sulfadoxine		P*
	Microgametocytaemia	Macrogametocytaemia		Microgametocytaemia	Macrogametocytaemia	
Day 0						
No. with gametocytaemia	3	1		1	1	
Geometric mean level of gametocytaemia	15	12	-	12	12	-
Range (gametocytes/μl)	12 - 24	-		-		
Day 3						
No. with gametocytaemia	3	3		3	3	
Geometric mean level of gametocytaemia	17	17	1.0	17	24	0.65
Range (gametocytes/μl)	12 - 24	12 - 24		12 - 24	12 - 48	
Day 5						
No. with gametocytaemia	9	8		3	3	
Geometric mean level of gametocytaemia	21	27	0.83	30	51	0.1
Range (gametocytes/μl)	12 - 24	12 - 36		24 - 48	36 - 60	
Day 7						
No. with gametocytaemia	22	7		29	17	
Geometric mean level of gametocytaemia	115	28	0.02	96	43	0.01
Range (gametocytes/μl)	60 - 852	12 - 108		24 - 564	12 - 313	
Day 11						
No. with gametocytaemia	20	3		16	4	
Geometric mean level of gametocytaemia	64	29	0.18	38	12	0.18
Range (gametocytes/μl)	24 - 180	24 - 36		12 - 96	12 - 12	

* Wilcoxon sign rank test

Disposition kinetics of micro- and macro-gametocytaemia

As, at each time-point investigated, the sex ratio and levels of gametocytaemia for the PSP treated children were similar to those treated with PS (see Figure 8.1 and Table 8.2), the data for both groups were pooled for analysis of the disposition kinetics (Table 8.3). The AUC and the $t_{1/2}$ for microgametocytaemia were significantly higher than those for macrogametocytaemia, and the clearance of macrogametocytaemia was two and a half folds higher than for microgametocytaemia.

TABLE 8.3. Disposition kinetics of *P. falciparum* micro and macrogametocyte following pyrimethamine-sulfadoxine-probenecid or pyrimethamine-sulfadoxine treatment in children

Parameter*	Microgametocytaemia (N= 8)	Macrogametocytaemia (N= 4)	P values
AUC (sexual forms/µl.h)			
Mean and (S.E)	17619 (3831)	4728 (691)	0.017†
Range	5040 – 40017	3034 – 6250	
95% confidence interval	8561 – 26678	2530 - 6927	
$t_{1/2}$ (h)			
Mean and (S.E)	265.6 (59.4)	74.3 (12.8)	0.05
Range	66.3 – 624.0	37.1 - 96.0	
95% confidence interval	125.1 – 406.1	33.5 – 115.1	
CL_{app} (µl/kg.h			
Mean and (S.E)	0.00008 (0.00003)	0.0002 (0.00008)	0.14
Range	0.00002 – 0.00015	0.00002 – 0.0004	
95% confidence interval	0.000009 – 0.00016	-0.00007 – 0.00048	

*Calculations were from the time to attain a male: female sex ratio of 1
†Wilcoxon signed rank test
AUC, Area under the curve of gametocytaemia *v.* time : $t_{1/2}$, apparent half-life of gametocytaemia; CL_{app} , volume of blood completely cleared of micro- or macro-gametocytes per unit time.

Discussion

An ideal chemosensitizer of *P. falciparum* to antimalarial drugs *in vivo* should not only accelerate the clearance of asexual parasitaemia and symptoms of infections without producing undue toxicity, but also reduce the frequency of gametocyte carriage and level of gametocytaemia. In addition, it should produce temporal changes in gametocyte sex ratios that reduce gametocyte infectivity to mosquito.

The present results show that, compared to pre-treatment, at the dose level of probenecid used, both PSP and PS, significantly increased the frequency of gametocyte carriage and levels of gametocytaemia post-treatment. With the exception of the levels of gametocytaemia on day 5 post-treatment, both these parameters were also similar in both treatment groups. It is unclear why there was a significant decrease in the level of gametocytaemia in those treated with PSP on day 5, but it may not be unrelated to the relatively rapid clearance of asexual parasites induced by the addition of probenecid. As commitment to gametocyte development occurs prior to schizont maturation (Silvestrini et al., 2000), and delay in the time taken to clear initial asexual parasitaemia (> 2 d, Sowunmi, unpublished) in children in southwestern Nigeria, is associated with increased risk of gametocyte carriage, it appears rapid clearance of asexual parasitaemia by PSP temporarily, reduced the progression of the committed populations of asexual parasites to sexual forms. This effect, however, was of short duration as levels of gametocytaemia were subsequently similar thereafter.

The gametocyte sex ratios in the small population of children at enrolment was male-biased, and at variance with earlier report (Sowunmi and Fateye, 2003 c). The relatively lower level of gametocytaemia at presentation, as a form of fertility insurance (Gardner et al., 2003), may be partly responsible. In addition, the children could have been exposed to other sex ratio modifying factors prior to presentation. These underscore an important fact: gametocyte sex ratios in *P. falciparum* may be variable (Paul et al., 2002, Robert et al., 2003). These, in turn, may explain why in some of the children SR1 was reached before, at or shortly after presentation.

In general, following both treatment regimens, there was significant increase in ♀ ratio. In a loogitudinal follow-up of gametocyte carriers in a village in Senegal, obert et al. (2003) found a density-dependent relationship with sex ratios. Peaks of unetocytaemia were sometimes associated with minimum sex ratio. The finding of ncreasing sex ratio despite increasing level of gametocytaemia (and peak ametocytaemia on day 7) following treatment with PS in our small cohort of children is n agreement with previous report (Sowunmi and Fateye, 2003 c), suggesting that PS, cting singly or in concert with other factors, may substantially increase gametocyte maleness. The similarity of temporal changes in sex ratios between PSP- and PS-treated children indicates that the addition of probenecid to PS had little or no effects on gametocyte sex ratios.

The findings of the present study support the notion that microgametocytes persist longer in circulation than macrogametocytes, or are longer lived (Ponnudurai et al., 1986; Reece et al., 2003; Sowunmi and Fateye, 2003 e). However, the addition of probenecid to PS did not alter micro- and macro-gametocyte disposition in the population of children. Given that levels of gametocytaemia and a male-biased sex ratio correlate with gametocyte infectivity to mosquito feeding on humans (Tchuinkam et al., 1993; Robert et al., 1996 b), it would appear that, the combination of probenecid at the present dose level with PS is unlikely to decrease the potential for transmission of malaria in the population, whether the gametocytes arise from sensitive or resistant PS infections.

Chapter 9

Comparative effects of pyrimethamine-sulphadoxine, chloroquine plus chlorpheniramine and amodiaquine plus pyrimethamine-sulphadoxine on gametocytes during and after treatment of acute, uncomplicated malaria

CHAPTER 9

Comparative effects of pyrimethamine-sulphadoxine, chloroquine plus chlorpheniramine and amodiaquine plus pyrimethamine-sulphadoxine on gametocytes during and after treatment of acute, uncomplicated malaria

Introduction

As resistance to chloroquine (CQ) increases in extent and severity, alternative regimens available to control programmes in developing endemic countries including pyrimethamine-sulphadoxine (PS), amodiaquine (AQ) (Olliaro et al., 1996; Brasseur et al., 1999; Sowunmi et al., 2001) or combination of AQ with PS (AQPS) (Sowunmi 2002) or other suitable combinations have become increasingly used in the treatment of CQ-resistant falciparum infections. These alternatives have varying effects on clearance of asexual parasitaemias or sexual forms of *P. falciparum*. For example, PS may (Puta and Manyando, 1997) or may not (Hogh et al., 1995) enhance gametocyte carriage during treatment of acute falciparum infections. Although the presence of gametocytes in peripheral blood after antimalarial treatment is no proof of viability, their generation is required for the transmission of the infection from the vertebrate to the anopheline host. In order to improve the management of paediatric cases of malaria and reduce transmission in our area of study, the effects of these drugs on gametocyte production needs urgent assessment. In addition, it is not clear whether the enhancement or non-enhancement of gametocyte production by PS will be influenced by its use in combination with other antimalarial drugs. It is noteworthy that antifolates are ineffective in the treatment of uncomplicated falciparum malaria in South America, for example, in Brazil (Fontes et al., 2002).

Resistance to CQ in *P. falciparum* can be reversed by chlorpheniramine (CP) *in vitro* and *in vivo* (Sowunmi et al., 1997, 1998 a, b, c). It has been recently shown that

the presence in peripheral blood of very young gametocytes (PYG) 72 h after commencing CQ may be used as indicator of response to CQ (Sowunmi et al., 2003). However, it is unclear whether the addition of CP to CQ will alter the use of PYG as an indicator of response to CQ or indeed as an indicator of failure of reversal of CQ resistance *in vivo* by CP. Although the combination of CQ with CP will not be employed by control programmes in Africa in the very near future, it is still essential to study PYG and peripheral mature gametocyte (PMG) generation during treatment with CQCP in the event that this or other similar combination become available.

In other to address these issues, gametocyte generation during treatment of falciparum malaria in children with PS, CQCP and AQPS have been evaluated. The main aims of our study were: (i) to evaluate the effects of PS, CQCP and AQPS on gametocyte generation during treatment with these drugs, (ii) to determine whether or not the addition of PS to AQ will influence the generation of gametocytaemia by PS and, (iii) to evaluate PYG as an indicator of response to PS, CQCP or AQPS.

Patients and methods

Study site

The study site, Ibadan, is a hyperendemic area for malaria in southwestern Nigeria (Salako et al., 1990). In the area, it is difficult to distinguish, clinically, re-infection from recrudescence after day-14 of treatment because of intense transmission. Antimalarial drugs have therefore generally, until recently, been evaluated on the basis of data recorded up to day 14, rather than the customary day 28 (Ekanem et al., 1990, Sowunmi and Salako, 1992).

Patients

The study took place at the University College Hospital in Ibadan, Nigeria. Overall, 166 children who presented with acute, symptomatic, uncomplicated *P. falciparum* malaria were enrolled in the study between September 1999 and September 2001.

The study was designed to elicit a 20% difference in cure rates between AQPS/CQCP on one hand and PS on the other hand with 80% power and at 95% level of confidence. The minimum number of patients required for each treatment arm is 45. In general, to be enrolled, the children had to be aged 0.5-10 years, and have symptoms compatible with acute, falciparum malaria (with fever or history of fever in the 24-48 h preceding presentation) and a pure *P. falciparum* parasitaemia of > 2000 asexual forms/μl blood. Those who had taken antimalarial drugs in the 2 weeks preceding presentation, provided a urine sample found positive for 4 aminoquinolines or sulphonamides (by the Dill-Glazko and lignin tests, respectively), or who had a concomitant illness, such as sickle-cell anaemia, or severe or complicated malaria (Warrell et al., 1990, WHO, 2000) were excluded. The informed consent of a parent or guardian was obtained for each child included in the study. A child was withdrawn from the study if she/he developed concomitant illness during the follow-up period, or if his/her parent or guardian requested it. The study received ethical approval from the local ethics committee.

Before enrolment in the study, a medical history of each child was obtained from an accompanying parent/guardian and each was physically examined. Body weight and oral or rectal temperature were recorded, and thick and thin films were prepared from finger-prick blood samples. These smears were Giemsa-stained for parasite identification and quantification of any peripheral parasitaemias.

Drug treatment

Children were randomly allotted to one of 3 treatment groups. One group received PS at presentation (day 0) at a dose 25 mg/kg of the sulphonamide component. Each tablet of PS contained 500 mg of sulphadoxine and 25 mg pyrimethamine. The other groups received chloroquine base, 30 mg/kg of body weight over 3 days (days 0-2) plus chlorpheniramine maleate, 6 mg at presentation followed by 4 mg every 8 h for 7 days (days 0-6) if the child was aged < 5 years, or 8 mg at presentation followed by 6 mg every 8 h if the child was ≥ 5 years, or a single dose of PS at presentation plus AQ 30 mg/kg over 3 days (days 0-2). All drugs were given by a physician orally and each child was observed for at least 3 h after each such supervised drug treatment, in order to ensure that the drug was not vomited. If it was, the child was excluded from the study. Additional management of some children included the administration of an antipyretic (e.g. 10-15 mg paracetamol/kg, every 8 h for 24 h) and fanning and tepid sponging when necessary.

Evaluation of response

Clinical observations were recorded daily for 8 days (days 0-7) and then on day 14. Thick and thin blood films, for quantification of parasitaemia, were prepared at the same times. At each follow-up, the guardians or parents (and, when possible, the children) were actively questioned, using a standard questionnaire, and the children were examined for the presence of adverse reactions to drugs.

Giemsa-stained blood films were examined by light microscopy under an oil-immersion objective, at X 1000 magnification, by two independent assessors who did not know the drug treatment of the patients. Parasitaemia in thick films was estimated

by counting asexual parasites relative to 1000 leukocytes, or 500 asexual forms, whichever occurred first. From this figure, the parasite density was calculated assuming a leukocyte count of 6000/µl blood. Young gametocytes (stage I-III) and mature gametocytes (stage IV and V) (Sinden, 1998) were also counted in thick blood films against 1000 leukocytes on days 0, 3, 4, 5, 6, 7, and 14. The responses to drug treatment were classified according to World Health Organization (1973) criteria. Treatment was considered a failure if the day-3 parasitaemia was > 25% of the day 0 value, if parasitaemia did not clear by day 7, or if parasitaemia cleared before day 7 but re-appeared before day 28. The parasite clearance time (PCT) was delined as the time elapsing from drug administration until there was no patent parasitaemia. The fever clearance time (FCT) was delined as the time from drug administration until the oral or rectal temperature fell to ≤ 37.4°C and remained so for at least 72 h. (This definition was necessary because of the routine use of paracetamol during the first 36 h of treatment in some children). Cure rates were defined as the proportions of patients who remained free of parasitaemia on day 14 of follow-up.

Re-treatment of drug treatment failures

All treatment failures were re-treated with AQPS on day 14 provided they were not symptomatic before this time. Patients with profound clinical (hyperpyrexia, oral fluid intolerance) and parasitological deterioration during follow-up were treated with artemether, 9.6 mg/kg of body weight over five days and were regarded as treatment failures.

Statistical analysis

Data were analysed using version 6 of the Epi-Info software (Anon. 1994). Proportions were compared by calculating χ^2 with Yates' correction or by Fisher exact tests. Normally distributed, continuous data were compared by Student's t-tests and analysis of variance (ANOVA). Data not conforming to a normal distribution were compared by the Mann-Whitney U-tests and the Kruskal-Wallis tests (or by Wilcoxon rank sum test). The values presented below are generally means and standard deviations (sd). P-values of < 0.05 were taken to indicate significant differences.

Results

Clinical and parasitological characteristics at enrolment and therapeutic responses

A total of 166 children was enrolled in the study. Fifty one, 52 and 63 children were enrolled in the PS, CQCP, and AQPS groups, respectively. Of these, 49, 48, and 60 children in the PS, CQCP, and AQPS groups, respectively completed the mandatory 14-day follow-up period and were analysed. The clinical and parasitological characteristics at enrolment were similar in all groups (Table 9.1). The therapeutic responses to drug treatment are also summarized in Table 9.1. AQPS was significantly more effective than CQCP or PS in clearing fever and parasitaemia and with a significantly higher cure rate on day 14. Direct comparison of PS and CQCP showed that fever (2.2 ± 1.1 *vs* 1.6 ± 0.8 day, P = 0.008) but not parasite clearance in those with sensitive response (2.7 ± 1.1 *vs* 2.5 ± 0.8 day, P = 0.33) was significantly faster with CQCP than with PS. The cure rate on day 14 was also significantly higher with CQCP than with PS (80.8 *vs* 59.2%, P = 0.03).

Gametocytaemia during follow-up

The prevalence and intensities of gametocytaemia before, during and after treatment are summarized in Table 9.2. Gametocyte carriage on days 3, 7, and 14 or days 3, 7, and 14 combined were significantly higher in the PS group than in the other treatment groups. However, the geometric mean gametocyte densities (GMGD) were similar in all the treatment groups.

The median survival time for peripheral young gametocytes (PYG) (Figure 9.1) in PS, CQCP, and AQPS treatment groups were 3.5, 1.5, and 1.5 days, respectively. There was a significant difference in the overall comparison of the survival experience using Wilcoxon (Gehan) statistics (χ^2 = 14.7, P = 0.0006). The ratios of the densities (per µl blood) of peripheral young gametocytes (PYG) to peripheral mature gametocytes are summarized in Table 9.3. The ratios were consistently below 1 in the CQCP and AQPS groups up till day 7. However, in the PS group, this ratio rose progressively to

TABLE 9.1. Clinical and parasitological parameters of the children enrolled in the study

	PS (n =49)	CQCP (n = 48)	AQPS(n = 60)	P value
Age (years)				
mean + s.d.	5.1 ± 2.7	6.0 ± 2.3	5.5 ± 2.5	0.52
range	0.6-10	2.0-10	1.2 – 10	
Weight (kg)				
mean ± s.d.	15.4 ± 5.6	16.8 ± 5.2	15.5 ± 4.7	0.32
range	6.5-26	8.1-35	6.26	
Duration of illness (d)				
mean ± s.d.	3.1 ± 1.4	3.6 ± 2.4	2.8 ± 1.3	0.06
range	1-7	2-14	1-8	
Presenting body temp. (°C)				
mean ± s.d.	38.5 ± 1.2	38.6 ± 1.2	38.1 ± 1.0	0.05
range	35.8–40.5	36.2-40.5	36.40.2	
Parasitaemia (per µl)				
geometric mean	37858	29248	30482	0.56
range	3310-375476	2511-219600	878-716000	
Fever clearance time (d)				
mean ± s.d	2.2 ± 1.1	1.6 ± 0.8	1.2 ± 0.9	0.000001
range	1-5	1-4	1-3	
Parasite clearance time (d)				
mean ± s.d.	2.7 ± 1.1	2.5 ± 0.8	2.2 ± 0.7	0.012
range	1-6	1-4	1-4	
No of children				
cured	29	38	60	0.000001
RI	10	10	-	
RII	7	-	-	
RIII	3	-	-	

95%CI: 95% confidence interval. PS: pyrimethamine-sulphadoxine, CQCP: chloroquine plus chlorpheniramine, AQPS: amodiaquine plus pyrimethamine-sulphadoxine

TABLE 9.2. Gametocytaemias before, during and after the treatment, with pyrimethamine-sulphadoxine (PS), chloroquine plus chlorpheniramine (CQCP) or amodiaquine plus pyrimethamine-sulphadoxine (AQPS), of *Plasmodium falciparum* infections in children

	PS (n = 49)	CQCP (n = 48)	AQPS (n = 60)	P value
Day 0 Gametocytaemia				
Geometric mean (/µl)	36	22	40	0.49
Mean ± S.E.	87 ± 52.1	26 ± 5.3	74 ± 43.3	
Range	12-444	12-36	12-288	
Day 3 Gametocytaemia				
Geometric mean (/µl)	36	44	40	0.92
Mean ± S.E.	100 ± 43.5	127 ± 83.5	86 ± 54.1	
Range	12-876	12-612	12-408	
Day 5 Gametocytaemia				
Geometric mean (/µl)	70	61	43	0.74
Mean ± S.E.	243 ± 112.9	118 ± 64.3	141 ± 121.0	
Range	12-468	12-518	12-504	
Day 7 Gametocytaemia				
Geometric mean (/µl)	135	39	36	0.38
Mean ± S.E.	399 ± 141.2	136 ± 73.6	98 ± 74.1	
Range	12-3520	12-696	12-468	
Day 14 Gametocytaemia				
Geometric mean (/µl)	83	34	19	0.21
Mean ± S.E.	139 ± 29	60 ± 30.1	24 ± 12	
Range	12-480	12-168	12-48	
No of patients with gametocytaemia on *				
Day 0	9	8	6	0.42
Day 3	25	8	7	0.000004
Day 7	26	8	6	0.000002
Day 14	20	4	3	0.000001
Day 3, 7 & 14	32	11	3	0.000000
Day 7 & 14	27	11	3	0.000000

*Wilcoxon (Gehan) for survival analysis ($\chi^2 = 14.7$, P = 0.0006)

FIGURE. 9.1. Micrograph of peripheral young gametocyte (arrows a -stage II. b –stage IV) obtained in thick blood smear of a child treated with pyrimethamine sulfadoxine

TABLE 9.3 Prevalence and intensities of peripheral young gametocytes and peripheral mature gametocytes in children treated with pyrimethamine-sulphadoxine (PS), chloroquine plus chlorpheniramine (CQCP) or amodiaquine plus pyrimethamine-sulphadoxine (AQPS)

	PS		CQCP		AQPS	
Day	GMPYGD (/µl)	GMPMGD (/µl)	GMPYGD (/µl)	GMPMGD (/µl)	GMPYGD (/µl)	GMPMGD (/µl)
0	21 (5)[a]	65 (4)	12 (4)	36 (1)	42 (2)	51 (2)
	12-36[b]	12-408	12-12	-	24-72	12-216
	1[c]	3.1[c]	1	3	1	1.2
3	28 (20)	57 (9)	55 (4)	106 (2)	27 (2)	348 (1)
	12-372	12-552	12-144	24-468	12-60	
	1	2.0	1	1.9	-*	-*
5	37 (21)	54 (17)	46 (6)	58 (4)	23 (3)	39 (3)
	12-640	12-1840	12-348	12-180	12-84	12-420
	1	1.5	1	1.3	1	1.7
7	79 (24)	85 (21)	42 (7)	97 (4)	28 (3)	87 (2)
	12-1320	12-2210	12-216	12-600	12-156	24-312
	1	1.1	1	2.3	1	3.1
14	50 (14)	78 (12)	21 (4)	35 (3)	-**	19 (3)
	12-240	12-444	12-120	12-72	-**	12-48
	1	1.6	1	1.7	-	

GMPYGD Geometric mean peripheral young gametocyte density; GMPMGD Geometric mean peripheral mature gametocyte density; a values in parentheses represent number of children with gametocytaemia; b range; c GMPYGD:GMPMGD ratio. * not calculated. ** no peripheral young gametocytes observed

l on day 7 indicating continuing production (or generation or mobilization) of young gametocytes. PYG-PMG density ratio increased significantly from day 0-14 in those treated with PS and CQCP (χ^2 = 76, P = 0.000001 and χ^2 = 42.2, P = 0.00001, respectively) but decreased significantly in those treated with AQPS (χ^2 = 53.2, P = 0.000001) (Figure 9.2).

Relationship between PYG and responses to drug treatment

None of the children successfully treated with CQCP had PYG during the follow-up. In children who had sensitive response to PS treatment (n = 29), PYG was present on days 0, 3, 5, 7, and 14 in 5, 12, 13, 13, and 7 children, respectively. Similarly in those successfully treated with AQPS (n = 60), PYG was present on days 0, 3, 5, 7, and 14 in 2, 2, 3, 3, and 0 patient, respectively. The PYG rates were significantly higher in those treated with PS than in those treated with AQPS at all times during follow up (P ≤ 0.006 in all comparisons). Post Hoc Turkey HSD test for repeated measure of the effect of PYG generation over the 14 day follow up showed significant differences in the comparisons of PS *vs* AQPS and PS *vs* CQCP (P = 0.0001 and 0.0001 respectively). There was no significant difference in the comparison of PYG generated by those treated with AQPS and CQPS (P = 0.08).

Relationship between PYG and outcomes of treatment in the children treated with PS and CQCP

The relationship between treatment outcomes and presence of PYG in children treated with PS and CQCP are shown in Tables 9.4 and 9.5. PYG rates were similar in those with sensitive or resistant responses to PS (18 of 29 *vs* 13 of 20, χ^2 = 0.04, P = 0.93) and the rates were similar from days 0-14. In contrast, PYG was seen only in those with resistant response to CQCP. In those without gametocytaemia at presentation, but who subsequently developed PYG 72 h after commencement of CQCP, the presence of PYG was associated with treatment failure on or before day 14 (Table 9.5).

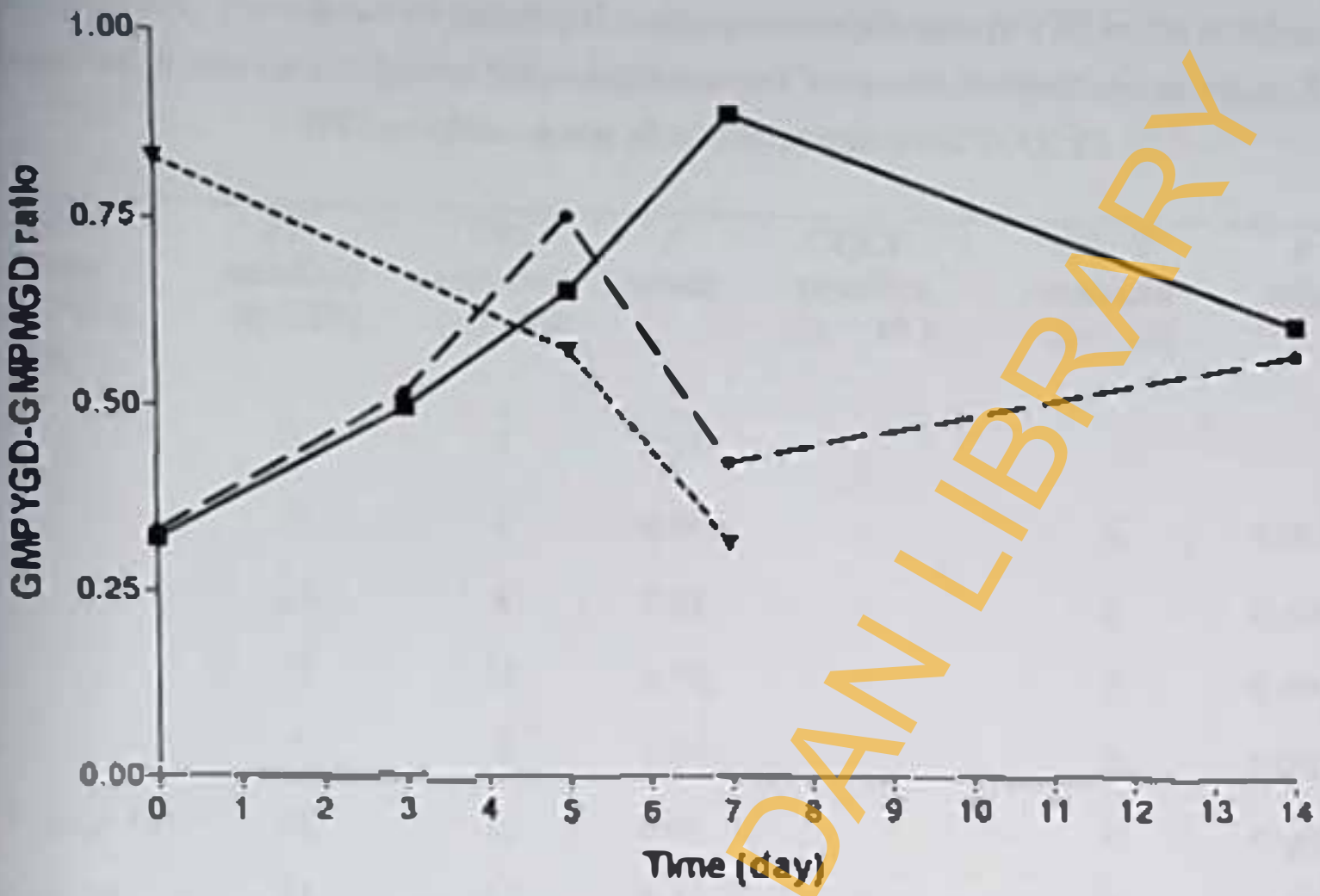


FIGURE 9.2 Changes in geometric mean peripheral young gametocytes(GMPYGD) and geometric mean peripheral mature gametocytes(GMPMG) ratio in children treated with pyrimethamine-sulphadoxine (-■-PS), chloroquine plus chlorpheniramine (-●-CQCP) or amodiaquine plus pyrimethamine-sulphadoxine (-◀-AQPS)

TABLE 9.4. Prevalence of peripheral young gametocytaemia (PYG) in the children with sensitive or resistant response following treatment with oral pyrimethamine-sulphadoxine (PS) or chloroquine plus chlorpheniramine (CQCP)

No. of children with PYG on day	PS-sensitive (n = 29)	PS-resistant (n = 20)	P value	CQCP-sensitive (n = 38)	CQCP-resistant (n = 10)	P value
0	5	4	0.81	-	3	-
3	12	8	0.96	-	4	0.001
5	13	8	0.85	-	6	0.00002
7	13	11	0.38	-	7	0.000002
14	7	7	0.34	-	4	0.001
3, 7 &/or 14	18	13	0.65	-	8	0.000002
7 &/or 14	13	13	0.11	-	7	0.0000001

TABLE 9.5. Peripheral young gametocyte (PYG) carriage at or after day 3 in children treated with oral pyrimethamine-sulphadoxine (PS) or chloroquine plus chlorpheniramine (CQCP) and who did not have gametocytaemia on presentation

No. of children with PYG on day	PS-sensitive (n = 21)	PS-resistant (n = 15)	P value	CQCP-sensitive (n = 38)	CQCP-resistant (n = 4)	P value
3	5	6	0.46	-	-	-
4	6	6	0.72	-	2	0.008
5	9	7	0.91	-	1	0.08
6	10	9	0.69	-	1	0.08
7	11	10	0.61	-	2	0.008
14	7	7	0.64	-	2	0.008

Discussion

The ideal antimalarial drugs or drug combinations for the treatment of falciparum malaria should not only promptly clear parasitaemia, fever or other symptoms of malaria, but should also prevent the generation of gametocytes from asexual forms during treatment. In the present study, PS was significantly less effective than CQCP or AQPS in clearing parasitaemia or fever in children with acute falciparum infections. This is not surprising since progressive decline in sensitivity of *P. falciparum* to PS has been reported from the area of study from the late 1990s (Falade et al., 1997; Sowunmi et al., 1998a). The decline in sensitivity of the parasite to PS has also occurred in many areas of Africa (Sibley et al., 2001).

In addition to their effects on the sexual forms, gametocyte carriage may be influenced to a considerable extent by the sensitivity of the asexual parasites to the drugs used for the treatment of infections. For example, as resistance of the asexual parasites to the 4-aminoquinolines, CQ, and AQ, increases, gametocyte carriage also increases (Strickland et al., 1986; Hogh et al., 1995). In these studies, gametocyte carriage rates 28 days after PS treatment were significantly less than those of CQ and AQ since PS was more effective than the 4-minoquinolines on asexual parasites in the settings of these studies. However, increased carriage may also be related to decreased sensitivity to PS in certain circumstances (Sowunmi et al., 1998a; Tjitra et al. 2002). In this cohort of children, gametocyte carriage was significantly higher at all times after treatment with PS than in the other treatment groups. In addition, PYG rates were similar in both PS-sensitive and -resistant infections supporting a known fact that PS enhanced generation or release of gametocytes during treatment of acute falciparum infections (Puta and Manyando, 1997). However, GMGD were similar in all the treatment groups.

Many antimalarial drugs appear to reduce gametocytaemia by clearing the asexual stage infections. This clearance, if exceptionally rapid, may reduce transmissibility particularly in areas of low transmission. For example, the artemisinin derivatives have reduced transmissibility in some parts of Thailand by this process (Price et al., 1996).

In order to determine the influence of treatment with antimalarial drugs on gametocyte production and densities, both young and mature gametocytes were quantified and were expressed as ratios. The ratios of PYG to PMG were consistently below 1 up to day 7 in those treated with CQCP and AQPS, but rose to 1 by day 7 in those treated with PS irrespective of the sensitivity of the asexual parasite to PS. This showed continuing and enhanced production or, preferential mobilization of gametocytes by PS irrespective of the sensitivity of the asexual parasites to PS. This process of continuing or preferential mobilization of young gametocytes by PS may explain why gametocytes persist longer in some patients treated with PS. This is plausible because the young gametocytes must grow and run the normal time-course of survival of the normal mature gametocytes.

Given that gametocyte density may correlate with mosquito infectivity and therefore transmission success (Tchuinkam et al., 1993; Drakeley et al., 1999), the effects of PS on gametocytes carriage and mobilization have implications for malaria control programmes with respect to the use of this drug. Recent WHO recommendations (WHO, 2001a, b) have focused on the use of combination antimalarial therapy (CT), particularly artemisinin-based combination therapy (ACT). Although several control programmes in Africa have switched to CT, some programmes use PS-based combination, for example, AQPS (Sowunmi, 2002). The modulating effect of AQ on enhanced production of PYG by PS may provide supporting argument for the use of combination therapy. However, the reduced generation of PYG by PS despite its combination with other drugs suggests that generation of gametocyte is an inherent property of antifolate antimalarials (Hamel et al., 2005).

In a recent study, it was shown that the detection of PYG 72 h after the start of CQ therapy may be used as an indicator of response to this drug (Sowunmi et al., 2003). The results of the present study show that PYG may also be used as an indicator of response to CQCP. Failure of the enhancement of the antimalarial efficacy of CQ by CP *in vivo* was associated with the presence, in peripheral blood, of young gametocytes. However, PYG was not an indicator of response to PS, since both PS-sensitive and -resistant infections generated PYG. In addition, the presence of PS in combination with

AQ also generated PYG and was clearly not an indicator of response to AQPS since the cure rate in this group was 100%.

The limitation of the present study is the fewer number of gametocyte carriers in the AQPS and CQCP groups following treatment. Therefore caution is required with the interpretation of the data from these two groups.

Chapter 10

Comparative effects of antifolates- trimethoprim-sulfamethoxazole and pyrimethamine-sulfadoxine on gametocytes in children with acute, symptomatic, uncomplicated, Plasmodium falciparum malaria

CHAPTER 10

Comparative effects of antifolates- trimethoprim-sulfamethoxazole and pyrimethamine-sulfadoxine on gametocytes in children with acute, symptomatic, uncomplicated, *Plasmodium falciparum* malaria

Introduction

The antifolate antimalarial, pyrimethamine-sulfadoxine (PS), has become increasingly used as first line treatment of falciparum malaria in several African countries because of increasing resistance in *Plasmodium falciparum* to chloroquine (CQ). In spite of frequent use and of *in vivo* and *in vitro* studies (Hogh et al., 1998, Sowunmi and Fateye, 2003 b), its effects on gametocytes in children with falciparum infections remain incompletely understood.

With increasing use, resistance in *P. falciparum* to PS is increasing (Sibley et al., 2001) probably as a consequence of long half lives of its components. It has recently been suggested that, trimethoprim-sulfamethoxazole (TS), an antifolate antimalarial with relatively short half-lives of its components compared to PS, may be used as alternative to the latter for the treatment of uncomplicated falciparum infections in children because it is as efficacious as PS (Omar et al., 2001 a; Fchintola et al., 2004). It is assumed that the relatively short half-life of TS may, when compared with PS, reduce the chances of engendering resistance in *P. falciparum* to this drug and may provide additional advantage with transmission of drug resistance infections over PS.

However, while the effects on PS on gametocytes and gametocyte sex ratios (GSR) are known (GSR may influence infectivity to mosquitoes and transmission- see

Robert et al , 1996 b, Sowunmi and Fateye, 2003 c), the effects of TS on gametocytes are relatively unknown in African children with falciparum malaria. It is hypothesized that PS and TS have similar effects on gametocyte prevalence, density and sex ratio, and possess similar effects on gametocyte survival in children treated with these drugs. This hypothesis was tested in a group of children with acute symptomatic uncomplicated *P. falciparum* malaria who were randomized to and who received PS and TS for the treatment of their infections.

Patients and methods

Patients

Between June and August 1999, a randomized trial of TS and PS for the treatment of uncomplicated falciparum malaria was conducted in 102 children at the University College Hospital in Ibadan, a hyperendemic area for malaria in southwestern Nigeria (Salako et al., 1990). Ethical clearance for the study was provided by the local ethics committee. In general, to be enrolled, the children had to be aged 0.5–12 years, and have symptoms compatible with acute, falciparum malaria (with fever or history of fever in the 24–48 h preceding presentation) and a pure *Plasmodium falciparum* parasitaemia of > 2000 asexual forms/μl blood. Those who had taken antimalarial drugs in the 2 weeks preceding presentation, provided a urine sample found positive for 4 aminoquinolines or sulfonamides (by the Dill-Glazko and lignin tests, respectively), or who had a concomitant illness, such as sickle-cell anaemia, or severe or complicated malaria (WHO, 2000) were excluded. The informed consent of a parent or guardian was obtained for each child included in the study. A child was withdrawn from the study if she/he developed concomitant illness during the follow-up period, or if his/her parent or guardian requested it. Thick and thin blood films from all patients who participated in the study were examined for the presence and density of asexual and sexual parasites at enrolment and start of treatment (day 0), and at follow-up at days 1–7, and then on day 14. TS was given as 20 mg/kg of the sulfamethoxazole component twice daily for 5 days (day 0–4); PS was given as the 25 mg/kg of the sulfadoxine component at presentation (day 0). All drugs were administered orally.

Assessment of parasitaemia and gametocyte sex ratio

Thick and thin blood films prepared from a finger prick were Giemsa-stained and were examined by light microscopy under an oil-immersion objective, at x 1000 magnification, by two independent assessors who did not know the drug treatment of the patients. Parasitaemia in thick films was estimated by counting asexual parasites relative to 1000 leukocytes, or 500 asexual forms, whichever occurred first. From this figure, the parasite density was calculated assuming an average leukocyte count of 6000/μL of blood (Shaper and Lewis, 1971; Ezeilo, 1971; Sowunmi et al., 1995). Gametocytes were also

counted in thick films against 1000 leukocytes assuming an average leukocyte count of 6000/μL of blood at enrolment (day 0) and on days 3, 5, 7 and 14. Gametocytes were sexed if gametocytaemia was ≥ 12 sexual forms/μl. Gametocyte sex determination was based on following criteria (Carter and Graves, 1988; Robert et al., 1996 b): males are smaller than females; the nucleus is bigger in males than females; the ends of the cells are round in males and angular in females; the cytoplasm stains pale purple in males and deep blue in females; and the granules of malaria pigment are centrally located in females and more widely scattered in males. Gametocyte sex ratio was defined as the proportion of gametocytes in peripheral blood that were microgametocytes (Pickering et al., 2000; West et al., 2001).

Statistical analysis

Data were analysed using version 6 of the Epi-Info software (Anon., 1994), and the statistical program SPSS for Windows version 10.01 (SPSS, 1999). Proportions were compared by calculating χ^2 with Yates' correction or Fisher exact test or Mantel Haenszel test. Normally distributed, continuous data were compared by Student's t-tests and analysis of variance (ANOVA). Data not conforming to a normal distribution were compared by the Mann-Whitney U-test and the Kruskal-Wallis test (or by Wilcoxon rank sum test). Kaplan-Meier analysis was used to estimate the cumulative probability of remaining free of gametocytes during follow-up for all cases of malaria combined and for those cases that were free of gametocytaemia at enrolment. Differences in survival time were assessed by inspection of Kaplan-Meier curves and pair wise log-rank tests. P-values of ≤ 0.05 were taken to indicate significant differences.

Results

Demographic characteristics and therapeutic responses

A total of 104 children were enrolled into the study. Two children, one in each treatment group were excluded from the study due to parental relocation. These children were cleared of their peripheral parasitaemia at the time of exclusion. The demographic characteristics of the children enrolled in the study and the therapeutic responses to the treatment given are summarized in Table 10.1. These were similar in the two treatment groups. However, parasite clearance was significantly shorter in those treated with TS than PS.

Prevalence of gametocytaemia

The prevalence of gametocytaemia before and after treatment with PS, and before, during and after treatment with TS is shown in Table 10.2. Gametocyte carriage was similar on days 0-7 in both treatment groups and it peaked at day 7 in both TS and PS groups. Gametocyte carriage was significantly lower on day 14 in those treated with TS than PS ($\chi^2 = 5.7$, $P = 0.017$). Eleven and 19 children treated with TS and PS, respectively were gametocyte carriers on both days 7 and 14. The difference between these proportions was significant ($\chi^2 = 4.0$, $P = 0.046$).

In general, compared to pre-treatment, both drugs significantly increased gametocyte carriage post-initiation of treatment ($\chi^2 = 20.9$, $P = 0.003$ for TS and $\chi^2 = 28.4$, $P = 0.0001$ for PS, see Table 10.2). In children without patent gametocytaemia at enrolment, there was a greater propensity to be gametocyte-positive by day 7 with a significantly greater proportion of children treated with PS having gametocytes by day 14 of follow up compared with TS (30/47 [63.8%] vs 16/48 [43.3%], $\chi^2 = 7.66$, $P = 0.005$).

TABLE 10.1 Summary of clinical characteristics at enrolment and therapeutic responses in patients with acute falciparum malaria treated with trimethoprim-sulfamethoxazole or pyrimethamine-sulphadoxine

Parameter	TS (n = 53)	PS (n = 49)	P value
Age (y)			
mean ± sd	6.3 ± 2.9	6.3 ± 2.8	0.9
range	1.5 – 12.0	0.8 – 10.5	
Weight (kg)			
mean ± sd	18.2 ± 6.4	17.6 ± 5.2	0.6
range	7.5 – 34.5	7.0 – 28.0	
Temperature (°C)			
mean ± sd	38.1 ± 1.3	38.4 ± 1.4	0.2
range	35.7 – 40.9	35.9 – 41.0	
Parasite density (/µl)			
Geometric mean	36543	34983	0.29
Range	2200-349636	2552-652800	
Gametocyte density (/µl)			
Geometric mean	15 (n = 3)	17 (n = 2)	0.8
Range	12 – 24	12 – 24	
PCT (d)			
mean ± sd	2.5 ± 0.9 (n = 50)	3.2 ± 1.2 (n = 44)	0.002
range	1-5	1-6	
FCT (d)			
mean ± sd	2.0 ± 1.0	2.3 ± 1.3	0.20
range	1 – 4	1 – 6	
Day 14 responses*			
No of infections			
Cured (%)	47 (88.7)	43 (87.7)	0.88
RI	6	5	
RII	0	0	
RIII	0	1	

*Using WHO (1973) criteria
TS, trimethoprim-sulfamethoxazole; PS, pyrimethamine-sulphadoxine; FCT, fever clearance time; PCT, parasite clearance time; SD standard deviation. All comparisons were two-tailed

TABLE 10.2. Intensity and prevalence of *P. falciparum* gametocytaemia following treatment of uncomplicated malaria with trimethoprim-sulfamethoxazole or pyrimethamine-sulfadoxine of 102 malarious children

	[illegible] (n = 53)	[illegible] (n = 49)	[illegible] value
Day 0	18 [12 – 48]*	17 [12 - 24]	1.0***
	5/ 53 (9.4%)**	2/ 49 (4.1%)	0.44¶
Day 3	27 [12 – 120]	27 [12 – 144]	1.0
	13/ 53 (25.0%)	12/ 49 (24.5%)	1.0
Day 5	33 [12 – 420]	45 [12 – 1872]	0.49
	21/51(41.2%)	25/48 (52.1%)	0.31
Day 7	42 [12 – 444]	71 [12 – 2316]	0.27
	28/49 (57.1%)	34/ 46 (73.9%)	0.13
Day 14	33 [12 – 120]	43 [12 – 1200]	0.52
	13/37 (35.1%)	23/35 (65.7%)	0.018

* Geometric mean [range] ** Gametocyte positive/ No of patients examined, values in parentheses represents percentage of patients with gametocytaemia *** Mann Whitney test ¶ χ^2 square test

Gametocytaemia

Gametocytaemia before and after treatment with PS, and before, during and after treatment with TS is shown in Table 10.2. Gametocytaemia was similar throughout the duration of the study in both TS and PS-treated children with peak gametocytaemia occurring in both treatment groups on day 7. Peak gametocytaemia (on day 7) was significantly higher than day 3 gametocytaemia in both treatment groups (t = 0.066, P = 0.018 for TS; t = 0.08, P = 0.017, by Wilcoxon sign rank test for paired data). Gametocytaemias occurring on days 3-14 were not compared with pre-treatment gametocytaemia because of the small number of patients in both groups. However, multiple comparison of gametocytaemia using Friedman test showed that there was significant increase in gametocytaema with time on days 3, 5, 7 and 14 in those treated with PS (P = 0.011). In comparison, there was no significant increase in gametocytaemia with time on days 3, 5, 7 and 14 in those treated with TS (P = 0.29).

The Kaplan-Meier survival curve of the cumulative probability of remaining gametocyte-free in children who were agametocytaemic at enrolment is shown in Figure 9.1. By day 7 of follow up, children treated with PS had a significantly higher propensity to have developed gametocytes than in TS-treated children (Log-rank statistic 5.35, df = 1, P = 0.02).

Temporal changes in gametocyte sex ratios

In TS-treated children, 7, 28, 104, 134 and 44 gametocytes were counted on days 0, 3, 5, 7 and 14, respectively and approximately 77% of these gametocytes could be sexed. In PS-treated children, 7, 34, 230, 293 and 168 gametocytes were counted on days 0, 3, 5, 7 and 14, respectively and approximately 76% of these gametocytes could be sexed. The data on GSR at enrolment were pooled because of the small number of gametocyte carriers observed pre-treatment (five among TS-treated children and 2 among

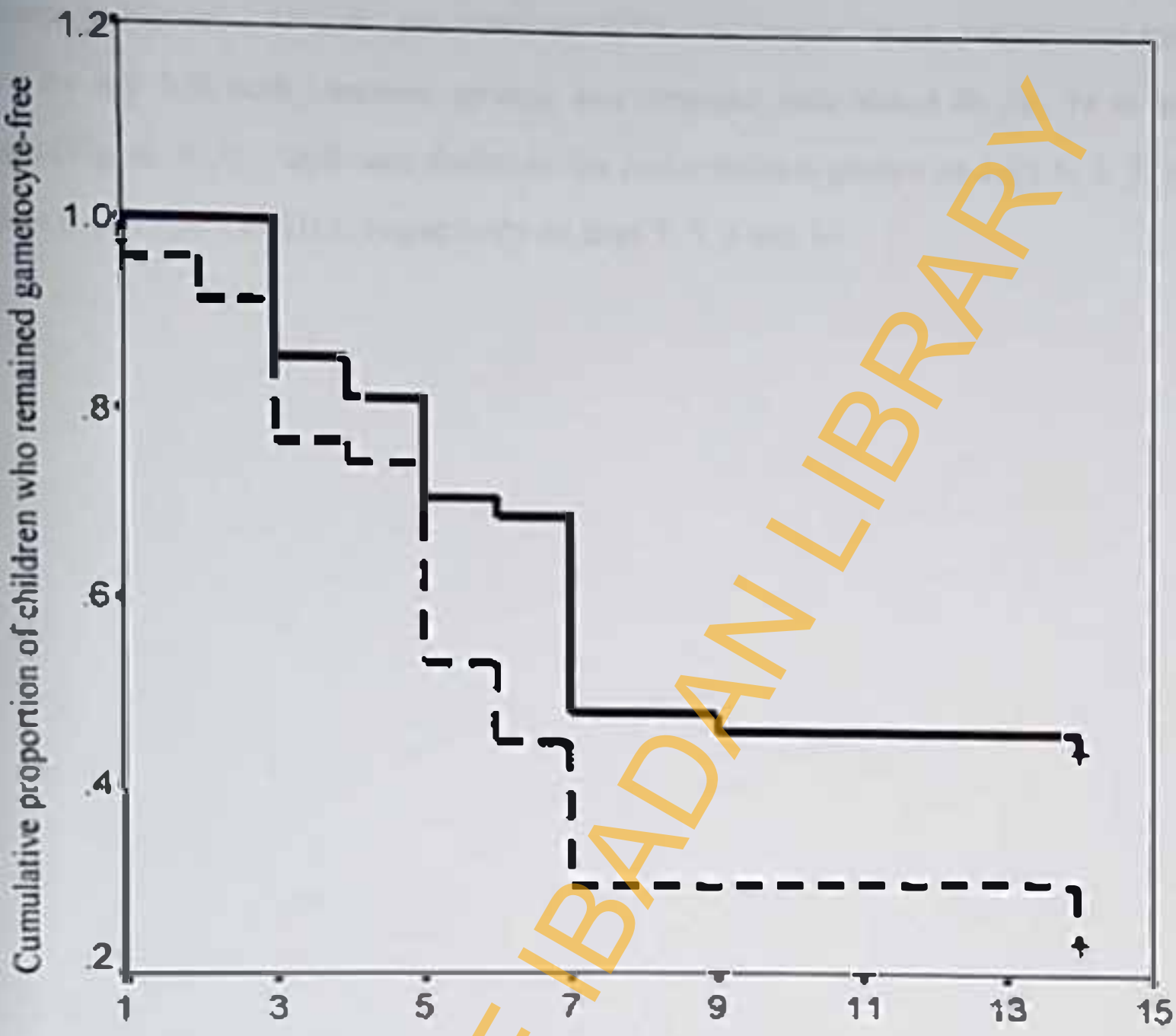


FIGURE 10.1 Figure 1. Kaplan-Meier plot (survival curve) of cumulative probability of remaining gametocyte-free in 95 children who were agamctocytaemic at enrolment following treatment with trimethoprim-sulfamethoxazole (TS, broken line) or pyrimethamine-sulphadoxine (PS, solid line)

PS-treated children). Overall, pre-treatment GSR was female-biased, but became male-biased by day 3 in both treatment groups, and remained male-biased till day 14 in both groups (Figure 10.2). GSR was similar in the two treatment groups on days 3, 5, 7, and 14 (P = 0.4, 0.7, 0.7 and 0.2, respectively on days 3, 5, 7 and 14.

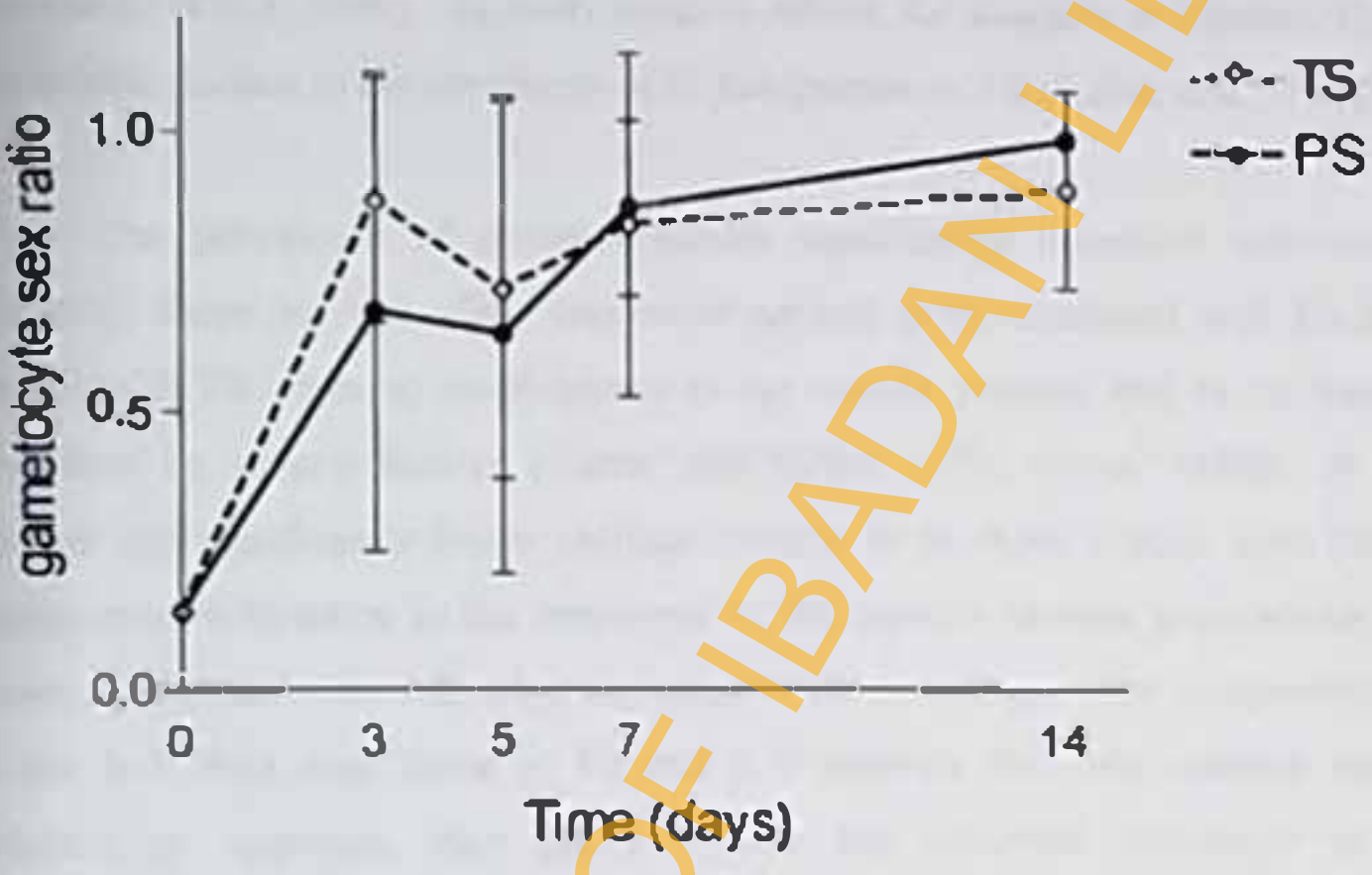


FIGURE 10.2 Changes in sex ratio of gametocytes before and after treatment with pyrimethamine-sulfadoxine (PS), and before, during and after treatment with trimethoprim-sulfamethoxazole (TS) in children with acute, uncomplicated, falciparum malaria. The vertical lines indicate standard error.

Discussion

TS and PS were both effective in the treatment of uncomplicated falciparum malaria in children from this endemic area of southwest Nigeria. Apart from a significantly shorter parasite clearance in the TS-treated children, none of the outcome measures, clinical or parasitological, differed between the two antifolate drug combinations. The results support those of recent findings from the same area (Fehintola et al., 2004) and are in agreement with those from Kenya (Omar et al., 2001 a). However, the results are contrary to the suggestion that TS is less effective than PS for the treatment of malaria (WHO, 1996). In many areas in Africa, for example in Uganda, there has been appreciable decline in the sensitivity of *P. falciparum* to TS (Kilian et al., 1998).

The prevalence of gametocytaemia significantly increased following treatment with both drugs but this effect was more marked in those treated with PS than in those treated with TS. Sexual development in the malaria parasite and its modulation may be influenced by several factors (Carter and Miller, 1979, Mons, 1985). It is not clear whether the significantly lower carriage on day 14 in those treated with TS was due to fundamental differences in the responses of the asexual parasite populations to switch to gametocyte production following exposure to the two drugs. The components of TS have shorter half lives than those of PS and it is possible that this, coupled with individual variation in response, may partly explain the observed difference in gametocyte prevalence between the two drugs.

Although there were no significant differences in gametocyte density in the two treatment groups, the significant increases in gametocyte prevalence with time, the greater proportion of children with patent gametocytaemia on both days 7 and 14 among children treated with PS, and the significantly higher propensity to have developed gametocytes by day 7 in PS compared with TS treated children (see Figure 10.1) suggest a more marked effects of PS on gametocyte production. These findings with PS are in agreement with previous observations from the same area (Sowunmi and Fateye, 2003 b, c). Thus, the significantly reduced effects of TS on gametocyte retention may be an advantage for the use of TS over PS in endemic setting.

Despite lower gametocyte prevalence and insignificant increase in gametocytaemia with time in TS treated children, both TS and PS appear to have similar effects on GSR. None of the post-treatment initiation GSR data differ between the two antifolate drug combinations; both drugs favoured gametocyte maleness. It is not clear whether the effects of the drugs on gametocytaemia are fundamentally different from their effects on GSR. Since GSR may be influenced by several factors (West et al., 2002; Gardner et al., 2003), this may impact on the temporal changes in GSR. The male-biased sex ratio after PS treatment is in agreement with recent findings from the same area (Sowunmi and Fateye, 2003 c). The gametocyte maleness seen after initiation of treatment with both drugs suggests that antifolates, in general, may favour gametocyte maleness. Since gametocyte infectivity to mosquito is increased by gametocyte maleness (Robert et al., 1996 b) and infectivity correlates with gametocyte density (Tchuinkam et al., 1993; Robert et al., 2000), both TS and PS by enhancing gametocyte maleness, gametocyte carriage and gametocytaemia, may markedly enhance malaria transmission whether the treated patients have antifolate sensitive or resistant infections. This a demerit for the use of these drugs alone for the treatment of malaria.

Conclusions and Recommendations

Conclusions and Recommendations

The studies presented in this dissertation have shown that

- Children are uniformly susceptible to gametocyte carriage and that longer duration of illness, absence of fever, male gender and parasitaemia < 5000 /µl are risk factors for gametocyte carriage.
- Apart from male gender, the risk factors associated with gametocyte carriage are little affected by season
- Children with CQ- resistant infections and those treated with PS irrespective of outcome were significantly at risk of gametocyte carriage
- PS treatment significantly increased PYG. PMG, but significant increases in this ratio were found only with CQCP resistant infections. AQPS significantly decreased the ratio
- Presence of PYG was an indicator of response to CQCP but not to PS or AQPS.
- PPS and TS, like TS alone, enhanced gametocyte carriage and gametocyte maleness, but TS has a lower propensity to cause gametocyte maleness
- Recently developed molecular assays are more sensitive than microscopic method. Therefore, the estimates of the prevalence of gametocytaemia in the studies reported in this thesis are likely to be underestimates.

Further studies are needed in the following areas:

a. The effects of other antimalarial drugs, for example amodiaquine, a drug similar in action to chloroquine, or its combination with artesunate on gametocyte carriage and sex ratio
b. Evaluation of the value of PYG as an indicator of resistance to amodiaquine
c. Molecular and cellular basis of the mobilization of gametocytes to peripheral blood by pyrimethamine sulfadoxine
d. Infectivity to mosquitoes, of gametocytes obtained after treatment with various antimalarial drugs studied in this dissertation.

e. The prevalence of submicroscopic gametocytaemia before, during and after treatment with various antimalarial drugs and combination therapies by molecular assays.

f. Infectivity to mosquitoes of blood obtained from children with submicroscopic gametocytaemia before, during and after treatment with various antimalarial drugs and combination therapies.

g. Distinguishing between male and female gametocytes using molecular assay techniques.

References

REFERENCES

Adjuik, M., Agnamey, P., Babiker, A., Borrmann, S., Brasseur, P., Cisse, M., Cobelens, F., Diallo, S., Faucher, J.F., Garner, P., Gikunda, S., Kremsner, P.G., Krishna, S., Lell, B., Loolpapit, M., Matsiegui, P-B., Missinou, M.A., Mwanza, J., Ntoumi, F., Olliaro, P., Osimbo, P., Rezbach, P., Some, R. and Taylor, W.R. 2002. Amodiaquine-artesunate versus amodiaquine for uncomplicated *Plasmodium falciparum* malaria in African children: a randomized multicentre trial. *Lancet* 359: 1365-1372.

Akim, N.I.J., Drakeley, C., Kingo, T., Simon, B., Senkoro, K. and Sauerwein, R.W. 2000. Dynamics of *Plasmodium falciparum* gametocytaemia in symptomatic patients in an area of intense perennial transmission in Tanzania. *American Journal of Tropical Medicine and Hygiene* 63: 199-203.

Anon. 1994. Epi Info Version 6. A Word Processing Data Base and Statistics Program for Public Health on IBM-compatible Microcomputers. Atlanta, GA: Centers for Disease Control and Prevention.

Babalola, C.P., Bolaji O.O., Dixon, P.A. and Ogunbona, F.A. 1993. Column liquid chromatographic analysis of quinine in human plasma, saliva and urine. *Journal of Chromatography* 616: 151-154.

Basco, L.K., Same-Ekobo, A., Ngane, V.F., Ndounga, M., Metoh, T., Ringwald, P. and Soula, G. 2002. Therapeutic efficacy of sulfadoxine-pyrimethamine, amodiaquine and the sulfadoxine-pyrimethamine-amodiaquine combination

against uncomplicated *Plasmodium falciparum* malaria in young children in Cameroon. *Bulletin of the World Health Organization* 80: 538-545.

Billingsley, P.F. 1990. The midgut ultrastructure of haematophagous insects. *Annual Review Entomology* 35 219-248.

Billingsley, P.F. and Hecker, H. 1991. Blood digestion in the mosquito, *Anopheles stephensi* Liston (Diptera: Culicidae): activity and distribution of trpsin, aminopeptidase, and alpha-glucosidase in the midgut. *Journal of Medical Entomology* 28: 865-871.

Birago, C., Dore, E., Frontali, C. and Zenobi, P. 1982. Mosquito infectivity is directly related to the proportion of repetitive DNA in *Plasmodium berghei*. *Molecular Biology and Parasitology* 6: 1-12.

Bonnet, S., Gouagna, L.C., Paul, R.E., Safeukui, I., Meunier, J.-Y. and Boudin, C. 2003. Estimation of malaria transmission from humans in two neighbouring villages in south Cameroon: evaluation and comparison of several indices. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 97: 53-59.

Borst, P., Evers, R., Kool, M. and Wijnbolds, J. 2000. A family of drug transporters: the multidrug resistance-associated proteins. *Journal of National Cancer Institute* 92: 1295-1302.

Bousema, J.T., Schneider, P., Gouagna, L.C., Drakeley, C.J., Tostmann, A., Houben, R., Githure, J.I., Ord, R., Sutherland, C.J., Omar, S.A. and Sauerwein R.W. 2006. Moderate effect of artemisinin-based combination therapy on transmission of *Plasmodium falciparum*. *Journal of Infectious Disease* 193: 1151-1159.

Boyd, M.F., Stratman-Thomas, W.F. and Kitchen, S.F. 1935. On the relative susceptibility of *Anopheles quadrimaculatus* to *Plasmodium vivax* and *Plasmodium falciparum*. *American Journal of Tropical Medicine and Hygiene* 15: 485-493.

Brasseur, P., Guiguemde, R., Diallo, S., Guiyedi, V., Kombila, M., Ringwald, P. and Olliaro P. 1999. Amodiaquine remains effective for the treating uncomplicated malaria in West and Central Africa. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 93: 645-650.

Briegel, H. and Lea, A.O. 1975. Relationship between protein and proteolytic activity in the midgut of mosquitoes. *Journal of Insect Physiology* 21: 1597-1604.

Bruce Chwatt, L.J. 1951. Malaria in Nigeria. *Bulletin of the World Health Organization* 15: 775-784.

Bruce, M.C., Alano, P., Duthie, S. and Carter, R. 1990. Commitment of the malaria parasite *Plasmodium falciparum* to sexual and asexual development. *Parasitology* 100: 191-200.

Buckling, A.G.J., Taylor, L.H., Carlton, J.M.R. and Read, A.F. 1997. Adaptive changes in *Plasmodium* transmission strategies following chloroquine chemotherapy. *Proceedings of Royal Society London* B 264: 553-559.

Buckling, A., Ranford-Cartwright, L.C., Miles, A. and Read, A.F. 1999. Chloroquine increases *Plasmodium falciparum* gametocytogenesis *in vitro*. *Parasitology* 118: 339-346.

Butcher, G.A. 1997. Antimalarial drugs and the mosquito transmission of *Plasmodium*. *International Journal of Parasitology* 27: 975-987.

Carter, R. and Miller, L.H. 1979. Evidence for environmental modulation of gametocytogenesis in *Plasmodium falciparum* in continuous culture. *Bulletin of the World Health Organization* 57: 37–52.

Carter, R., Graves, P.M., Quakyi, I. and Good, M.F. 1989. Restricted or absent immune responses in human populations to *Plasmodium falciparum* gamete antigens that are targets of malaria transmission-blocking antibodies. *Journal of Experimental Medicine* 169: 135-147.

Carter, R. and Graves, P.M. 1988. Gametocytes. In: *Malaria. Principles and practice of malariology*, edited by Wernsdorfer, W.H. and McGregor, Sir I. Edinburgh, Chuchill Livingstone. pp 253- 306.

Carter, R. and Nijhout, M.M. 1977. Control of gamete formation (exflagellation) in malaria parasites. *Science* 195: 407-409.

Chege, G.M.M., Pumpuni, C.B. and Beier, J.C. 1996. Proteolytic enzyme activity and *Plasmodium falciparum* sporogonic development in three species of *Anopheles* mosquitoes. *Journal of Parasitology* 82: 11-16.

Chutmongkonkul, M., Maier, W.A. and Seitz, H.M. 1992. *Plasmodium falciparum:* effect of chloroquine, halofantrine and pyrimethamine on the infectivity of gametocytes for *An. stephensi* mosquitoes. *Annals of Tropical Medicine and Parasitology* 86: 103-110.

Coleman, R.E., Vaughan, J.A., Hayes, D.O., Hollingdale, M.R. and Do Rosario, V.E. 1988. Effects of mefloquine and artemisinin on the sporogonic cycle of *Plasmodium berghei* ANKA in *Anopheles stephensi* mosquitoes. *Acta Leidensia* 57: 61- 74.

Day, K.P., Karamalis, F., Thompson, J., Barnes, D.A., Peterson, C., Brown, H., Brown, G.V. and Kemp, D.J. 1993. Genes necessary for expression of a virulence determinant and for transmission of *Plasmodium falciparum* are located on a 0.3 megabase region of chromosome 9. *Proceeding National Academy of Science* 90: 8292-829.

Day, K.P., Hayward, R.E. and Dyer, M. 1998. The biology of *Plasmodium falciparum* transmission stages. *Parasitology* 116: S95-S109.

Diourté, Y., Djimdé, A., Doumbo, O.K., Sagara, I., Coulibaly, Y., Dicko, A., Diallo, M., Diakite, M., Cortese, J.F. and Plowe, C.V. 1999. Pyrimethamine-sulphadoxine efficacy and selection for mutations in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase in Mali. *American Journal of Tropical Medicine and Hygiene* 60: 475-478.

Do Rosario, E., Vaughan, J.A., Murphy, M.L., Harrod, V. and Coleman, R. 1988. Effects of chloroquine on the sporogonic cycle of *Plasmodium falciparum* and *Plasmodium berghei* in anopheline mosquitoes. *Acta Leidensia* 57: 53-60.

Drakeley, C.J., Secka, I., Correa, S., Greenwood, B.M. and Targett, G.A. 1999. Host haematological factors influencing the transmission of *Plasmodium falciparum* gametocytes to *Anopheles gambiae s.s.* mosquitoes. *Tropical Medicine and International Health* 4: 131-138.

Drakeley, C.J., Jawara, M., Targett, G.A.T., Walraven, G., Obisike, U., Coleman, R., Pinder, M. and Sutherland, C.J. 2004. Addition of artesunate to chloroquine for the treatment of *P. falciparum* malaria in Gambian children causes a significant but short-lived reduction in infectiousness for mosquitoes. *Tropical Medicine and International Health* 9: 53-61.

Duraisingh, M.T., Drakeley, C.J., Muller, O., Bailey, R., Snounou, G., Targett, G.A., Greenwood, B.M. and Warhurst, D.C. 1997. Evidence for selection for the tyrosine-86 allele of the *pfmdr 1* gene of *Plasmodium falciparum* by chloroquine and amodiaquine. *Parasitology* 114: 205-211.

Dyer, M. and Day K.P. 2000. Commitment to gametocytogenesis in *Plasmodium falciparum*. *Parasitology Today* 16: 102–107.

Eichner, M., Diebner, H.H., Molineaux, L., Illins, W.G., Jeffiery, G.M. and Dietz, K. 2001. Genesis, sequestration, and survival of *Plasmodium falciparum* gametocyte: parameter, estimate from filling a model malaria therapy data. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 95: 497-501.

Ekanem, O.J. 1985. *Plasmodium falciparum* infection not responding to chloroquine. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 79: 141-142.

Ekanem, O.J., Weisfeld, J.S., Salako, L.A., Nahlen, B.L., Ezedinachi, E.N.U., Walker, O., Breman, J.G., Laoye, O.J. and Hedberg, K. 1990. Sensitivity of *Plasmodium falciparum* to chloroquine and sulphadoxine-pyrimethamine in Nigerian children. *Bulletin of the World Health Organization* 68: 45-52.

Eyles, D.E. 1952. Studies on *Plasmodium gallinaceum* II. Factors in the blood of the vertebrate host influencing mosquito infection. *American Journal of Tropical Medicine and Hygiene* 5: 276- 290.

Ezeilo, G.C. 1971. Neutropenia in Africans. *Tropical and Geographical Medicine* 23: 264-267.

Falade, C.O., Salako, L.A., Sowunmi, A., Oduola, A.M.J. and Larcier, P. 1997. Comparative efficacy of halofantrine, chloroquine and sulphadoxine-pyrimethamine in the treatment of acute uncomplicated falciparum malaria in Nigerian children. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 91: 58-62.

Federal Ministry of Health. 2004. National antimalaria treatment policy, Federal Ministry of Health National Malaria and Vector Control Division, Abuja, Nigeria, p. 1-28.

Feldmann, A.M. and Ponnudurai, T. 1989. Selection of Anopheles stephensi for refractoriness and susceptibility to *Plasmodium falciparum*. *Medical and Veterinary Entomology* 3: 41-52.

Feldmann, A.M., Billingsley, P.F. and Sawerkoul, E. 1990. Bloodmeal digestion by strains of *Anopheles stephensi liston* (Diptera: Culicidae) of differing susceptibility to *Plasmodium falciparum*. *Parasitology* 101: 193–200.

Fehintola, F.A., Adedeji, A.A., Tambo, E., Fateye, B.B., Happi, T.C. and Sowunmi, A. 2004. Cotrimoxazole in the treatment of acute uncomplicated falciparum malaria in Nigerian children: a controlled clinical trial. *Clinical Drug Investigation* 24: 149-155.

Feng, Z. Hoffman, K.N., Nussenzweig, R.S., Tsuji, M., Fujioka, H., Aikawa,M., Lensen, T.H.W., Ponnudurai, T. and Pologe, L.G. 1993. Pfs2400 can mediate antibody-dependent malaria transmission inhibition and may be the *Plasmodium falciparum* 11-1 gene product. *Journal of Experimental Medicine* 177: 273-81.

Field, J W and Shute, P G 1956. In: A morphological study of the erythrocytic parasites. Kuala Lumpur: Government Press; The microscopic diagnostic of human malaria; p. 142.

Fontes, C.J., Ribeiro, L.C. and Pang, L.W. 2002. Proguanil plus sulfamethoxazole in the treatment of acute uncomplicated *Plasmodium falciparum* malaria. *Southeast Asian Journal of Tropical Medicine and Public Health* 33: 685-688.

Fowler, R.E., Billingsley P.F., Pudney, M. and Sinden, R.E. 1994. Inhibitory action of the anti-malarial Atovaquone (566C80) against *Plasmodium berghei* ANKA in the mosquito *Anopheles stephensi*. *Parasitology* 108: 383-388.

Fowler, R.E., Sinden, R.E. and Pudney, M. 1995. Inhibitory activity of the anti-malarial Atovaquone (566C80) against ookinete, oocysts and sporozoites of *Plasmodium berghei*. *Journal of Parasitology* 8: 452-458.

Frontali, C. 1994. Genome plasticity in *Plasmodium*. *Genetica* 94: 91-100.

Gardner, A., Reece, S.E. and West, S.A. 2003. Even more extreme fertility insurance and sex ratios of protozoan blood parasites. *Journal of Theoretical Biology* 223: 515-521.

Garnham, P.C.C. 1966. Malaria parasites and other haemosporisia. Oxford (UK): Blackwell Scientific.

Gasasira, A.F., Dorsey, G., Nzarubara, B., Staedke, S.G., Nassali, A., Rosenthal, P.J. and Kamya, M.R. 2003. Comparative efficacy of aminoquinoline-antifolate combinations for the treatment of uncomplicated falciparum malaria in

Kampala, Uganda. *American Journal of Tropical Medicine and Hygiene* 68: 127-132.

Gass, R.F. 1977. Influences of blood digestion on the development of *Plasmodium gallinaceum* (Brumpt) in the midgut of *Aedes aegypti* (L.). *Acta Tropica* 34: 127-140.

Gass, R.F. and Yeates, R.A. 1979. *In vitro* damage to cultured ookinetes of *Plasmodium gallinaceum* by digestive proteinases from susceptible *Aedes aegypti*. *Acta Tropica* 36: 243-252.

Gilles, H.M. and Warrell, D.A. 1993. Pathology and pathophysiology of human malaria. In Bruce-Chwatt's Essential Malariology 3rd edition. Great Britain: Edward Arnold. pp 50-56.

Githeko, A.K., Brandling-Bennet, A.D., Beier, M., Atieli, F., Owaga, M. and Collins, F.H. 1992. The reservoir of *Plasmodium falciparum* malaria in a holoendemic area of Western Kenya. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 86: 355-358.

Graf, R. and Briegel, H. 1982. Comparison between aminopeptidase and trypsin activity in blood-fed females of *Aedes aegypti*. *Revew Suis Zoology* 89: 845-850.

Graf, R., Raikhel, A.S., Brown, M.R., Lea, A.O. and Briegel, H. 1986. Mosquito trypsin: immunocytochemical localization in the midgut of blood-fed *Aedes aegypti* (L.). *Cell and Tissue Research* 245: 19-27.

GraphPad 1999. GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA.

Graves, P.M., Cater, R. and McNeil, K.M. 1984. Gametocyte production in cloned lines of *Plasmodium falciparum*. *American Journal of Tropical Medicine and Hygiene* 33: 1045-50.

Ginsburg, H. and Krugliak, M. 1992. Quinoline containing antimalarias- mode of action, drug resistance and its reversal. *Biochemical Pharmacology* 43: 63-70.

Griffin, J.E. and Wilson, J.D. 1991. Disorders of the testis. In *Harrison's Principles of Internal Medicine*, volume 2 (ed. Wilson, J.D., Braunwald, E., Isselbacher, K.J., Peterdorsf, R.G., Martin, J.B., Fauci, A.S. & Root, R.K. Mcgraw-Hill, New York. pp 1765-1776.

Hamel, M.J., Holtz, T., Mkandala, C., Kaimila, N., Chizani, N., Bloland, P., Kublin, J., Kazembe, P. and Steketee, R. 2005. Efficacy of trimethoprim-sulfamethoxazole compared with sulfadoxine-pyrimethamine plus erythromycin for the treatment of uncomplicated malaria in children with integrated management of childhood illness dual classification of malaria and pneumonia. *American Journal of Tropical Medicine and Hygiene* 73: 609-615.

Handunnetti, S.M., Gunewardena, D.M., Pathirana, P.P.S.L., Ekanayake, K., Weerasinghe, S. and Mendis, K.N. 1996. Features of recrudescent chloroquine-resistant *Plasmodium falciparum* infections confer a survival advantage on parasites and have implications for disease control. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 90: 563-567.

Happi, C.T., Thomas, S.M., Gbotosho, G.O., Falade, C.O., Akinboye, D.O., Gerena, L., Hudson, T., Sowunmi, A., Kyle, D.E., Milhous, W., Wirth, D.F. and Oduola, A.M.J. 2003. Point mutations in the *pfcrt* and *pfmdr-1* genes of *Plasmodium*

falciparum and clinical response to chloroquine, among malaria patients from Nigeria. *Annals of Tropical Medicine and Parasitology* 97: 439-451.

Hawking, F., Wilson, M.E. and Gammage, K. 1971. Evidence for cyclic short-lived maturity in the gametocytes of *Plasmodium falciparum*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 65: 549–559.

Hallett, R.L., Sutherland, C.J., Alexander, N., Ord, R., Jawara, M., Drakeley, C.J., Pinder, M., Walraven, G., Targett, G.A.T. and Alloueche, A. 2004. Combination therapy counteracts the enhanced transmission of drug resistant malaria parasites to mosquitoes. *Antimicrobial Agent and Chemotherapy* 48: 3940-3943.

Hien, T.T. and White, N.J. 1993. Quinghaosu. *Lancet* 341: 603-608.

Hogh, B., Thompson, R., Hetzel, C., Kruse, N.A., Jones, I., Dgedge, M., Barreto, J. and Sinden, R.E. 1995. Specific and nonspecific responses to *Plasmodium falciparum* blood-stage parasites and observations on the gametocytaemia in school children living in a malaria-endemic area of Mozambique. *American Journal of Tropical Medicine and Hygiene* 52: 50-59.

Hogh, B., Gamage-Mendis, A., Butcher, G.A., Thompson, R., Begtrup, K., Mendis, C., Enosse, S.M., Dgedge, M., Barreto, J., Eling, W. and Sinden, R.E. 1998. The differing impact of chloroquine and pyrimethamine/sulfadoxine upon the infectivity of malaria species to mosquito vector. *American Journal of Tropical Medicine and Hygiene* 58: 176-182.

Hooijberg, J.H., Broxterman, H.J., Kool, M., Assaraf, Y.G., Peters, G.J., Noordhuis, P., Scheper, R.J., Borst, P., Pinedo, H.M. and Jansen, J. 1999. Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2. *Cancer Research* 59: 2532-2535.

Ichimori, K., Curtis, C.F. and Targett, G.A.T. 1990. The effects of chloroquine on the infectivity of chloroquine-sensitive and - resistant populations of *Plasmodium yoelii nigeriensis* to mosquitoes. *Parasitology* 100: 377-381.

Inselburg, J. 1983. Stage-specific inhibitory effect of cyclic AMP on asexual maturation and gametocyte formation of *Plasmodium falciparum*. *Journal of Parasitology* 69: 592–597.

Janse, C.J., Van Der Klooster, P.F.J., Van Der Kaay, H.J., Van Der Ploeg, M. and Overdulve, J.P. 1986. DNA synthesis in *Plasmodium berghei* during asexual and sexual development. *Molecular Biochemical Parasitology* 20: 173–183.

Jeffery, G.M., Young, M.D. and Eyles, D.E. 1956. The treatment of *Plasmodium falciparum* infection with chloroquine, with a note on infectivity to mosquitoes of primaquine-and pyrimethamine-treated cases. *American Journal of Tropical Medicine and Hygiene* 6: 1- 11.

Kawamoto, F., Alejo Blanco, R., Fleck, S.L. and Sinden, R.E. 1991. *Plasmodium berghei* - Ionic regulation and the induction of gametogenesis. *Experimental Parasitology* 72: 33–42.

Kilian, A.H.D., Jelinek, T., Prislin, I., Kabagambe, W., Byamukama, G., Mpipika, R., Kotic, R., and Von Sonnenburg, F. 1998. Resistance *in vivo* of *Plasmodium falciparum* to co-trimoxazole in western Uganda. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 92: 197-200.

Kitchen, S F and Putman, P 1942 Observations on the mechanism of the parasite cycle in falciparum malaria *American Journal of Tropical Medicine and Hygiene* 22: 381-386

Koella, J C., Sorensen, F L and Anderson, R A 1998 The malaria parasite, *Plasmodium falciparum*, increases the frequency of multiple feeding of its mosquito vector, *Anopheles gambiae* *Proceeding of Biological Science* 265: 763-768

Krogstad, D J., Gluzman, I Y., Kyle, D E., Oduola, A M J., Martin, S K., Milhous, W K and Schlesinger, P H 1987 Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance *Science* 238: 1283-1285

Kumar, N and Carter, R 1984 Biosynthesis of the target antigens of antibodies blocking transmission of *Plasmodium falciparum* *Molecular Biochemical Parasitology* 13: 333-342

Langreth, S G., Jensen, J B., Reese, R T and Trager, W 1978 Fine structure of human malaria *in vitro* *Journal of Protozoology* 25: 443-452

Lines, J D., Wilkes, T J and Lyimo, E O 1991 Human malaria infectiousness measured by age-specific sporozoite rates in *An gambiae* in Tanzania *Parasitology* 102: 167-177

Lingueti, A., Nargos, G., Meier, W A and Seitz, H M 1993 The effects of hormones on the gametocytogenesis of *Plasmodium falciparum in vitro* *Applied Parasitology* 34: 153-160

MacPherson, G.G., Warrell, M.J., White, N.J., Looareesuwan, S. and Warrell, D.A. 1985. Human cerebral malaria: A quantitative ultrastructural analysis of parasitised erythrocyte sequestration. *American Journal of Pathology* 119: 385-401.

Martin, S.K., Miller, L.H., Nijhout, M.M. and Carter, R. 1978. *Plasmodium gallinaceum*: induction of male gametocyte exflagellation by phosphdiesterase inhibitors. *Experimental Parasitology* 44: 239-242.

Maswoswe, S.M., Peters, W. and Warhurst, D.C. 1985. Corticosteroid stimulation of the growth of *Plasmodium falciparum* gametocytes *in vitro*. *Annals of Tropical Medicine and Parasitology*, 79, 607-616.

McElroy, P.D., Beier, J.C., Oster, C.N., Beadle, C., Sherwood, J.A., Oloo, A.J. and Hoffman, S.L. 1994. Predicting outcome of malaria: correlation between rate of exposure to infected mosquitoes and level of *Plasmodium falciparum* parasitaemia. *American Journal of Tropical Medicine and Hygiene* 51: 523-532.

Molineaux, L.G. 1980. The Garki Project. World Health Organization, Geneva.

Mons, B. 1985. Induction of sexual differentiation in malaria. *Parasitology Today* 1: 87-89.

Mons, B. 1986. Intraerythrocytic differentiation of *Plasmodium berghei*. *Acta Leidensia*, 54: 1-83.

Nacher, M., Singhasivanon, P., Silachamroon, U., Treeprasertsuk, S., Krudsood, S., Gay, F., Mazier, D. and Looareesuwan, S. 2002. Association of helminth infections with increased gametocyte carriage during mild falciparum

malaria in Thailand. *American Journal of Tropical Medicine and Hygiene* 65: 644-647.

Nacher, M., Carrara, V.I., Ashley, E., McGready, R., Hutagalung, R., Nguen, J.V., Thwai, K.L., Looareesuwan, S. and Nosten, F. 2004. Seasonal variation in hyperparasitaemia and gametocyte carriage in patients with *Plasmodium falciparum* malaria on the Thai-Burmese border. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 98: 322-328.

Ndounga, M. and Basco, L. K. 2003. Rapid clearance of *Plasmodium falciparum* hyperparasitaemia after oral amodiaquine treatment in patients with uncomplicated malaria. *Acta Tropica* 88: 27-32.

Nee, S., West, S.A. and Read, A.F. 2002. Inbreeding and parasite sex ratios. *Proceedings of Royal Society, Biological Science* 269: 755-60.

Nijhout, M.M. 1979. *Plasmodium gallinaceum*: exflagellation stimulated by a mosquito factor. *Experimental Parasitology* 48: 75-80.

Nijhout, M.M. and Carter, R. 1978. Gamete development in malarial parasites: bicarbonate-dependent stimulation by pH *in vitro*. *Parasitology* 76: 39-53.

Nzila, A., Mberu, E., Bray, P., Kokwaro, G., Winstanley, P., Marsh, K. and Ward, S. 2003. Characterization of *Plasmodium falciparum* by probenecid *in vitro*. *Antimicrobial Agents and Chemotherapy* 47: 2108-2112.

Olliaro, P., Nevill, C., Le Bras, J., Ringwald, P., Mussano, P., Garner, P. and Brasseur, P. 1996. Systematic review of amodiaquine treatment of uncomplicated malaria. *Lancet* 348: 1196-1201.

Omar, S.A., Bakari, A., Owiti, A., Adagu, I.S. and Warhurst, D.C. 2001 a. Co-trimoxazole compared with sulfadoxine-pyrimethamine in the treatment of uncomplicated malaria in Kenyan children. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 95: 657-660.

Omar, S.A., Adagu, I.S., Gump, D.W., Ndaru, N.P. and Warhurst, D.C. 2001 b. *Plasmodium falciparum* in Kenya: high prevalence of drug-resistance-associated polymorphism in hospital admissions with severe malaria in an epidemic area. *Annals of Tropical Medicine and Parasitology* 95: 661-669.

Ono, T., Onhnishe, Y., Nagamune, K. and Kano M. 1993. Gametocytogenesis induction by Berenil in cultured *Plasmodium falciparum*. *Experimental Parasitology* 77: 74-78.

Ono, T., Nakai, T. and Nakabayashi, T. 1986. Induction of gametocytogenesis in *Plasmodium falciparum* by culture supernatant of hybridoma cells producing anti-*P. falciparum* antibody. *Biken Journal* 29: 77-81.

Otto, S.P. and Lenormand, T. 2002. Resolving the paradox of sex and recombination. *Nature Review Genetic* 3: 252-261.

Paul, R.E.L., Coulson, T.N., Raibaud, A. and Brey, P.T. 2000. Sex determination in malaria parasite. *Science* 287: 128-131.

Paul, R.E.L., Brey, P.T. and Robert, V. 2002. *Plasmodium* sex determination and transmission to mosquitoes. *Trends in Parasitology* 18: 32-38.

Paul, R.E. and Brey, P.T. 2003. Malaria parasites and red blood cells: from anaemia to [illegible]. *Molecules and Cells* 15: 139-49.

Pickering, J.A. Read, A.F., Guerrero, S, and West, S.A. 2000. Sex ratio and virulence in two species of lizard malaria parasites. *Evolutionary Ecology Research* 2: 171-184.

Plowe, C.V., Cortese, J.F., Djimde, A., Nwanyanwu, O.C., Watkins, W.M., Winstanley, P.A., Estrada-Franco, J.G., Mollinedo, R.E., Avila, J.C., Cespedes, J.L., Carter, D. and Doumbo, O.K. 1997. Mutations in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthetase and the epidemiologic patterns of pyrimethamine-sulfadoxine use and resistance. *Journal of Infectious Diseases* 176: 1590-1596.

Pologe, L.G. 1994. Aberrant transcriptions and the failure of *Plasmodium falciparum* to differentiate into gametocytes. *Molecular and Biochemical Parasitology* 68: 35-43.

Ponnudurai, T., Lensen, A.H.W., Meis, J.F.G.M., and Meuwissen, J.H.E.T. 1986. Synchronization of *Plasmodium falciparum* gametocytes using automated suspension culture. *Parasitology* 93: 263-274.

Ponnudurai, T., Lensen, A.H.W., Van Gemert, G.J.A., Bensink, M.P.E., Bolmer, M. and Meuwissen, J.H.E.T. 1989. Infectivity of cultured *Plasmodium falciparum* gametocytes to mosquitoes. *Parasitology* 98: 165-173.

Price, R.N., Nosten, F., Luxemburger, C., ter Kuile, F.O., Paiphun, L., Chongsuphajaisiddhi, T. and White, N.J. 1996. Effects of artemisinin derivatives on malaria transmissibility. *Lancet* 347: 1654-1658.

Price, R., Nosten, F., Simpson, J.A., Luxemburger, C., Paiphun, L., ter Kuile, F., van Vugt, M., Chongsuphajaisiddhi, T. and White, N.J. 1999. Risk factors for gametocyte carriage in uncomplicated falciparum malaria. *American Journal of Tropical Medicine and Hygiene* 60: 1019-1023.

Puta, C. and Manyando, C. 1997. Enhanced gametocyte production in Fansidar-treated *Plasmodium falciparum* malaria patients: implications for malaria transmission control programmes. *Tropical Medicine and International Health* 2: 227-229.

Rawlings, D.J., Fujioka, H., Fried, M., Keister, D.B., Aikawa, M. and Kaslow, D.C. 1992. Alpha-tubulin formation by gliding malaria sporozoites. *Journal of Protozoology* 39, 663-671.

Read, A.F., Narara, A., Nee, S., Keymer, A.E. and Day, K.P. 1992. Gametocyte sex ratio as indirect measures of outcrossing rates in malaria. *Parasitology* 104: 387-395.

Reece, S.E., Duncan, A.B., West, S.A. and Read, A.F. 2003. Sex ratios in the rodent malaria parasite, *Plasmodium chabaudi*. *Parasitology* 127: 419-425.

Robert, V. and Trape, J.F. 1998. Dynamics of *Plasmodium falciparum* gametocytemia as a function of therapeutic response to chloroquine in a mesoendemic malaria zone. *Bulletin de la Société de Pathologie Exotique* 91: 142-145.

Robert, V., Molez, J-F. and Trape, J-F. 1996 a. Short report: gametocytes, chloroquine pressure, and the relative parasite survival advantage of resistant strains of falciparum malaria in West Africa. *American Journal of Tropical Medicine and Hygiene* 55: 350-351.

Robert, V., Read, A.F., Essong, J., Tchuinkam, T., Mulder, B., Verhave, J.P. and Carnevale, P. 1996 b. Effect of gametocyte sex ratio on infectivity of *Plasmodium falciparum* to *Anopheles gambiae*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 90: 621-624.

Robert, V., Awono-Ambene, H.P., Le Hesran, J-Y. and Trape, J-F. 2000. Gametocytaemia and infectivity to mosquitoes of patients with uncomplicated *Plasmodium falciparum* malaria attacks treated with chloroquine or sulfadoxine-pyrimethamine. *American Journal of Tropical Medicine and Hygiene* 62: 210-216.

Robert, V., Sokhna, C.S., Rogier, C., Ariey, F. and Trape J-F. 2003. Sex ratio of *Plasmodium falciparum* gametocytes in inhabitants of Dielmo, Senegal. *Parasitology* 127: 1-8.

Rosenberg, R, Koontz, L.C., Alston, K. and Friedman, F.K. 1984. *Plasmodium gallinaceum*: erythrocyte factor essential for zygote infection of *Aedes aegypti*. *Experimental Parasitology* 57: 158-164.

Rosenberg, R., Andre, R.G. and Somchit, L. 1990. Highly efficient dry season transmission of malaria in Thailand. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 84: 22-28.

Rudin, W., Billingsley, P.F. and Saludin, S. 1991. The fate of *Plasmodium gallinaceum* in *Anopheles stephensi* Liston and possible barriers to transmission. *Annales de la Société Belge de Médecine Tropicale* 71: 167-177.

Salako, L.A. and Adeyemumi, A.F. 1987. *In vitro* chloroquine and mefloquine-resistant *Plasmodium falciparum* in Nigeria. *Lancet* I: 572-573.

Salako, L.A., Ajayi, F.O., Sowunmi, A. and Walker, O. 1990. Malaria in Nigeria: a review. *Annals of Tropical Medicine and Parasitology* 84: 435 - 445.

Sanchez, C.P., Wunsch, S. and Lanzer, M. 1997. Identification of chloroquine importer in *Plasmodium falciparum*: differences in import kinetics are genetically linked with the chloroquine resistant phenotype. *Journal of Biological Chemistry* 272: 2652-2658.

Schall, J.J. 1989. The sex ratio of *Plasmodium* gametocytes. *Parasitology* 98: 343–350.

Schall, J.J. 2000. Transmission success of the malaria parasite *Plasmodium mexicanum* into its vector: role of gametocyte density and sex ratio. *Parasitology* 121: 575-580.

Scherf, A., Petersen, C., Carter, R., Alano, P., Nelson, R., Aikawa, M., Mattei, D. and Pereira da Silva, L. (1992). Characterization of a *Plasmodium falciparum* mutant that has deleted the majority of the gametocyte-specific Pf11-1 locus. *Memorias Instituto Oswaldo Cruz* 87 (Suppl. III): 91-94.

Scherf, A., Behr, C., Sarthou, J.L., Pla, M., Rogier, C., Trape, J.F., Pereira da Silva, L. and Dubois, P. 1993. Immune response in mouse and malaria-exposed humans to peptides derived from Pf11-1, a highly repetitive merozoite protein of *Plasmodium falciparum*. *European Journal Immunology* 23: 1574-1581.

Schwetz, S., Maier, W.A. and Seitz, H.M 1991. Haemolysis of infected erythrocytes—a trigger for formation of Plasmodium gametocytes. *Parasitology Research* 77: 458-460.

Shahabuddin, M., Toyoshima, T., Aikawa, M. and Kaslow D.C. 1993. Transmission-blocking activity of a chitinase inhibitor activation of malarial parasite chitinase by mosquito protease. *Proceeding of National Academy of Science USA* 90: 4266-4270.

Shaper, A.G. and Lewis, P. 1971. Genetic neutropenia in people of African origin. *Lancet* ii: 1021-1023.

Shekalaghe, S.A., Bousema, J.T., Kunei, K.K., Lushino, P., Masokoto, A., Wolters, L.R., Mwakalinga, S., Mosha, F.W., Sauerwein, R.W. and Drakeley, C.J. 2007. Submicroscopic *Plasmodium falciparum* gametocyte carriage is common in an area of low and seasonal transmission in Tanzania. *Tropical Medicine and International Health* 12: 547-553.

Shute P.G. and Marion, M. 1951. A study of gametocytes in a West Africa strain of *Plamodium falciparum*. *Transactions of the Royal Society of Tropical Medicine Hygiene* 44: 421-438.

Shute, P.G. and Maryon, M. 1954. The effect of pyrimethamine (Daraprim) on the gametocytes and oocysts of *Plasmodium falciparum* and *Plasmodium vivax*. *Transactions of the Royal Society of Tropical Medicine Hygiene* 48: 50-63.

Sibley, C.H., Hyde, J.E., Sims, P.G.F., Plowe, C.V., Kublin, J.G., Mberu, E.K., Cowman, A.F., Winstanley, P.A., Watkins, W.M. and Alecis, M.N. 2001. Pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum*: what next? *Trends in Parasitology* 17: 582-588.

Silvestrini, F., Alano, P. and Williams, J.L. 2000. Commitment to the production of male and female gametocytes in the human malaria parasite *Plasmodium falciparum*. *Parasitology* 121: 465-471.

Silvestrini, F., Bozdech, Z., Lanfrancotti, A., Giulio, E.D., Bultrini, E., Picci, L., deRisi, J.L., Pizzi, E. and Alano, P. 2005. Genome-wide identification of genes upregulated at the onset of gametocytogenesis in *Plasmodium falciparum*. *Molecular and Biochemical Parasitology* 143: 100-110.

Sinden, R.E. 1982. Gametocytogenesis of *Plasmodium falciparum in vitro*: an electron microscopic study. *Parasitology* 84: 1-11.

Sinden, R.E. 1983. The cell biology of sexual development in *Plasmodium*. *Parasitology* 86: 7-28.

Sinden, R.E. 1984. The biology of *Plasmodium* in the mosquito. *Experientia* 40: 1330-1343.

Sinden, R.E. 1989. Recent advances in the parasitology of malaria. *Transactions of the Royal Society of Tropical Medicine Hygiene*. 83,Suppl: 3-9.

Sinden, R.E. 1998. Gametocytes and sexual development, in *Malaria: Parasite Biology, Pathogenesis and Protection*, Sherman I.W. (ed), Washington, DC: ASM Press. pp 25-48.

Sinden, R.E, and Croll, N.A. 1975. Cytology and kinetics of microgametogenesis and fertilization of *Plasmodium yoelii nigeriensis*. *Parasitology* 70: 53-65.

Sinden, R.E and Smalley, M.E. 1979. Gametocytogenesis of *Plasmodium falciparum in vitro*: the cell-cycle. *Parasitology* 79: 277-296.

Sinden, R.E., Canning, E.U., Bray, R.S. and Smalley, M.E. 1978. Gametocyte and gamete development in *Plasmodium falciparum*. *Proceedings of the Royal Society of London Series B-Biological Sciences* 201: 375-399.

Smalley, M.E. 1976. *Plasmodium falciparum* gametocytogenesis *in vitro*. *Nature* 264: 271-272.

Smalley, M.E. 1977. *Plasmodium falciparum* gametocytes: the effect of chloroquine on their development *in vitro*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 71: 525-529.

Smalley, M.E. and Sinden, R.E. 1977. *Plasmodium falciparum* gametocytes: their longevity and infectivity. *Parasitology* 74: 1-18.

Smalley, M.E., Abdalla, S. and Brown, J. 1980. The distribution of *Plasmodium falciparum* in the peripheral blood and bone marrow of Gambian children. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 75: 103–105.

Smalley, M.E., Brown, J. and Basset, N.M. 1981. The rate of production of *Plasmodium falciparum* gametocytes during natural infections. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 75: 317-319.

Smith, T.G., Lourenco, P., Carter, R., Walliker, D. and Ranford-Cartwright, L.C. 2000. Commitment to sexual differentiation in the human malaria parasite, *Plasmodium falciparum*. *Parasitology* 121: 127-33

Sowunmi A. 2002. A randomized comparison of chloroquine, amodiaquine and their combination in the treatment of acute uncomplicated falciparum malaria in children. *Annals of Tropical Medicine and Parasitology* 96: 227 - 238.

Sowunmi, A. 2003. A randomized comparison of chloroquine and chloroquine plus ketoulfen in the treatment of acute, uncomplicated, *Plasmodium falciparum* malaria in children. *Annals of Tropical Medicine and Parasitology* 97: 103-117.

Sowunmi, A. and Salako, L.A. 1992. Evaluation of the relative efficacy of various antimalarial drugs in Nigerian children under five years of age suffering

from acute uncomplicated falciparum malaria. *Annals of Tropical Medicine and Parasitology* 86: 1-8.

Sowunmi, A. and Fateye, B.A. 2003 a. Asymptomatic, recrudescent, chloroquine-resistant, *Plasmodium falciparum* infections in Nigerian children: clinical and parasitological characteristics and implications for transmission of drug-resistant infections. *Annals of Tropical Medicine and Parasitology* 97: 469-479.

Sowunmi, A. and Fateye, B.A. 2003 b. *Plasmodium falciparum* gametocytaemia in Nigerian children: before, during and after treatment with antimalarial drugs. *Tropical Medicine and International Health* 8: 783-792.

Sowunmi, A. and Fateye, B.A. 2003 c. Gametocyte sex ratios in children with asymptomatic, recrudescent, pyrimethamine-sulfadoxine-resistant, *Plasmodium falciparum* malaria. *Annals of Tropical Medicine and Parasitology* 97: 671-682.

Sowunmi, A. and Fateye, B.A. 2003 d. Changes in *Plasmodium falciparum* gametocytaemia in children with chloroquine-sensitive asexual infections. *Parasite* 10: 363-369.

Sowunmi, A., Walker, O. and Salako, L.A. 1992. Hyperparasitaemia not a reliable indicator of severity or poor prognosis in falciparum malaria in children from endemic African countries. *Annals of Tropical Paediatrics* 12: 155-158.

Sowunmi, A., Ogundele, A.M.J., Salako, L.A., Akindele, J.A., Omigbodun, A.O. and Omilowoju, G.O. 1993. Intramuscular sulphadoxine-pyrimethamine in chloroquine-resistant *Plasmodium falciparum* malaria during pregnancy. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 84: 472.

Sowunmi A., Akindele J.A. and Balogun M.A. 1995. Leukocyte counts in falciparum malaria in African children from an endemic area. *African Journal of Medicine and Medical Sciences* 24: 145-149.

Sowunmi, A., Oduola, A.M.J., Ilesanmi, A.O. and Salako, L.A. 1996. Open comparison of artemether and mefloquine in uncomplicated *Plasmodium falciparum* hyperparasitaemia in children. *Annals of Tropical Paediatric* 16: 5-9.

Sowunmi, A., Oduola, A.M.J., Ogundahunsi, O.A.T., Falade, C.O., Gbotosho, G.O. and Salako, L.A. 1997. Enhanced efficacy of chloroquine-chlorpheniramine combination in acute uncomplicated falciparum malaria in children. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 91: 63-67.

Sowunmi, A., Oduola, A.M.J., Ogundahunsi, O.A.T. and Salako, L.A. 1998 a. Comparative efficacy of chloroquine plus chlorpheniramine and pyrimethamine/sulphadoxine in acute uncomplicated falciparum malaria in Nigerian children. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 92: 77-81.

Sowunmi, A., Fehintola, F.A., Ogundahunsi, O.A.T. and Oduola, A.M.J. 1998 b. Comparative efficacy of chloroquine plus chlorpheniramine and halofantrine in acute uncomplicated falciparum malaria in Nigerian children. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 92: 441-445.

Sowunmi, A., Oduola, A.M.J., Ogundahunsi, O.A.T. and Salako, L.A. 1998 c. Enhancement of the antimalarial effect of chloroquine by chlorpheniramine *in vivo*. *Tropical Medicine and International Health* 3: 177-183.

Sowunmi, A., Adedeji, A.A., Sowunmi, C.O., Falade, A.G., Sijuade, A.O. and Oduola, A.M.J. 2000 a. Comparative clinical characteristics and response to oral therapy of children with and without *Plasmodium falciparum* hyperparasitaemia in an endemic area. *Annals Tropical Medicine and Parasitology* 94: 549-558.

Sowunmi, A., Adedeji, A.A., Fehintola, F.A. and Oduola, A.M.J. 2000 b. *Plasmodium falciparum* kinetics during treatment with antimalarial drugs in children. *Clinical Drug Investigation* 20, 43-51.

Sowunmi, A., Ayede, A.I., Falade, A.G., Ndikum, V.N., Sowunmi, C.O., Adedeji, A.A., Falade, C.O., Happi T.C. and Oduola A.M.J. 2001. Randomized comparison of chloroquine and amodiaquine in the treatment of acute uncomplicated, *Plasmodium falciparum* malaria in children. *Annals of Tropical Medicine and Parasitology* 95: 549-558.

Sowunmi, A., Fateye, B.A., Happi, T.C., Gbolosho, G.O. and Oduola, A.M.J. 2003. *Plasmodium falciparum* gametocytaemia in Nigerian children: peripheral immature gametocytaemia as indicator of a poor response to chloroquine treatment and its relationship to molecular determinants of chloroquine resistance. *Annals of Tropical Medicine and Parasitology* 97: 453-468.

SPSS 1999. *SPSS for Windows Release 10.01 (Standard Version)*. SPSS Inc, Chicago, IL, USA.

Strickland, G.T., Fox, E., Sarwar, M., Khaliq, A.A. and Macdonald M. 1986. Effects of chloroquine, amodiaquine and pyrimethamine-sulphadoxine on *Plasmodium falciparum* gametocytaemia. *American Journal of Tropical Medicine and Hygiene* 35: 259-262.

Subtrier, A., Janse, C.J., Mons, B., Fleck S.L., Nicholas, J., Davies, C.S and Sinden, R.E. 1987. The complete development of the vertebrate stages of *Plasmodium*

herghei in vitro. *Transactions of Royal Society of Tropical Medicine and Hygiene* 81: 907-910.

Sutherland, C.J., Alloueche, A., Curtis, J., Drakeley, C.J., Ord, R., Duraisingh, M., Greenwood, B.M., Warhurst, D.C. and Targett G.A.T. 2002. Gambian children successfully treated with chloroquine can harbor and transmit *Plasmodium falciparum* gametocytes carrying resistant genes. *American Journal of Tropical Medicine and Hygiene* 67: 575-585.

Sutherland, C.J., Drakeley, C.J., Obisike, U., Coleman, R., Jawara, M., Targett, G.A.T., Milligan, P. Pinda, M. and Walraven, G. 2003. The addition of artesunate to chloroquine for the treatment of *Plasmodium falciparum* malaria in Gambian children delays but does not prevent treatment failure. *American Journal of Tropical Medicine and Hygiene* 69: 19-25.

Talman, A.M., Paul, R.E.L., Sokhna, C.S., Domarle, O., Ariey, F., Trape, J-F. and Robert, V. 2004 a. Influence of chemotherapy on *Plasmodium* gametocytes sex ratio of mice and man. *American Journal of Tropical Medicine and Hygiene* 71: 739-44.

Talman, A.M., Domarle, O., McKenzie, F.E., Ariey, F. and Robert, V. 2004 b. Gametocytogenesis: the puberty of *Plasmodium falciparum*. *Malaria Journal* 3: 1-14.

Taylor, L.H. and Read, A.F. 1997. Why so few transmission stages? Reproductive restraint by malaria parasite. *Parasitology Today* 13: 135-140.

Tchuinkam T., Mulder, B., Dechering, K., Stoffels, H., Verhave, J.P., Cot, M., Camevale, P., Meuwissen, J.H. and Robert, V. 1993. Experimental infections of *Anopheles gambiae* with *Plasmodium falciparum* of naturally infected

gametocyte carriers in Cameroon: factors influencing the infectivity to mosquitoes. *Tropical Medicine and Parasitology* 44: 271-276.

Thompson, J. and Sinden, R.E. 1994. *In situ* detection of Pbs21 mRNA during sexual development of *Plasmodium berghei*. *Molecular and Biochemical Parasitology* 68: 189-96.

Thompson, J.G., and Robertson, A. 1935. The structure and development of *Plasmodium falciparum* gametocytes in the internal organs and peripheral circulation. *Transactions of Royal Society of Tropical Medicine and Hygiene* 29: 31-40.

Thomson, D. 1911. I. - A research into the production, life and death of crescents in malignant tertian malaria, in treated and untreated cases, by an enumerative method. *Annals of Tropical Medicine and Parasitology* 5: 57-82.

Tjitra, E., Suprianto, S. and Anstey, N.M. 2002. Higher gametocyte prevalence following failure of treatment of *Plasmodium falciparum* malaria with sulfadoxine-pyrimethamine and the combination of chloroquine plus sulfadoxine-pyrimethamine: implications for progression of anti-folate resistance. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 96: 434-437.

Trape, J-F. 2001. The public health impact of chloroquine resistance in Africa. *American Journal of Tropical Medicine and Hygiene* 64 Suppl: 12-17.

Trape, J.-F., Pison, G., Preziosi, M-P., Enel, C., Du Lou, A.D., Delaunay, V., Samb, B., Lagarde, E., Molez, J.-F. and Simondon, F. 1998. Impact of chloroquine resistance on malaria mortality. *Life Sciences* 321: 689-697.

Van Dillen, J., Custers, M., Wensink, A., Wouters, B., Van Voorthuizen, T., Voorn, W., Khan, B., Muller, L. and Nevill C. 1999. A comparison of amodiaquine and sulfadoxine-pyrimethamine as first-line treatment of falciparum malaria in Kenya. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 93: 185-188.

Von Seidlein, L., Drakeley, C., Greenwood, B., Walraven, G. and Targett, G. 2001. Risk factors for gametocyte carriage in Gambian children. *American Journal of Tropical Medicine and Hygiene* 65: 523-526.

Von Seidlein, L., Milligan, P., Pinder, M., Bojang, K., Anyalebechi, C., Gosling, R., Coleman, R., Ude, J.I., Sadiq, A., Duraisingh, M., Warhurst, D., Alloueche, A., Targett, G., Meadam, K., Greenwood, B., Walraven, G., Olliaro, P. and Doherty, T. 2000. Efficacy of artesunate plus pyrimethamine-sulfadoxine for uncomplicated malaria in Gambian children. *Lancet* 355: 352-357.

Wang, P., Lee, C.-S., Bayoumi, R., Djimde, A., Doumbo, O., Swedberg, G., Dao, L.D., Mshinda, H., Tanner, M., Watkins, W.M., Sims, P.F.G., and Hyde, J.E. 1997. Resistance to antifolates in *Plasmodium falciparum* monitored by sequence analysis of dihydropteroate synthetase and dihydrofolate reductase alleles in a large number of field samples of diverse origin. *Molecular and Biochemical Parasitology* 89: 161-177.

Warrell, D.A., Molyneux, M.E. and Beales, P.F. 1990. Severe and complicated malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 80 (suppl): 1-50.

West, S.A., Reece, S.E. and Read, A.F. 2001. Evolution of sex ratios in malaria and related apicomplexan (protozoan parasites). *Trends in Parasitology* 17: 525-531.

West, S.A., Smith, T.G., Nee, S. and Read, A.F. 2002. Fertility insurance and the sex ratios of malaria and related hemosporin blood parasites. *Journal of Parasitology* 88: 258-263.

White, N.J. 1997. Assessment of the pharmacodynamic properties of antimalarial drugs *in vivo*. *Antimicrobial Agents and Chemotherapy* 41: 1413-1422.

Wilkinson, R.N., Colwell, E.J. and Neoypatimanond, S. 1973. Effect of sulphamethoxazole-trimethoprim on the viability of *Plasmodium falciparum* gametocytes. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 67: 148-149.

Wilkinson, R.N., Nocypatimanondh, S. and Gould, D.J. 1976. Infectivity of falciparum malaria patients for anopheline mosquitoes before and after chloroquine treatment. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 70: 306-307.

Woklay, D., Kireab, T., Bukenya, D. and Hodes, R. 1995. Sensitivity of *Plasmodium falciparum in vivo* to chloroquine and pytimethamine-sulfadoxine in Rwanda patients in refugee camp in Zaire. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 89: 654-655.

World Health Organization. 1973. *Chemotherapy of Malaria and Resistance to Antimalarials*. Technical Report Series No. 529. WHO, Geneva.

World Health Organization. 1996. Management of uncomplicated malaria and the use of antimalarial drugs for the protection of travellers. Report of an informal consultation. WHO/MAL 96.1075Rev.1. WHO, Geneva.

World Health Organization. 2000. Severe falciparum malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 94 Suppl 1: 1-59.

World Health Organization. 2001 a. *The use of Antimalarial drugs. Report of a WHO informal Consultation.* Document WHO/CDS/RBM/2001.35. WHO, Geneva.

World Health Organization 2001b. *Antimalarial Dug Combination Therapy. Report of a WHO Technical Consultation.* Document WHO/CDS/RBM/2001.35. WHO, Geneva.

World Health Organization. 2003. Assessment and monitoring antimalarial drug efficacy for the treatment of uncomplicated falciparum malaria. World Health Organisation. WHO/HTM/RBM/2003.50. WHO, Geneva.

Appendices

APPENDIX 1

Tests for the presence of antimalarial in Urine (Dill-Glazko and Lignin test)

A. Dill- Glazko for 4- aminoquinoline

Reagents:

Eosine powder	50mg
Reagent grade chloroform	100ml
Hydrochloric acid	1mol/L

Procedure

1. Add the 50mg eosine to the 100ml chloroform and 1ml HCL (1 mol/l) in a glass stoppered separating funnel
2. Shake gently for few minutes until the chloroform becomes light yellow in colour
3. Separate the chloroform layer and store in dark brown glass stoppered bottle
4. Add 10 drops of chloroform solution to 2 ml urine in a test-tube and mix vigorously or a few moments
5. The presence of chloroquine in urine is indicated by a change in the colour of the precipitated chloroform layer from light yellow to violet red

B. Lignin test

This is a simple qualitative field test for the detection of sulfonamides in urine

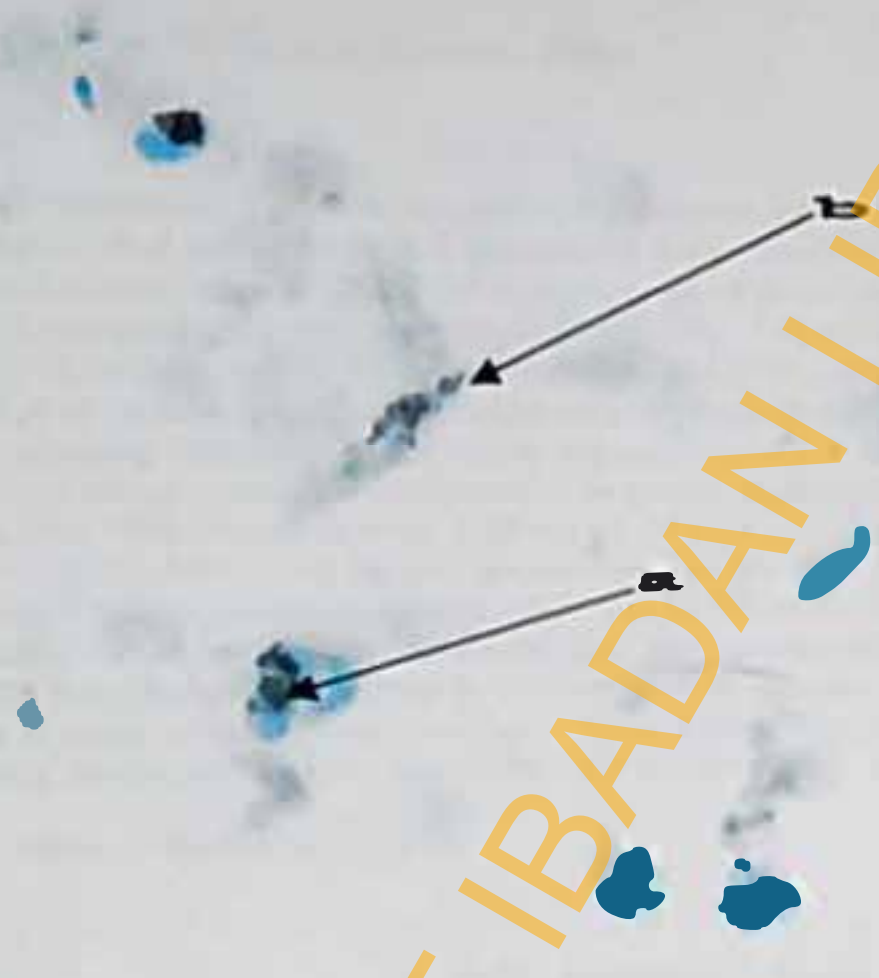
Reagents

Paper towel or blank newspaper strips

Hydrochloric acid, HCL (3mol/l)

Procedure

1. Place one or two drops of urine on a blank strip of newspaper or paper towel
2. Add a small drop of HCL (3 mol/l) to the center of the moistured area. The immediate appearance of a yellow to orange colour indicates the presence of a sulfonamide compound. The test becomes positive 1 hour after the ingestion of sulfonamides and stays positive for 3 days. (Caution- paper of bond quality or filter paper cannot be used).



Appendix 2. Micrograph of peripheral mature male (arrow a) and female (arrow b) obtained in thick blood smear of a child treated with antimalarial drug.

Risk factors for gametocyte carriage in uncomplicated falciparum malaria in children

A. SOWUNMI*, B. A. FATEYE, A. A. ADEDEJI, F. A. FEHINTOLA and T. C. HAPPI

Department of Pharmacology and Therapeutics and Institute for Medical Research and Training, University of Ibadan, Ibadan, Nigeria

(*Received 23 September 2003, revised 19 February 2004; accepted 24 February 2004*)

SUMMARY

The risk factors associated with gametocytaemia at presentation and after treatment with different antimalarial drug regimens were evaluated in 767 children enrolled prospectively in 5 antimalarial drug trials between July 1996 and December 2002 in a hyperendemic area of southwestern Nigeria. The children were assigned to one of 6 treatment groups: chloroquine (CQ) only, pyrimethamine-sulfadoxine (PS) only; amodiaquine (AQ) only, CQ combined with chlorpheniramine (CQCP) or PS combined with CQ (CQPS) or AQ (AQPS). At enrolment, 115 (15%) of 767 children were gametocyte carriers. During follow-up, 15·6% of all patients (i.e. 120 patients) developed patent gametocytaemia, which in 85% (102 patients) had developed by day 7 following treatment. In a multiple regression model, 4 factors were found to be independent risk factors for the presence of gametocytaemia at enrolment: male gender [adjusted odds ratio (AOR) = 0·55, 95% confidence interval (CI) 0·36–0·83, $P = 0·005$], absence of fever (AOR = 1·61, 95% CI 1·05–2·5, $P = 0·03$), duration of illness >3 days (AOR = 1·57, 95% CI 1·0–2·4, $P = 0·047$), and asexual parasite densities less than 5000/μl (AOR = 0·42, 95% CI 0·24–0·73, $P = 0·002$). The presence of patent gametocytaemia at enrolment (AOR = 0·04, 95% CI 0·02–0·07, $P < 0·001$) and recrudescence of asexual parasites within 14 days were associated with the presence of gametocytaemia 7 or 14 days after enrolment (AOR = 0·5, 95% CI 0·3–0·8, $P = 0·007$). Delay in the time taken to clear the initial parasitaemia (>2 days) was associated with increased risk of subsequent gametocyte carriage. These findings may have implications for malaria control efforts in sub-Saharan Africa where control of the disease depends almost entirely on chemotherapy.

Key words: gametocyte carriage, children, risk factors, Nigeria.

INTRODUCTION

The transmission of *Plasmodium falciparum* from humans to mosquitoes can only occur through the gametocyte, the sexual stage that develops from proliferating asexual parasite. Gametocytes, in turn, are essential for the infection of new hosts by the mosquito (Sinden *et al.* 1978; Carter & Graves, 1988). Although the mechanisms of the switch from asexual to sexual stage, and its modulation, are complex and incompletely understood (Carter & Miller, 1979; Mons, 1985), the process, and the infectivity of the gametocytes arising from the switch may be influenced by antimalarial drugs (Wilkinson *et al.* 1976; Butcher, 1997; Huckling *et al.* 1999).

In sub-Saharan Africa, increasing drug resistance in *P. falciparum* has led to increases in malaria-related morbidity and mortality (Trape *et al.* 1998; Trape, 2001) and is thought to be associated with increases in gametocyte carriage and gametocyte infectivity to mosquitoes (Robert *et al.* 1996, 2000; Hogh *et al.* 1998). In West African children, pre-treatment gametocyte carriage in those with acute falciparum infections may reach 14–17% (von Seidlein *et al.* 2001; Sowunmi & Fateye, 2003*a*), and children, in general, are thought to constitute a significant reservoir of infection in sub-Saharan Africa (Githeko *et al.* 1992; Bonnet *et al.* 2003).

A recent study from The Gambia (von Seidlein *et al.* 2001), an area of lesser intensity of malaria transmission than Nigeria (Salako *et al.* 1990), has shown that anaemia, absence of fever and parasitaemia less than 100 000 asexual forms per μl were independent risk factors for gametocyte carriage at presentation in Gambian children. In addition, treatment with pyrimethamine-sulfadoxine (PS) alone was associated with increased risk of gametocyte carriage 7 days after treatment compared to chloroquine (CQ) or artemisinin-based combination therapy. It is unclear whether these factors, alone or in addition to others, are associated with gametocyte carriage in Nigerian children.

Although with increasing antimalarial drug resistance (Falade *et al.* 1997; Sowunmi *et al.* 1998*a*, *b*; Sowunmi, 2002) there have been associated increases in gametocyte carriage in Nigerian children (Sowunmi & Fateye, 2003*a*), there is little information on the risk factors associated with gametocyte carriage pre- or post-treatment in Nigerian children. Such information is necessary as it may potentially harness the efforts aimed at the management and control of drug resistance in the community. In the

* Corresponding author. Tel: +234 2 2412101, 234-2-2411904. Fax: +234 2 2411843. E-mail: malaria.iba@alpha.linkserve.com; akinsowunmi@hotmail.com

Parasitology (2004), **129**, 255–262. © 2004 Cambridge University Press
DOI: 10.1017/S0031182004005669 Printed in the United Kingdom

present study we evaluated the factors that influence the production of gametocytes in children presenting with acute, symptomatic, uncomplicated, *P. falciparum* malaria in a hyperendemic area of malaria in southwest Nigeria.

PATIENTS AND METHODS

Patients

The study took place between July 1996 and December 2002 in patients presenting at the University College Hospital in Ibadan, a hyperendemic area for malaria in southwestern Nigeria (Salako *et al.* 1990). Ethical clearance was provided by the local ethics committee. During the period, a series of antimalarial drug studies were conducted to evaluate the efficacy and safety of different treatment regimens. Studies on CQ were done during the entire 6-year period, those of chloroquine plus chlorpheniramine (CQCP) in the first 3 years, those of PS in the first 2 years and the last 2 years, those of amodiaquine (AQ) alone in the last 3 years, and those of combination antimalarials in the last 2 years. However, there was a considerable degree of overlap in the study periods. Details of the studies have been described before (Sowunmi *et al.* 1998*a*,*b*; Sowunmi, 2002, 2003; Sowunmi & Fateye, 2003*a*). Briefly, children with symptoms compatible with acute falciparum malaria who fulfilled the following criteria were enlisted in the study: age below 120 months, pure *P. falciparum* parasitaemia greater than 2000 asexual forms/μl of blood, negative urine tests for antimalarial drugs (Dill-Glazko and lignin tests), absence of concomitant illness, no evidence of severe malaria (WHO, 2000) and written informed consent given by parents or guardians. After enrolment and start of treatment (day 0), follow-up with clinical and parasitological evaluation was at days 1–7, and then on day 14, and when necessary, on days 21 and 28, for example, in patients who received PS combined with CQ (CQPS) or AQ (AQPS). Clinical evaluation consisted of a general clinical examination including measurement of weight, core temperature and physical examination.

Assessment of parasitaemia

Thick and thin blood films prepared from a finger prick were Giemsa-stained and were examined by light microscopy under an oil-immersion objective, at ×1000 magnification, by 2 independent assessors. Parasitaemia in thick films was estimated by counting asexual parasites relative to 1000 leukocytes, or 500 asexual forms, whichever occurred first. From this figure, the parasite density was calculated assuming a leukocyte count of 6000/μl of blood. Gametocytes were also counted in thick blood films against 1000 leukocytes assuming an average leukocyte count of 6000/μl of blood (Shapiro & Lewis, 1971; Ezeilo, 1971; Sowunmi, Akindele & Balogun, 1995). A haematocrit was done at enrolment in 124 of the patients treated with CQPS or AQPS in order to evaluate the safety of combination antimalarial therapy.

Evaluation of response to drug treatment

Response to drug treatment was assessed using World Health Organization (WHO) criteria (WHO, 1973) as follows: S = sensitive, clearance of parasitaemia without recurrence; RI (mild resistance) = parasitaemia disappears but reappears within 7 to 14 days; RII (moderate resistance) = decrease of parasitaemia but no complete clearance from peripheral blood; RIII (severe resistance) = no pronounced decrease or increase in parasitaemia at 48 h after treatment. In those with sensitive or RI response, parasite clearance time (PCT) was defined as the time elapsing from drug administration until there was no patent parasitaemia for at least 72 h. Asexual parasite reduction ratio [PRR] (White, 1997) was defined as the ratio of day 0/day 2 parasitaemia.

Statistical analysis

Data were analysed using version 6 of the Epi-Info software (Anon., 1994), and the statistical program SPSS for Windows version 10.01 (SPSS, 1999). Proportions were compared by calculating χ^2 with Yates' correction. Normally distributed, continuous data were compared by Student's *t*-tests and analysis of variance (ANOVA). In the drug treatment groups *post-hoc* comparisons were done using Tukey honestly significant difference (Tukey HSD). Data not conforming to a normal distribution were compared by the Mann–Whitney *U*-test and the Kruskal–Wallis test (or by Wilcoxon rank sum test). A multiple logistic regression model was used to test the association between gametocytaemia (yes or no at presentation or during follow up) and factors that were significant at univariate analysis: male gender, presence of fever, duration of illness before presentation, asexual parasitaemia at presentation, drug treatment, and recrudescence of asexual parasites within 14 days of initiating treatment. Because the study was conducted over a period of 6 years, time in study years since the commencement of trials was included as a covariate in the model for pre-treatment gametocytaemia. The values presented below are generally means and standard deviations (S.D.) or standard error (S.E.) or median with interquartile range [IQR]. *P* values of <0.05 were taken to indicate significant differences.

RESULTS

Patent gametocytaemia (geometric mean 26, range 6–1344/μl) was present in 115 (15%) of the 767 children at enrolment.

Risk factors for gametocyte carriage at enrolment

The responses of the asexual parasitaemia to drug treatment and gametocyte carriage during and/or after treatment are shown in Table 1. PRR in children treated with AQPS or CQPS was significantly higher than all other treatment groups ($P<0.001$) with the exception of the AQ and PS groups, which, compared to CQPS, did not differ significantly ($P=0.099$ and 0.30 respectively, Tukey HSD). PCT was significantly shorter in those treated with AQPS and CQPS compared to other treatment groups ($P<0.001$) except AQ ($P=0.052$ and 0.25, respectively, Tukey HSD). PCT was also significantly shorter in those treated with AQ compared to CQ ($P=0.019$, Tukey HSD). Factors associated with gametocytaemia at enrolment are presented in Table 2. Male gender, absence of fever, duration of illness >3 days, and asexual parasite densities less than 5000/μl were related to the presence of gametocytaemia at enrolment. Neither age nor packed cell volume at presentation was an independent risk factor for gametocyte carriage (Table 2).

Risk factors for gametocyte carriage during follow up

During follow-up, 15·6% of all patients (i.e. 120 patients) developed patent gametocytaemia which, in 85% (102 patients), had developed by day 7 following treatment. Gametocyte densities at enrolment were similar in all treatment groups, were significantly higher on day 14 in those treated with PS, and a significantly higher proportion of children treated with PS carried gametocytes throughout the duration of the study (Table 3). In the cohort of children in whom gametocytes were not detected at enrolment, 16 of 259 (13·9%) children treated with CQ, 9 of 82 (11·0%) treated with CQCP, 3 of 93 (3·2%) treated with AQ, 1 of 64 (1·6%) treated with CQPS, 3 of 64 (4·7%) children treated with AQPS, and 5 of 90 (5·6%) children treated with PS developed patent gametocytaemia within 7 days of enrolment. Thus, the proportion of children who developed gametocytaemia following treatment were significantly higher in those treated with PS compared with other treatment regimens ($Chi^2=136.9$, $P=<0.001$).

The presence of patent gametocytaemia at enrolment, and recrudescence of asexual parasites within 14 days were associated with the presence of gametocytaemia 7 or 14 days after enrolment (Table 4). Delay in the time taken to clear the initial parasitaemia was associated with increased risk of subsequent gametocyte carriage, but this association was not significant following multivariate analysis (Table 4). Children treated with AQ, AQPS or CQPS were significantly less likely to have delayed day 7 asexual parasite clearance compared with those treated with CQ or PS alone ($Chi^2=41.7$, degree of freedom (d.f.) $=5$, $P<0.001$).

The presence of gametocytes on day 7 or 14 was significantly associated with treatment outcome by day 14 in children treated with CQ ($Chi^2=18.3$, d.f. $=1$, $P=<0.001$) and CQCP ($Chi^2=10.1$, d.f. $=1$, $P=0.001$), but not PS ($Chi^2=0.21$, d.f. $=1$, $P=0.64$), and AQ ($Chi^2=0.24$, d.f. $=1$, $P=0.62$) and AQPS or CQPS in which all children were clinically cured.

DISCUSSION

Gametocytes are often detectable in peripheral blood for a variable period after acute falciparum infection, with morphologically mature gametocytes being detectable in the blood 10–14 days after originating from merozoites (Thomson, 1911; Smalley, 1976). Carriage rates may vary widely and are dependent on several factors. In the current study, gametocyte prevalence was much higher than those reported from western Thailand (2%, Price *et al.* 1999) and Tanzania (8%, Akim *et al.* 2000) but similar to that from The Gambia (17%, von Seidlein *et al.* 2001) in the same region of Africa. However, despite regional differences in prevalence rates, the risk factors associated with gametocyte carriage were remarkably similar.

Gametocyte prevalence in the study area before the 1990s, a period of full sensitivity to CQ, was less than 2% (L. A. Salako, unpublished observation). Presently, in the area, CQ treatment of CQ-resistant infections is associated with significant gametocyte carriage and gametocytaemia and slower clearance of gametocytaemia (Sowunmi & Fateye, 2003*a*,*b*). Therefore, it would appear that the present relatively high prevalence rate may, in part, be due to increasing CQ resistance. Seventy percent of all cases of acute malaria infections in our area of study occur in children aged less than 10 years (Salako *et al.* 1990); the similar gametocyte carriage in all age groups suggests that children aged below 10 years were uniformly susceptible to gametocyte carriage. In other studies involving a broader age range than we evaluated, a younger age was associated with increased gametocyte prevalence, for example, in Tanzania (Akim *et al.* 2000).

It is unclear why male gender is a risk factor for gametocyte carriage at presentation, despite similar duration of illness and other characteristics in both gender groups (data not shown). To our knowledge, this is the first report of the association between male gender and gametocyte carriage in African children with falciparum malaria. Could this simply be a chance finding? Plasma testosterone is often significantly raised in pre-pubertal-male than -female children (Griffin & Wilson, 1991), and testosterone and other corticosteroids may stimulate *P. falciparum* gametocytogenesis *in vitro* (Meshnick, Peters & Warhurst, 1985; Lensen *et al.* 1993). It seems possible that differences in sex hormone levels

Table 1. Responses of asexual parasitaemia and gametocyte carriage following treatment with antimalarial drugs

(Standard doses of drugs were given at presentation (day 0) and asexual parasitaemia quantification was done daily for 8 days (days 0–7) and then on day 14. Gametocyte carriage was assessed on days 0, 7 and 14. PRR, parasite reduction ratio; PCT, parasite clearance time; PS, pyrimethamine-sulfadoxine; CQ, chloroquine; CQCP, chloroquine plus chlorpheniramine; AQ, amodiaquine, pyrimethamine-sulfadoxine combined with chloroquine; AQPS, pyrimethamine-sulfadoxine combined with amodiaquine; RI = parasitaemia disappears but reappears within 7 to 14 days; RII = decrease of parasitaemia but no complete clearance from peripheral blood; RIII = no pronounced decrease or increase in parasitaemia at 48 h after treatment; S = sensitive response.)

	CQ (n = 315)	CQCP (n = 104)	AQ (n = 104)	PS (n = 109)	CQPS (n = 65)	AQPS (n = 70)	*P* value
% of children with gametocytes							
at enrolment	17·8 (n = 56)	21·1 (n = 22)	10·6 (n = 11)	17·4 (n = 19)	1·5 (n = 1)	8·6 (n = 6)	0·001
on day 7	24·8 (n = 78)	23·1 (n = 24)	10·6 (n = 11)	61·5 (n = 67)	3·1 (n = 2)	10·0 (n = 7)	0·001
on day 14	17·1 (n = 54)	10·6 (n = 11)	7·7 (n = 8)	48·6 (n = 53)	—	4·3 (n = 3)	0·001
PRR							
Median	2·27	2·30	2·75	3·18	3·94	3·70	<0·001*
Interquartile range	1·42–3·97	1·73–3·93	1·85–4·11	1·91–4·13			
Range	−0·7–5·77	−0·10–5·32	−0·40–5·10	−0·21–5·6	−1·0–5·66	0·72–5·65	
PCT (days)	2·9±0·9	2·8±0·8	2·6±0·8	2·9±1·1	2·3±0·8	2·2±0·8	0·001**
	1–6	1–5	1–5	1–6	1–4	1–4	
S (no. of patients)	198	97	102	78	65	70	0·001
RI	87	6	2	18	—	—	
RII	15	—	—	9	—	—	
RIII	15	1	—	4	—	—	
Cure rate (%)	62·9	93·2	98·1	71·5	100	100	0·001

* PRR of AQPS- and CQPS-treated children were significantly higher than in other treatment groups except those treated with AQ or PS (compared with CQPS, $P = 0·09$ and 0·10).

** PCT was significantly shorter in those treated with AQPS and CQPS compared to other treatment groups ($P \leq 0·001$) except AQ ($P = 0·052$ and 0·25), respectively. PCT was also significantly shorter in those treated with AQ compared to CQ ($P = 0·019$, Tukey HSD).

Table 2. Risk factors for *Plasmodium falciparum* gametocytaemia at enrolment*

		No. of children with gametocytes	Crude odds ratio (95% CI)	P value	Adjusted OR (95% CI)	P value
Age (y)						
<5	420	69	1			
≥5	347	46	0·78 (0·52–1·2)	0·26	—	—
Gender						
male	354	66	1		1	
female	413	49	0·6 (0·4–0·9)	0·01§	0·55 (0·36–0·83)	0·005
Parasitaemia (/μl)						
<5000	82	21	1		1	
≥5000	685	94	0·46 (0·26–0·83)	0·007	0·42 (0·24–0·73)	0·002
Fever†						
Febrile	533	73	1		1	
Afebrile	208	42	1·6 (1·06–2·13)	0·01§	1·61 (1·05–2·5)	0·03
Duration of illness						
≤3 days	575	76	1		1	
>3 days	162	39	1·7 (1·1–2·7)	0·01§	1·57 (1·1–2·4)	0·047
PCV††						
≤25%	24	3	1		—	—
>25%	100	10	0·78 (0·52–1·2)	0·71		

* Time was included as a covariate in the analysis.
† Fever, axillary temperature ≥37·5 °C.
†† PCV, packed cell volume.
§ Chi with Yate's correction.

Table 3. Gametocyte densities at enrolment and following treatment with antimalarial drugs

(GMGD, Geometric mean gametocyte density; PS, pyrimethamine-sulfadoxine; CQ, chloroquine; CQCP, chloroquine plus chlorpheniramine; AQ, amodiaquine; COM, pyrimethamine-sulfadoxine combined with chloroquine or amodiaquine.)

	CQ (n=315)	CQCP (n=101)	AQ (n=104)	PS (n=102)	CQPS* (n=65)	AQPS (n=70)	P value
Gametocytaemia							
At enrolment GMGD (/μl)	25 (n=56)	24 (n=22)	20 (n=11)	24 (n=19)	132 (n=1)	40 (n=6)	0·55
Range	6–1344	12–576	12–740	6–444	132	12–288	
On day 7							
GMGD (/μl)	34 (n=28)	43 (n=24)	34 (n=11)	75 (n=67)	54 (n=2)	31 (n=7)	0·054
Range	6–1476	12–606	12–630	6–35211	24–120	12–408	
On day 14							
GMGD (/μl)	21 (n=54)	41 (n=11)	10 (n=5)	50 (n=53)	—	19 (n=3)	0·001
Range	6–144	12–168	12–30	6–180		12–48	
Proportion (%) of children with gametocytaemia on days 0, 7 and 14	47 (n=29)	77 (n=8)	38 (n=4)	12·8 (n=14)	—	1·4 (n=1)	0·030

* CQPS not included in the comparison due to small number.

may be contributory, but hormone concentrations were not measured in the children. Gender-related differences as risk factors for gametocyte carriage require further evaluation in African children.

As was expected, duration of illness longer than 3 days was associated with increased risk of gametocyte carriage on presentation. In areas of low transmission, duration of illness longer than 2 days has also been associated with gametocyte carriage (Price et al. 1999). As longer established *P. falciparum* infections are more likely to produce gametocytes (Smalley, Brown & Bassett, 1981), it is likely that longer duration of illness before presentation allowed sufficient time for the progression of committed asexual parasites to gametocytes. Since absence of fever is associated with increased risk of gametocyte carriage, afebrile children may have harboured the infection for a longer period. Alternatively, children with longer duration of illness may have had a relatively shorter duration of fever resulting in reduced

Table 4. Risk factors for *Plasmodium falciparum* gametocytaemia 7 days after treatment†

(PS, pyrimethamine–sulfadoxine; CQ, chloroquine; CQCP, chloroquine plus chlorpheniramine; AQ, amodiaquine; COPS, pyrimethamine–sulfadoxine combined with chloroquine or amodiaquine.)

Status at enrolment	Total no.	No. of children with gametocytes on day 7	Crude odds ratio (95 % CI)	*P* value	Adjusted odds ratio (95 % CI)	*P* value
Gametocytes						
Present	115	86	1		1	
Absent	652	102	0·06 (0·04–0·09)	<0·0001§	0·04 (0·02–0·07)	<0·001
PCT‡						
≤2 days	298	61	1		1	
>2 days	469	127	1·4 (1·03–2·07)	0·03§	1·4 (0·9–2·1)	0·20
Patent asexual parasitaemia within 14 days						
Present	157	68	1		1	
Absent	610	121	0·32 (0·22–0·47)	<0·001	0·50 (0·3–0·8)	0·007
Drug treatment*						
PS	109	67	1		1	
CQ	315	78	4·8 (3·0–7·9)	<0·001	8·5 (4·9–14·6)	<0·001
CQCP	104	24	5·3 (2·8–10·1)	<0·001	9·4 (4·5–19·7)	<0·001
AQ	104	11	13·5 (6·2–30·2)	<0·001	17·4 (7·3–41·0)	<0·001
AQPS	70	6	14·4 (5·7–38·0)	<0·001	14·9 (5·5–40·2)	<0·001
COPS	65	1	50·2 (11·2–313·7)	<0·001	35·6 (7·8–160·5)	<0·001

* Values of OR represent chances of being gametocyte free.
† PCT, parasite clearance time.
‡ Chi² with Yate's correction.

noxious effects of fever on gametocyte development. Low parasitaemia (as in the present study) and anaemia are also significantly associated with gametocyte carriage (Price *et al.* 1999; von Seidlein *et al.* 2001) but haematocrit values less than 25 % were not associated with gametocyte carriage in our cohort of children. We have no clear explanation for this observation. Anaemia in uncomplicated falciparum malaria may be enhanced by pre-existing helminth infections (Nacher *et al.* 2002), and both conditions may enhance gametocyte carriage (von Seidlein *et al.* 2001; Nacher *et al.* 2002), frequently co-exist and, are common in tropical endemic regions.

Despite lower efficacy, CQ treatment resulted in lower gametocyte carriage than PS. A similar observation has been made in Senegal and The Gambia (Robert *et al.* 2000; von Seidlein *et al.* 2001). The ability to release more gametocytes into the circulation following PS treatment may, in part, be independent of parasite sensitivity to PS (Sowunmi & Fateye, 2003*c*) and may partly explain this observation. Irrespective of treatment regimen given, children with patent gametocytaemia at presentation were significantly more likely to be gametocytaemic 7 days later than children without patent gametocytaemia. This suggests that the drugs evaluated had little or no effect on mature circulating gametocytes.

As was expected, recrudescent infections were associated with higher gametocyte prevalence, as was delay in peripheral parasite clearance as parasites develop resistance to drugs. The increase in gametocyte carriage and density as resistance develops to antimalarial drugs may confer survival and propagation advantages on the parasite in the population (Handunnetti *et al.* 1990; Robert *et al.* 1996; Sutherland *et al.* 2002; Sowunmi & Fateye, 2003*a*, *b*). In the current study, delayed clearance of peripheral parasitaemia and increased recrudescence rates were most frequently seen in those treated with CQ or PS and least frequent in those treated with AQ, AQPS or COPS. Similar observations have been made elsewhere (Price *et al.* 1999; Robert *et al.* 2000; Akim *et al.* 2000; von Seidlein *et al.* 2001). The significantly higher gametocyte density in those treated with PS than CQ at recrudescence of asexual parasitaemia would suggest that the former may have a higher propensity for the transmission of drug-resistant infections than the latter since gametocyte infectivity to mosquitoes may correlate with level of gametocytaemia (Tchuinkam *et al.* 1993; Robert *et al.* 2000). Since leukocyte counts may vary widely, one of the possible sources of errors in our estimation of gametocyte density is assuming an average leukocyte count of 8000/μl of blood.

The findings of the present study may have potential implications for the management of acute infections in this endemic area. Prompt treatment of falciparum infections with effective drugs is often associated with low gametocyte carriage (and may invariably reduce transmission of gametocytes to

mosquitoes), treatment of acute infections should, preferably, employ rapidly acting schizontocide to reduce the development of gametocytes. The artemisinin derivatives may reduce transmissibility by this mode of action (Price *et al.* 1996). Finally, the findings may have important implications with respect to malarial control in sub-Saharan Africa, where combination antimalarial therapy (WHO, 2001*a*,*b*) is presently being proposed for the treatment of malaria in the region.

This study received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. A.S. was supported by a WHO/TDR Career Development Award.

REFERENCES

AKIM, N.I.J., DRAKELEY, C., KINGO, T., SIMON, B., SENKORO, K. & SAUERWEIN, R.W. (2000). Dynamics of *Plasmodium falciparum* gametocytaemia in symptomatic patients in an area of intense perennial transmission in Tanzania. *American Journal of Tropical Medicine and Hygiene* 63, 199–203.

ANON. (1994). *Epi Info Version 6. A Word Processing Data Base and Statistics Program for Public Health on IBM-compatible Microcomputers*. Atlanta, GA: Centers for Disease Control and Prevention.

BONNET, S., GOUAGNA, L.C., PAUL, R.E., SAFEUKUI, I., MEUNIER, J.-Y. & BOUDIN, C. (2003). Estimation of malaria transmission from humans to mosquitoes in two neighbouring villages in south Cameroon: evaluation and comparison of several indices. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 97, 53–59.

BUCKLING, A., RANFORD-CARTWRIGHT, L.C., MILES, A. & READ, A.F. (1999). Chloroquine increases *Plasmodium falciparum* gametocytogenesis *in vitro*. *Parasitology* 118, 339–346.

BUTCHER, G.A. (1997). Antimalarial drugs and the mosquito transmission of *Plasmodium*. *International Journal for Parasitology* 27, 975–987.

CARTER, R. & MILLER, L.H. (1979). Evidence for environmental modulation of gametocytogenesis in *Plasmodium falciparum* in continuous culture. *Bulletin of the World Health Organization* 57 (Suppl. 1), 37–52.

CARTER, R. & GRAVES, P.M. (1988). Gametocytes. In *Malaria: Principles and Practice of Malariology*, vol. 1 (ed. Wernsdorfer, W.H. & McGregor, I.), pp. 253–306. Churchill Livingstone, Edinburgh.

EZEILO, G.C. (1971). Neutropenia in Africans. *Tropical and Geographical Medicine* 23, 264–267.

FALADE, C.O., SALAKO, L.A., SOWUNMI, A., ODUOLA, A.M.J. & LARCIER, P. (1997). Comparative efficacy of chloroquine, halofantrine and sulphadoxine-pyrimethamine in treatment of acute uncomplicated falciparum malaria in Nigerian children. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 91, 58–62.

GITHEKO, A.K., BRANDLING-BENNETT, A.D., BEIER, M., ATIELI, F., OWAGA, M. & COLLINS, F.H. (1992). The reservoir of *Plasmodium falciparum* malaria in a holoendemic area of western Kenya. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 86, 355–358.

GRIFFIN, J.E. & WILSON, J.D. (1991). Disorders of the testes. In *Harrison's Principles of Internal Medicine*, vol. 2 (ed. Wilson, J.D., Braunwald, E., Isselbacher, K.J., Petersdorf, R.G., Martin, J.B., Fauci, A.S. & Root, R.K.), pp. 1765–1776. McGraw-Hill, New York.

HANDUNNETTI, S.M., GUNEWARDENA, D.M., PATHIRANA, P.P.S.L., EKANAYAKE, K., WEERASINGHE, S. & MENDIS, K.N. (1996). Features of recrudescent chloroquine-resistant *Plasmodium falciparum* infections confer a survival advantage on parasites and have implications for disease control. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 90, 563–567.

HOGH, B., GAMAGE-MENDIS, A., BUTCHER, G.A., THOMPSON, R., BEGTRUP, K., MENDIS, C., ENOSSE, S.M.D., DGEDGE, M., BARRETO, J., ELING, W. & SINDEN, R.E. (1998). The differing impact of chloroquine and pyrimethamine/sulfadoxine upon the infectivity of malaria species to mosquito vector. *American Journal of Tropical Medicine and Hygiene* 58, 176–182.

LINGNAU, A., MARGOS, G., MAIER, W.A. & SEITZ, H.M. (1993). The effects of hormones on the gametocytogenesis of *Plasmodium falciparum in vitro*. *Applied Parasitology* 34, 153–160.

MASWOSWE, S.M., PETERS, W. & WARHURST, D.C. (1985). Corticosteroid stimulation of the growth of *Plasmodium falciparum* gametocytes *in vitro*. *Annals of Tropical Medicine and Parasitology* 79, 607–616.

MONS, B. (1985). Induction of sexual differentiation in malaria. *Parasitology Today* 1, 87–89.

NACHER, M., SINGHASIVANON, P., SILACHAMROON, U., TREEPRASERTSUK, S., KRUDSOOD, S., GAY, F., MAZIER, D. & LOOAREESUWAN, S. (2001). Association of helminth infections with increased gametocyte carriage during mild falciparum malaria in Thailand. *American Journal of Tropical Medicine and Hygiene* 65, 644–647.

PRICE, R.N., NOSTEN, F., LUXEMBURGER, C., TER KUILE, F.O., PAIPHUN, L., CHONGSUPHAJAISIDDHI, T. & WHITE, N.J. (1996). Effects of artemisinin derivatives on malaria transmissibility. *Lancet* 347, 1654–1658.

PRICE, R., NOSTEN, F., SIMPSON, J.A., LUXEMBURGER, C., PAIPHUN, L., TER KUILE, F., VAN VUGT, M., CHONGSUPHAJAISIDDHI, T. & WHITE, N.J. (1999). Risk factors for gametocyte carriage in uncomplicated falciparum malaria. *American Journal of Tropical Medicine and Hygiene* 60, 1019–1023.

ROBERT, V., MOLEZ, J.F. & TRAPE, J.F. (1996). Short report: gametocytes, chloroquine pressure, and the relative parasite survival advantage of resistant strains of falciparum malaria in West Africa. *American Journal of Tropical Medicine and Hygiene* 55, 350–351.

ROBERT, V., AWONO-AMBENE, H.P., LE HESRAN, J.-Y. & TRAPE, J.F. (2000). Gametocytemia and infectivity to mosquitoes of patients with uncomplicated *Plasmodium falciparum* malaria attacks treated with chloroquine or sulfadoxine/pyrimethamine. *American Journal of Tropical Medicine and Hygiene* 62, 210–216.

SALAKO, L.A., AJAYI, F.O., SOWUNMI, A. & WALKER, O. (1990). Malaria in Nigeria: a revisit. *Annals of Tropical Medicine and Parasitology* 84, 435–445.

SHAPER, A.G. & LEWIS, P. (1971). Genetic neutropenia in people of African origin. *Lancet* ii, 1021–1023.

SINDEN, R.E., CANNING, E.U., BRAY, R.S. & SMALLEY, M.E. (1978). Gametocyte and gamete development in

Plasmodium falciparum. *Proceedings of the Royal Society of London, Series B* 201, 375–399.

SMALLEY, M. E. (1976). *Plasmodium falciparum* gametocytogenesis in vitro. *Nature, London* 264, 271–272.

SMALLEY, M. E., BROWN, J. & BASSETT, N. M. (1981). The rate of production of *Plasmodium falciparum* gametocytes during natural infections. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 75, 318–319.

SOWUNMI, A. (2002). A randomized comparison of chloroquine, amodiaquine and their combination with pyrimethamine-sulfadoxine in the treatment of acute, uncomplicated, *Plasmodium falciparum* malaria in children. *Annals of Tropical Medicine and Parasitology* 96, 227–238.

SOWUNMI, A. (2003). A randomized comparison of chloroquine and chloroquine plus ketotifen, in the treatment of acute, uncomplicated, *Plasmodium falciparum* malaria in children. *Annals of Tropical Medicine and Parasitology* 97, 103–117.

SOWUNMI, A., AKINDELE, J. A. & BALOGUN, M. A. (1995). Leukocyte counts in falciparum malaria in African children from an endemic area. *African Journal of Medicine and Medical Sciences* 24, 145–149.

SOWUNMI, A. & FATEYE, B. A. (2003a). Asymptomatic, recrudescent, chloroquine-resistant, *Plasmodium falciparum* infections in Nigerian children: clinical and parasitological characteristics and implications for transmission of drug-resistant infections. *Annals of Tropical Medicine and Parasitology* 97, 469–479.

SOWUNMI, A. & FATEYE, B. A. (2003b). *Plasmodium falciparum* gametocytaemia in Nigerian children: before, during and after treatment with antimalarial drugs. *Tropical Medicine and International Health* 8, 783–792.

SOWUNMI, A. & FATEYE, B. A. (2003c). Gametocyte sex ratios in children with asymptomatic, recrudescent, pyrimethamine-sulfadoxine-resistant, *Plasmodium falciparum* malaria. *Annals of Tropical Medicine and Parasitology* 97, 671–682.

SOWUNMI, A., ODUOLA, A. M. J., OGUNDAHUNSI, O. A. T. & SALAKO, L. A. (1998a). Comparative efficacy of chloroquine plus chlorpheniramine and pyrimethamine-sulphadoxine in acute uncomplicated falciparum malaria in Nigerian children. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 92, 77–81.

SOWUNMI, A., ODUOLA, A. M. J., OGUNDAHUNSI, O. A. T. & SALAKO, L. A. (1998b). Enhancement of the antimalarial effect of chloroquine by chlorpheniramine in vivo. *Tropical Medicine and International Health* 3, 177–183.

SPSS (1999). *SPSS for Windows Release 10.01 (Standard Version)*. SPSS Inc., Chicago, IL, USA.

SUTHERLAND, C. J., ALLOUECHE, A., CURTIS, J., DRAKELEY, C. J., ORD, R., DURAISINGH, M., [redacted], B. M., WARHURST, D. C. & TARGETT, G. A. T. (2002). Gambian children successfully treated with chloroquine can harbor and transmit *Plasmodium falciparum* gametocytes carrying resistant genes. *American Journal of Tropical Medicine and Hygiene* 67, 578–585.

TCHUINKAM, T., MULDER, B., DECHERING, K., STOFFELS, H., VERHAVE, J.-P., COT, M., CARNEVALE, P., MEUWISSEN, J. H. E. T. & ROBERT, V. (1993). Experimental infections of *Anopheles gambiae* with *Plasmodium falciparum* of naturally infected gametocyte carriers in Cameroon: factors influencing the infectivity to mosquitoes. *Tropical Medicine and Parasitology* 44, 271–276.

THOMSON, D. (1911). I. A research into the production, life and death of crescents in malignant tertian malaria, in treated and untreated cases, by an enumerative method. *Annals of Tropical Medicine and Parasitology* 5, 57–82.

TRAPE, J.-F., PISON, G., PREZIOSI, M.-P., ENEL, C., DU LOU, A. D., DELAUNAY, V., SAMB, B., LAGARDE, E., MOLEZ, J.-F. & SIMONDON, F. (1998). Impact of chloroquine resistance on malaria mortality. *Life Sciences* 321, 689–697.

TRAPE, J.-F. (2001). The public health impact of chloroquine resistance in Africa. *American Journal of Tropical Medicine and Hygiene* 64 (Suppl.), 12–17.

VON SEIDLEIN, L., DRAKELEY, C., GREENWOOD, B., WALRAVEN, G. & TARGETT, G. (2001). Risk factors for gametocyte carriage in Gambian children. *American Journal of Tropical Medicine and Hygiene* 65, 523–526.

WHITE, N. J. (1997). Assessment of the pharmacodynamic properties of antimalarial drugs in vivo. *Antimicrobial Agents and Chemotherapy* 41, 1413–1422.

WILKINSON, R. N., NOEYPATIMANONDH, S. & GOULD, D. J. (1976). Infectivity of falciparum malaria patients for anopheline mosquitoes before and after chloroquine treatment. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 70, 306–307.

WORLD HEALTH ORGANIZATION (1973). *Chemotherapy of Malaria and Resistance to Antimalarials*. Technical Report Series No. 529. WHO, Geneva.

WORLD HEALTH ORGANIZATION (2000). Severe falciparum malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 94 (Suppl. 1).

WORLD HEALTH ORGANIZATION (2001a). *The Use of Antimalarial Drugs. Report of a WHO Informal Consultation*. Document WHO/CDS/RBM/2001.33. WHO, Geneva.

WORLD HEALTH ORGANIZATION (2001b). *The Use of Antimalarial Drugs. Report of a WHO Informal Consultation*. Document WHO/CDS/RBM/2001.35. WHO, Geneva.

Plasmodium falciparum hyperparasitaemia in children
Risk factors, treatment outcomes, and gametocytaemia following treatment

Sowunmi A., Adedeji A.A., Fateye B.A. & Babalola C.P.

Summary :

The risk factors associated with hyperparasitemia at presentation and after treatment with different antimalarial drug regimens were evaluated in 1,048 children enrolled prospectively in seven antimalarial drug trials between July 1996 and September 2003 in a hyperendemic area of southwestern Nigeria. The outcomes of treatment of hyperparasitaemia, and gametocyte carriage following treatment were also evaluated. The children were assigned to one of seven treatment groups : chloroquine (CQ) only ; pyrimethamine-sulfadoxine (PS) only ; amodiaquine (AQ) only ; CQ plus chlorpheniramine (CQCP) ; PS combined with CQ or AQ (COM) ; PS combined with probenecid (PPS) ; and halofantrine (HF). Hyperparasitaemia was found in 100 (9.5 %) of the 1,048 children at enrolment (day 0). Following oral therapy, 1.2 % of all patients (i.e. 13 patients) became hyperparasitaemic, which developed in all patients by day 1 of followup. In a multiple regression model, age ≤ 5 years, and a core temperature (oral or rectal) ≥ 39.5°C were found to be independent risk factors for hyperparasitaemia at enrolment. Following therapy, the cure rate on day 14 was significantly lower in those treated with CQ compared to other treatment groups. Severe resistance (RIII) response to treatment occured significantly more frequently in those with hyperparasitaemia at enrolment than in those without, and was seen in five and one child with hyperparasitaemia who were treated with CQ and CQCP, respectively. Gametocyte carriage was insignificantly lower at enrolment and at all times following treatment in children with hyperparasitaemia than in age and gender matched children without hyperparasitaemia who received the same treatment. The results are discussed in the light of management of uncomplicated hyperparasitaemia in children in endemic settings.

KEY WORDS : malaria, hyperparasitaemia, risk factors, gametocytaemia, children, Nigeria.

Résumé : *Hyperparasitémie à Plasmodium falciparum chez les enfants : facteurs de risque et gamétocytémie avant et après traitement*

Les facteurs de risque associés à l'hyperparasitémie à Plasmodium falciparum à l'admission et après le traitement avec sept protocoles antipaludéens différents par voie orale ont été évalués chez 1 048 enfants lors d'une étude prospective, menée entre juillet 1996 et septembre 2003 au Sud-Ouest du Nigéria, dans une zone hyperendémique. Les résultats du traitement (hyperparasitémie) et durée de la persistance des gamétocytes dans le sang ont été évalués. Les groupes traités étaient : chloroquine (CQ) ; pyriméthamine sulfadoxine (PS) ; amodiaquine (AQ) ; chloroquine plus chlorphéniramine (CQCP) ; pyriméthamine sulfadoxine plus chloroquine ou amodiaquine (COM) ; pyriméthamine sulfadoxine plus probénécid (PPS) ; halofantrine (HF). L'hyperparasitémie a été retrouvée chez 100 enfants lors de l'admission (9,5 %). Après 24 heures de traitement, 1,2 % des enfants (n = 13) sont devenus hyperparasités. Avec un programme de régression multiple, nous avons montré qu'un âge ≤ 5 ans et une température centrale ≥ 39,5°C sont des facteurs de risque indépendants pour l'hyperparasitémie à l'admission. Après deux semaines de traitement, le pourcentage de guérison est significativement plus bas dans le groupe CQ. Une résistance sévère à ce traitement (RIII) apparaît plus fréquemment chez ceux qui sont hyperparasités à l'admission. Le nombre de gamétocytes circulants était plus bas à l'admission et pendant le traitement chez les enfants hyperparasitémiques que chez ceux du même âge et de même sexe avec le même traitement, mais sans hyperparasitémie. Les résultats sont discutés dans le but d'améliorer le traitement des accès palustres non compliqués avec hyperparasitémie dans les zones d'endémie.

MOTS CLÉS : *paludisme, hyperparasitémie, facteur de risque, gamétocytémie, enfant, Nigéria.*

INTRODUCTION

Plasmodium falciparum infections may result in rapid multiplication of asexual parasites and massive increases in circulating peripheral parasites particularly in the relatively non-immune or, less frequently, in the semi-immune. These massive increases may reach or surpass a threshold referred to as hyperparasitaemia. Hyperparasitaemia, defined as 5 % or more parasitized erythrocytes or a parasitaemia greater than 250,000/µl blood, is considered one of the several features of severe malaria (WHO, 1990, 2000 a). Hyperparasitaemia still accompanied by other features of severe malaria (uncomplicated hyperparasitaemia) often pose management problems in patients residing in endemic areas. Apart from a general recommendation of parenteral antimalarials (WHO, 2000 b), there are no other clearcut guidelines for the management of uncomplicated hyperparasitaemia in children residing in such areas. However, it has been suggested that

* Department of pharmacology & therapeutics and Institute for medical research and training, and Department of pharmaceutical chemistry, University of Ibadan, Ibadan, Nigeria.
Correspondence : A. Sowunmi, Department of Clinical Pharmacology, University College Hospital, Ibadan, Nigeria.
Tel. : 234-2-2412101, 234-2-2411904 – Fax : +234-2 2411843
E-mail : malaria_tea@alpha.linkserve.com
akinsowunmi@hotmail.com

uncomplicated hyperparasitaemia in children in these endemic areas be treated with oral antimalarial drugs providing the drug is rapidly absorbed and the parasites are fully sensitive to the antimalarial drug(s) given (Sowunmi *et al.*, 1992, 1996, 2000 a). Such a suggestion needs review in view of the increasing resistance in *P. falciparum* to many antimalarials drugs and the lack of facilities to monitor drug sensitivity of *P. falciparum* in vitro and in vivo in many endemic areas.

There is little information on, for example, the risk factors associated with uncomplicated hyperparasitaemia or the time-course of gametocytaemias following oral antimalarial treatment of uncomplicated hyperparasitaemias in African children. Such information is necessary in view of the increasing resistance in *P. falciparum* to chloroquine (CQ) and other commonly available antimalarials and the increasing morbidity and mortality associated with drug resistance (Trape *et al.*, 1998; Trape, 2001). In addition, it may improve the overall management of these cases. The present study was designed to address these issues. The main aims of the study were : to evaluate the risk factors associated with hyperparasitaemia in a group of children presenting with acute, symptomatic, apparently uncomplicated, *P. falciparum* malaria in an endemic area; to assess the outcome of oral antimalarial treatment of uncomplicated hyperparasitaemia; and to follow the temporal changes in gametocytaemias in children with hyperparasitaemias who were treated with oral antimalarial drugs.

PATIENTS AND METHODS

PATIENTS

The study took place between July 1996 and September 2003 in patients presenting at the University College Hospital in Ibadan, a hyperendemic area for malaria in southwestern Nigeria (Salako *et al.*, 1990). Ethical clearance was provided by the local ethics committee. During the period, a series of antimalarial drug studies were conducted to evaluate the efficacy and safety of different treatment regimens. All antimalarial drugs were given orally. The details of the studies have been described before (Sowunmi *et al.*, 1999 a, b, c, 2000 a; Sowunmi, 2002, 2003; Sowunmi & Fateye, 2003). Briefly, children with symptoms compatible with acute falciparum malaria who fulfilled the following criteria were enlisted in the study : age below 12 years, pure *P. falciparum* parasitaemia greater than 2000 asexual forms/µl blood, negative urine tests for antimalarial drugs (Dill-Glazko and Ignis tests), absence of concomitant illness, no evidence of severe malaria (WHO, 2000 a) and written informed consent given by parents or guardians. After enrolment and start of treatment (day 0), follow-up with clinical and parasitological evaluation was at days 1-7, and then on days 14, and when necessary, on days 21 and 28, for example, in patients who received pyrimethamine-sulfadoxine (PS) (Fansidar®, Hoffmann La Roche) combined with chloroquine (Nivaquine®, May & Baker Plc, Nigeria) or amodiaquine (Camoquine®, Parke Davis, Senegal). Clinical evaluation consisted of a general clinical examination including measurement of weight, axillary temperature and physical examination.

ASSESSMENT OF PARASITAEMIA

Thick and thin blood films prepared from a finger prick were Giemsa-stained and were examined by light microscopy under an oil-immersion objective, at x 1,000 magnification, by two independent assessors. Parasitaemia in thick films was estimated by counting asexual parasites relative to 1,000 leukocytes, or 500 asexual forms, whichever occurred first. From this figure, the parasite density was calculated assuming an average leukocyte count of 6,000/µl of blood (Shapiro & Lewis, 1971; Ezedinma, 1971; Sowunmi *et al.*, 1995). Gametocytes were also counted in thick films against 1,000 leukocytes assuming an average leukocyte count of 6000/µl of blood at enrolment (day 0) and on days 7 and 14. Fractional gametocyte density (FGD) at enrolment was defined as gametocyte count divided by total asexual and sexual count (Price *et al.*, 1999). Haematocrit was done at enrolment in 121 of the patients treated with PS or CQPS, AQPS or PPS.

EVALUATION OF RESPONSE TO DRUG TREATMENT

Response to drug treatment was assessed using World Health Organization (WHO) criteria (WHO, 1973) as follows : S = sensitive, clearance of parasitaemia without recurrence; RI (mild resistance) = parasitaemia disappears but reappears within 7 to 14 days; RII (moderate resistance) = decrease of parasitaemia but no complete clearance from peripheral blood; RIII (severe resistance) = no pronounced decrease or increase in parasitaemia at 48 hours after treatment. In those with sensitive or RI response, parasite clearance time (PCT) was defined as the time elapsing from drug administration until there was no patent parasitaemia for at least 72 h. Asexual parasite reduction ratio (PRR) (Peters, 1997) was defined as the ratio of day 0/day 2 parasitaemia.

RE-TREATMENT OF TREATMENT FAILURES

All patients with RII and RIII responses were re-treated with intramuscular artemether (9.6 mg/kg, over five days). Patients with RI response were re-treated with oral mefloquine 25 mg/kg single dose and followed

up for another 14-28 days. Patients were retreated whenever they became symptomatic or when they show profound clinical (hyperpyrexia, oral fluid intolerance) or parasitological deterioration.

STATISTICAL ANALYSIS

Data were analysed using version 6 of the Epi-Info software (Anon., 1994), and the statistical program SPSS for Windows version 10.01 (SPSS, 1999). Proportions were compared by calculating χ^2 with Yates' correction or Fisher exact test. Normally distributed, continuous data were compared by Student's t-tests and analysis of variance (ANOVA). Data not conforming to a normal distribution were compared by the Mann-Whitney U-test and the Kruskal-Wallis test (or by Wilcoxon rank sum test). A multiple logistic regression model was used to test the association between hyperparasitaemia (yes or no at presentation or during follow up) and factors that were significant at univariate analysis : age ≤ 5 years and presence of fever (oral or rectal temperature) ≥ 39.5° C. Because the study was conducted over a period of seven years, time was included as a covariate in the analysis. P values of ≤ 0.05 were taken to indicate significant differences.

RESULTS

The demographic characteristics of the children enrolled in the study are summarized in Table I. At enrolment, 303, 173, 104, 203, 143, 78 and 44 of the 1,048 children were allotted to, and were subsequently treated with chloroquine (CQ) only, pyrimethamine-sulfadoxine (PS) only; amodiaquine (AQ) only; CQ plus chlorpheniramine (CQCP); PS combined with CQ or AQ (COM); PS combined with probenecid (PPS); and halofantrine (HF) (Halfan®, GlaxoSmithKline), respectively. Hyperparasitaemia was found in 100 (9.5 %) of the 1,048 children at enrolment.

RISK FACTORS FOR HYPERPARASITAEMIA AT ENROLMENT

Factors associated with hyperparasitaemia at enrolment are presented in Table II. Age ≤ 5 years, and oral or rectal temperature ≥ 39.5° C were independent risk factors for uncomplicated hyperparasitaemia at enrolment.

Variables	Value [mean (range)]
Age (years)	5.9 ± 3.3 (0.5-11.9)
M : F	495 : 553
Weight (kg)	19.1 ± 4.8 (6.6-27)
Presenting body temperature (°C)	38.6 ± 1.2 (35.6-40.0)
Duration of illness (d)	3.0 ± 1.3 (1-[illegible])
Asexual parasite density (per µL)	
Geometric mean	30,129
Range	2,000-2,341,000
No. > 250000	100

Table I – Summary of demographic and other characteristics of the 1,048 children enrolled in the trial

	Total no.	No. of children with hyperparasitaemia	Crude OR (95 % CI)	P. value	Adjusted OR (95 % CI)	P. value
Age (years)			1.85 (1.08-2.50)	0.025	1.65 (1.09-2.47)	0.016
≤ 5	533	62				
> 5	515	38				
Gender			1.1 (0.58-1.4)	0.7	–	–
male	495	43				
female	553	55				
Duration of illness (d)			0.98 (0.63-1.50)	0.9	–	–
≥ 3	685	66				
< 3	363	34				
Fever			1.88 (1.15-3.01)	0.0[illegible]	1.84 (1.17-2.89)	0.009
≥ 39.5 °C	314	51				
< 39.5 °C	734	49				
Convulsion			0.97 (0.43-1.30)	0.9	–	–
Yes	134	11				
No	914	89				

OR, odds ratio.
χ^2 with Yate's correction.
CI, confidence interval.

Table II – [illegible]

Hyperparasitaemia during follow up

Following oral therapy, 1.2 % of all patients (i.e. 13 of the 1,048 patients) became hyperparasitaemic, which developed in all patients by day 1 of follow-up. The 13 patients were treated with CQ (10 patients), PS (one patient) or COM (two patients), and following treatment, all but two had sensitive response. The two children in the COM group who became hyperparasitaemic on day 1 specifically received PS combined with CQ. The two children with resistance response (1 RI, 1 RII) were treated with CQ. Compared with other treatment groups, there was a significant difference in the proportion of children treated with CQ who became hyperparasitaemic on day 1 following treatment (p = 0.01).

Treatment outcomes of hyperparasitaemia

The clinical and parasitological characteristics of the 100 children who had hyperparasitaemia at enrolment and were treated with oral antimalarial drugs are summarized in Table III. Despite enrolment at different periods, these characteristics were similar (primarily because the criteria for enrolment into all studies were similar). No child with hyperparasitaemia was treated with AQ alone.

The responses of the initial hyperparasitaemia to drug treatment are shown in Table IV. The cure rate following treatment with CQ was significantly lower than the other treatment groups.

	CQ (n = 33)*	CQCP (n = 27)	PS (n = 32)	COM (n = 11)	PPS* (n = 5)	HF* (n = 1)	P. value
Age (years)							
Mean ± sd	4.3 ± 2.3	5.1 ± 2.5	4.6 ± 2.8	4.9 ± 2.2	7.9 ± 1.5	5.0	0.6
Range	1-5.9	0.7-10.5	0.5-10.5	[illegible]	6.5-10	-	
M : F	12 : 21	15 : 12	17 : 15	6 : 5	3 : 2	1 : 0	-
Duration of illness (d)							0.5
Mean	2.8 ± 1.2	3.2 ± 1.5	3.5 ± 2.5	3.0 ± 0.7	3.0 ± 0.0	3.0	
Range	1-6	1-6	1-14	2-4	3-3	-	
Parasitaemia (/µl)							
Geometric mean	438,649	467,090	[illegible]	[illegible]	730,304	-*	0.4
Range	253,091-1,500,000	253,600-2,341,000	253,091-1,[illegible],000	250,1[illegible]-716,000	414,730-1,[illegible],000		

PS, pyrimethamine-sulfadoxine; CQ, chloroquine; CQCP, chloroquine plus chlorpheniramine; COM, [illegible] combined with chloroquine or [illegible]; PPS, [illegible]; HF, halofantrine. [illegible]

[illegible]

Table III. – Clinical and parasitological characteristics of 100 children with *P. falciparum* hyperparasitaemia who were treated with oral antimalarial drugs.

	CQ (n = 33)	CQCP (n = 27)	PS (n = 25)	COM (n = 11)	PPS (n = 5)	HF (n = 1)	P. value
FCT (d)							
Mean ± sd	2.0 ± 0.9	2.3 ± 1.0	2.2 ± 1.2	1.6 ± 0.5	2.2 ± 1.3	1.0	0.5
Range	1-4	1-4	1-4	1-2	1-4	-	
PRR							
Median (× 10³)	5.6	1.0	3.1	13.1	93.2		0.5
Interquartile range (× 10³)	0.006-72.2	0.03-44.4	27.3-37.6	0.09-65.1		-	
PCT (d)							
Mean	2.8 ± 1.1	3.2 ± 0.8	3.0 ± 1.3	2.6 ± 0.5	2.6 ± 0.5	3.0	0.4
Range	2-6	2-5	2-4	2-3	2-4	-	
S (no. of patients)	18	22	23	11	5	1	
RI	[illegible]	3	5	0	0	0	
RII	3	1	0	0	0	0	
RIII	5	1	0	0	0	0	
Cure rate (%)	54.5	[illegible]	[illegible]	100	100	-	0.001

PRR, parasite reduction ratio; FCT, fever clearance time; PCT, parasite clearance time; PS, pyrimethamine-sulfadoxine; CQ, chloroquine; CQCP, chloroquine plus chlorpheniramine; COM, [illegible] combined with chloroquine or [illegible]; PPS, [illegible]; HF, halofantrine. [illegible] parasite clearance from peripheral blood [illegible]; S = sensitive response.

[illegible]

* Excluded [illegible] who had hyperparasitaemia at enrolment.

Table IV. – Therapeutic responses of 100 children with acute *P. falciparum* malaria [illegible]

COMPARISON OF OUTCOMES OF TREATMENT OF NON HYPERPARASITAEMIA AND HYPERPARASITAEMIA

Sixteen of 948 children without hyperparasitaemia had RIII response to treatment compared to six of 100 children with hyperparasitaemia. The difference between these proportions was significant ($\chi^2 = 6.22$, $P = 0.001$). Four children (three treated with CQ and one with PQ aged ≤ 3 years who had hyperparasitaemia progressed to cerebral malaria, while two of the 948 children without hyperparasitaemia had the same outcome. The difference between these two proportions was significant ($P = 0.001$, by Fisher exact test). The two children without hyperparasitaemia who progressed to cerebral malaria were treated with CQ. Adverse reactions reported following drug treatment were similar in children with hyperparasitaemia and in age- and gender- matched children without hyperparasitaemia who were treated with the same drugs (data not shown). For example, in those treated with CQ, pruritus occurred in five (of 33) and four (of 33) children with and without hyperparasitaemia, respectively.

GAMETOCYTE CARRIAGE AND GAMETOCYTAEMIA IN CHILDREN WITH HYPERPARASITAEMIA

In order to evaluate gametocyte carriage and gametocytaemia in those who were hyperparasitaemic at presentation, children with hyperparasitaemia were matched with those without hyperparasitaemia for time of presentation, age, gender, and drug treatment.

At enrolment gametocyte carriage was similar in children with hyperparasitaemia and in age- and gender-matched children without hyperparasitaemia who received the same drug treatment (6 of 100 vs 11 of 100 children, $\chi^2 = 1.03$, $P = 0.3$). Similarly following treatment, gametocyte carriage was similar on day 7 (16 of 100 vs 27 of 100 children, $\chi^2 = 2.9$, $P = 0.08$) and on day 14 (9 of 100 vs 17 of 100 children, $\chi^2 = 2.2$, $P = 0.14$).

At enrolment gametocytaemia was similar in children with hyperparasitaemia and in age- and gender- matched children without hyperparasitaemia who received the same drug treatment (geometric mean 12, range 6-24/uL vs 14 range 6-72, $P = 0.5$). Similarly following treatment, gametocytaemia was similar on day 7 (geometric mean 71, range 6-1320/uL vs 66, range 6-828, $P = 0.4$) and on day 14 (geometric mean 57, range 12-480/uL vs 70, range 12-360, $P = 0.7$).

Fractional gametocyte density was insignificantly lower in children with hyperparasitaemia compared with those without hyperparasitaemia (median 0.003, range 0.001- 0.096 vs 0.048, range 0.0015-2.3 %, $P = 0.24$).

RE-TREATMENT OF TREATMENT FAILURES

All treatment failures responded to re-treatment with intramuscular artemether or oral mefloquine with clearance of fever and parasitaemia within 72 h of commencing re-treatment and with no recurrence of parasitaemia during additional 14-28 days of follow-up.

DISCUSSION

Uncomplicated hyperparasitaemia is not uncommon in African children presenting with acute, symptomatic, *P. falciparum* malaria (Salako *et al*., 1990; Sowunmi *et al*., 1992, 1996, 2000 a). Prevalence rates in endemic and non endemic areas in Africa probably vary widely; in southwest Nigeria, the rate is approximately 10-12 % (Sowunmi, unpublished data). The 10 % prevalence recorded in the present study was similar to that previously reported from the same area in the early 1990's (Salako *et al*., 1990).

The risk factors associated with uncomplicated hyperparasitaemia at presentation are not frequently documented. In falciparum infections, younger age (< 3 years) has been associated with hyperparasitaemia and increased risk of progression to cerebral malaria (Sowunmi *et al*., 2000 a). In the present study, age ≤ 5 years and oral or rectal temperature ≥ 39.5°C were independent risk factors associated with hyperparasitaemia at presentation. In falciparum infections in young children, the general trend is for parasitaemia to increase with time, and more specifically, to be accompanied by increases in body temperature. However, in severe infections there may be hypothermia. In practice many children with lower oral or rectal temperatures than our model found may be hyperparasitaemic. This would be so because many parents or guardians have ready access to over the counter remedies including antipyretics before presentation. This 'blunting' of presenting oral or rectal temperature may mislead the attending health care provider and distract attention from the possible presence of hyperparasitaemia.

The responses of apparently uncomplicated hyperparasitaemia to oral therapy are less frequently reported, probably because of the dangers associated with oral therapy in a condition that may rapidly progress to a fatal outcome, and probably also because of increasing resistance in *P. falciparum* to antimalarial drugs leading to reluctance to try oral therapy. Providing the parasites are fully sensitive to the oral drugs chosen, responses to drug therapy appears to be independent of parasite load. Thus in a comparative study, therapeutic responses of those with and without hyperparasitaemia were similar in children from an endemic

area in West Africa (Sowunmi *et al.*, 2000 a). In addition, in drug sensitive infections, the disposition of parasitaemia appears to follow a first order kinetics (Sowunmi *et al.*, 2000 a, b). In our cohort of children, CQ was the least effective drug in children with hyperparasitaemia and clearly represented a significant decline in the sensitivity of *P. falciparum* to this drug. Thus with prevailing degree of CQ resistance, this drug may not be ideal for the treatment of malaria irrespective of parasite load. The significantly higher proportions of children without hyperparasitaemia who subsequently developed it following treatment with CQ or PS compared with the other treatment groups suggest slow onset of antimalarial action or reduced sensitivity to these drugs and a risk for development of post-treatment hyperparasitaemia.

The similar frequencies of pruritus (and other adverse drug reactions following treatment in those with and in those without hyperparasitaemia who were treated with the same drugs [data not shown]) suggest that hyperparasitaemia does not predispose to undue adverse drug reactions following treatment (Sowunmi *et al.*, 2000 a).

Relatively low asexual parasitaemia and absence of fever are some of the risk factors associated with gametocyte carriage in falciparum infections (Price *et al.*, 1999; Akim *et al.*, 2000; von Seidlein *et al.*, 2001). The lower gametocyte carriage and gametocytaemia following treatment of the children with hyperparasitaemia indicate that oral therapy of this condition is not associated with undue generation of gametocytes. However, it is not known whether gametocytes arising from patients who had hyperparasitaemia are more infectious to the mosquito than those arising from patients without hyperparasitaemia who were treated with the same drugs.

Hyperparasitaemia is a potentially life threatening condition, and with or without other features of severe malaria requires close clinical and parasitological monitoring. Its occurrence in children from this endemic area without other overt features of severe falciparum malaria suggests the presence of some degree of immunity, although these children are, in general, considered relatively non-immune compared with adults from the same endemic area, and are prone to multiple infections (Happi *et al.*, 2003). Should oral CQ or PS continued to be used for a potentially life threatening situation in view of increasing resistance of *P. falciparum* to these drugs in Africa? We feel otherwise. A recent study suggests that AQ, a drug more effective than CQ in both CQ- sensitive and resistant- *P. falciparum* infections, rapidly clears hyperparasitaemia (Ndounga & Basco, 2003). In the small number of children treated with a combination of PS plus AQ in our study population, neither clearance nor parasite reduction ratio was significantly faster or higher respectively than those of other treatments. In view of the fact that artemisinin and its derivatives clear parasitaemia more rapidly than most of the currently available antimalarials (Ilett & White, 1995), these drugs combined with, for example, AQ may be ideal for the management of uncomplicated hyperparasitaemia in children from Africa. This suggestion is predicated on the fact that AQ is a relatively safe drug (Olliaro *et al.*, 1996), and may be a suitable partner combination drug with the artemisinin derivatives, for example, artesunate for use in Africa (Adjuik *et al.*, 2002). Studies to assess the efficacy of such combinations in uncomplicated and complicated hyperparasitaemias are under way in our study area.

ACKNOWLEDGEMENTS

The study received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. AS was supported by a WHO/TDR Career Development Grant.

REFERENCES

ADJUIK M., AGNAMEY P., BABIKER A. *et al.* Amodiaquine-artesunate versus amodiaquine for uncomplicated *Plasmodium falciparum* malaria in African children: a randomized multicentre trial, *Lancet*, 2002, *359*, 1365-1372.

AKIM N.I.J., DRAKELEY C., KAKOLO T., SIMON B., SEMAKULA K. & SAUERWEIN R.W. Dynamics of *Plasmodium falciparum* gametocytaemia in symptomatic patients in an area of intense perennial transmission in Tanzania. *American Journal of Tropical Medicine and Hygiene*, 2000, *63*, 193-203.

ANON. Epi Info Version 6. A Word Processing Data Base and Statistics Program for Public Health on IBM-compatible Microcomputers. Atlanta, GA : Centers for Disease Control and Prevention, 1994.

EZIMO G.C. Neutropenia in Africans. *Tropical and Geographical Medicine*, 1971, *23*, 264-267.

HAPPI T.C., THOMAS S.M., GBOTOSHO G.O. *et al.* Point mutations in the *pfcrt* and *pfmdr-1* genes of *Plasmodium falciparum* and clinical response to chloroquine, among malaria patients from Nigeria. *Annals of Tropical Medicine & Parasitology*, 2003, *97*, 439-451.

ILETT T.T. & WHITE N.J. Quinghaosu. *Lancet*, 1995, *341*, 603-608.

NDOUNGA M. & BASCO L.K. Rapid clearance of *Plasmodium falciparum* hyperparasitaemia after oral amodiaquine treatment in patients with uncomplicated malaria. *Acta Tropica*, 2003, *88*, 27-32.

OLLIARO P., NEVILL C., LE BRAS J., RINGWALD P., MUSSANO P., GARNER P. & BRASSEUR P. Systematic review of amodiaquine treatment of uncomplicated malaria. *Lancet*, 1996, *348*, 1196-1201.

Price R.N., Nosten F., Luxemburger C., Ter Kuile F.O., Paiphun L., Chongsuphajaisiddhi T. & White N.J. Effects of artemisinin derivatives on malaria transmissibility. *Lancet*, 1996, *347*, 1654-1658.

Salako L.A., Ajayi F.O., Sowunmi A. & Walker O. Malaria in Nigeria: a revisit. *Annals of Tropical Medicine and Parasitology*, 1990, *84*, 435-445.

Shaper A.G. & Lewis P. Genetic neutropenia in people of African origin. *Lancet*, 1971, *ii*, 1021-1023.

Sowunmi A., Walker O. & Salako L.A. Hyperparasitaemia : not a reliable indicator of severity or poor prognosis in falciparum malaria in children from endemic African countries. *Annals of Tropical Paediatrics*, 1992, *12*, 155-158.

Sowunmi A., Akindele J.A. & Balogun M.A. Leukocyte counts in falciparum malaria in African children from an endemic area. *African Journal of Medicine and Medical Sciences*, 1995, *24*, 145- 149.

Sowunmi A., Oduola A.M.J., Ilesanmi A.O. & Salako L.A. Open comparison of artemether and mefloquine in uncomplicated *Plasmodium falciparum* hyperparasitaemia in children. *Annals of Tropical Paediatrics*, 1996, *16*, 5-9.

Sowunmi A., Oduola A.M.J., Ogundahunsi O.A.T. & Salako L.A. Comparative efficacy of chloroquine plus chlorpheniramine and pyrimethamine/sulphadoxine in acute uncomplicated falciparum malaria in Nigerian children. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 1998 a, *92*, 77-81.

Sowunmi A., Oduola A.M.J., Ogundahunsi O.A.T. & Salako L.A. Enhancement of the antimalarial effect of chloroquine by chlorpheniramine *in vivo*. *Tropical Medicine and International Health*, 1998 b, *3*, 177-183.

Sowunmi A., Fehintola F.A., Ogundahunsi O.A.T, Oduola A.M.J. & Salako L.A. Comparative efficacy of chloroquine plus chlorpheniramine and halofantrine in acute uncomplicated falciparum malaria in Nigerian children. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 1998 c, *92*, 441-445.

Sowunmi A., Adedeji A.A., Sowunmi C.O., Falade A.G., Sijuade A.O. & Oduola A.M.J. Comparative clinical characteristics and response to oral therapy of children with and without *Plasmodium falciparum* hyperparasitaemia in an endemic area. *Annals of Tropical Medicine and Parasitology*, 2000a, *94*, 549-558.

Sowunmi A., Adedeji A.A., Fehintola F.A. & Oduola A.M.J. *Plasmodium falciparum* kinetics during treatment with antimalarial drugs in children. *Clinical Drug Investigation*, 2000 b, *20*, 43-51.

Sowunmi A. A randomized comparison of chloroquine, amodiaquine and their combination with pyrimethamine-sulfadoxine in the treatment of acute, uncomplicated *Plasmodium falciparum* malaria in children. *Annals of Tropical Medicine and Parasitology*, 2002, *96*, 227-238.

Sowunmi A. A randomized comparison of chloroquine and chloroquine plus ketotifen in the treatment of acute, uncomplicated *Plasmodium falciparum* malaria in children. *Annals of Tropical Medicine and Parasitology*, 2003, *97*, 103-117.

Sowunmi A. & Fateye B.A. *Plasmodium falciparum* gametocytaemia in Nigerian children : before, during and after treatment with antimalarial drugs. *Tropical Medicine and International Health*, 2003, *8*, 783-792.

SPSS. *SPSS for Windows Release 10.01 (Standard Version)*. SPSS Inc, Chicago, IL, USA, 1999.

Trape J.F., Pison G., Preziosi M.P. *et al.* Impact of chloroquine resistance on malaria mortality. *Life Sciences*, 1998, *321*, 689-697.

Trape J.F. The public health impact of chloroquine resistance in Africa. *American Journal of Tropical Medicine and Hygiene*, 2001, *64*, (Suppl.), 12-17.

Von Seidlein L., Drakeley C.J., Greenwood B., Walraven G. & Targett G. Risk factors for gametocyte carriage in Gambian children. *American Journal of Tropical Medicine and Hygiene*, 2001, *65*, 523-526.

White N.J. Assessment of the pharmacodynamic properties of antimalarial drugs *in vivo*. *Antimicrobial Agents and Chemotherapy*, 1997, *41*, 1413-1422.

World Health Organization. Chemotherapy of Malaria and Resistance to Antimalarials. Technical Report Series No. 529. WHO, Geneva, 1973.

World Health Organization. Severe and complicated malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 1990, *84*, (Suppl.).

World Health Organization. Severe falciparum malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 2000 a, *94*, (Suppl. 1).

World Health Organization. Management of Severe Malaria. A Practical Handbook. Second edition. World Health Organization, Geneva, 2000 b.

Reçu le 19 janvier 2004
Accepté le 15 mars 2004

Journal of Tropical Pediatrics Advance Access published June 24, 2005

Plasmodium falciparum Malaria in Nigerian Children During High and Low Transmission Seasons: Gametocyte Carriage and Response to Oral Chloroquine

by A. A. Adedeji,[a] F. A. Fehintola,[a] R. A. Falade,[a] T. C. Happi,[a] A. O. I. Amoo,[a] G. O. Obafunke,[a] and A. Sowunmi[a]
[a]Department of Pharmacology & Therapeutics and Institute for Medical Research and Training, University of Ibadan, Ibadan, Nigeria
[b]Departments of [b]Pharmacology and [c]Medical Microbiology and Parasitology, Obafemi Awolowo College of Health Sciences, Olabisi Onabanjo University, Sagamu, Nigeria

Summary

Plasmodium falciparum malaria during high and low transmission seasons was evaluated in 1031 children treated with different antimalarial drug in a hyperendemic area of southwestern Nigeria. Seventy-three (10.5%) of 693 and forty (11.8%) of 338 children were gametocyte carriers in the high transmission seasons (HTS) and low transmission seasons (LTS), respectively. In a multiple regression model, two factors were found to be independent risk factors for the presence of gametocytemia at enrolment in the HTS: duration of illness >3d, and asexual parasite densities less than 10000/μl. Similarly male gender, duration of illness >3d and parasite density less than 5000/μl were found independent risk factors for presence of gametocytemia during LTS. The preceding parasitemia, parasite clearance times, intensity of gametocytemia and proportion carrying gametocytes post treatment differ significantly in the 333 (32.3%) of these children that were treated with chloroquine in the two seasons. These findings may be important in our understanding of *P. falciparum* transmission dynamics, response to chloroquine therapy and contribution of chloroquine to gametocyte carriage as seasonal changes occur.

Introduction

Incidence of *Plasmodium falciparum* malaria often has seasonal pattern. Gametocyte generation, carriage and infectivity to mosquitoes are crucial to successful transmission of falciparum malaria infection, particularly in endemic areas. Carter and Miller[1] demonstrated that the rate at which sexual differentiation occur in *Plasmodium falciparum* erythrocytic stages depends on certain environmental factors. Several other studies have reported immunological stress,[2,3] impact of host response to parasite[4-6] and chemotherapy[7] as important factors involved in the induction of gametocytogenesis.

Although some studies have reported seasonal influence on sectional opacity, gametocyte carriage and asymptomatic densities at the onset of dry or during rainy season in endemic area in Africa and Thailand,[14-19] little is known about the effect of seasonal variations on gametocyte carriage and response to chloroquine treatment in endemic area of southwest Nigeria. Such information is crucial to our understanding of the potential contribution of seasonal change to malaria transmission. Thus, in the present study, we evaluated the effect of low and high transmission seasons on gametocyte carriage and response of children to chloroquine during *P. falciparum* malaria infection in hyperendemic southwest Nigeria.

Acknowledgements

The study received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. AS was supported by a WHO/TDR Career Development Award.

Correspondence: Dr A. A. Adedeji, Department of Pharmacology, Obafemi Awolowo College of Health Sciences, Olabisi Onabanjo University, Sagamu, Nigeria. E-mail: <ahmedadel@yahoo.co.uk>.

Patients and Methods

Patients

The study took place between July 1999 and December 2002 in patients presenting at the University College Hospital in Ibadan, a hyperendemic area for malaria in south-western Nigeria.[16] Ethical clearance was provided by the local ethics committee. During the period, a series of antimalarial

drug studies were conducted to evaluate the efficacy and safety of different treatment regimens spanning the two periods of high (April–October) and low (November–March) transmission seasons known in the area. The details of the studies have been described before.[19,21] Briefly, children with symptoms compatible with acute falciparum malaria who fulfilled the following criteria were enlisted in the study: age 13 years or below, pure *P. falciparum* parasitemia greater than 2000 asexual forms/ml blood, negative urine tests for antimalarial drugs (Dill-Glazko and lignin tests), absence of concomitant illness, no evidence of severe malaria[22] and written informed consent given by parents or guardians. After enrolment and start of treatment (day 0), follow-up with clinical and parasitological evaluation was at days 1–7, and then on days 14, and when necessary, on days 21 and 28. Clinical evaluation consisted of a general clinical examination including measurement of weight, core temperature and physical examination.

Assessment of parasitemia and gametocytemia

Thick and thin blood films prepared from a finger prick were Giemsa-stained and were examined by light microscopy under an oil-immersion objective, at ×1000 magnification, by two independent assessors. Parasitemia in thick films was estimated by counting asexual parasites relative to 1000 leukocytes, or 500 asexual forms, whichever occurred first. From this figure, the parasite density was calculated assuming a leukocyte count of 6000/ml of blood. Gametocytes were also counted in thick blood films against 1000 leukocytes assuming an average leukocyte count of 6000/ml of blood.[23,24]

Evaluation of response to drug treatment

In order to evaluate the response of children to chloroquine treatment during the HTS and LTS, 25 mg/kg body weight of the drug over three days (10 mg/kg on day 1, 10 mg/kg on day 2 and 5 mg/kg on day 3) was administered to children. Response to drug treatment was assessed using World Health Organization (WHO) criteria[25] as follows: S = sensitive, clearance of parasitemia without recurrence, RI (mild resistance) = parasitemia disappears but reappears within 7–14 days; RII (moderate resistance) = decrease of parasitemia but no complete clearance from peripheral blood; RIII (severe resistance) = no pronounced decrease or increase in parasitemia at 48 h after treatment. In those with sensitive or RI response, parasite clearance time (PCT) was defined as the time elapsing from drug administration until there was no patent parasitemia for at least 72 h.

Statistical analysis

Data were analysed using version 6 of the Epi-Info software[27] and the statistical program SPSS for Windows version 10.01.[28] Proportions were compared by calculating χ^2 with Yates' correction or by Fisher exact or by Mantel Haenszel tests. Normally distributed, continuous data were compared by Student's t-tests and analysis of variance (ANOVA). Data not conforming to a normal distribution were compared by the Mann–Whitney U-test and the Kruskal–Wallis test (or by Wilcoxon rank sum test). A multiple logistic regression model was used to test the association between gametocytemia (Yes or No at presentation) and factors that were significant at univariate analysis: male gender, presence of fever, duration of illness before presentation and asexual parasitemia at presentation. The values presented below are generally means and standard deviations (SD) or standard error (SE). *p*-values of <0.05 were taken to indicate significant differences.

Results

Clinical and parasitological features at enrolment

The demographic parameters and other characteristics of the children enrolled in the study are summarised in Table 1. Of 1031 children enrolled into the studies, 693 and 338 children were recruited during the high and low transmission seasons respectively between 1996–2003. Patent gametocytemia (geometric mean 27, range 6–1344/μl) was present in 73 (10.5%) of 693 and 40 (11.8%) of 338 children at enrolment in both high and low transmission seasons, respectively. These proportions were not significantly different ($\chi^2 = 0.27$, $p = 0.6$). The parasite densities at enrolment in these children were 36 748 (Geometric mean, range 209–150 000) and 27 961 (Geometric mean, range 1116–565 333) in both high and low transmission seasons respectively ($p = 0.001$).

The response of the asexual parasitemia to drug treatments have been reported elsewhere. Factors associated with gametocytemia at enrolment during the high transmission season (HTS) are presented in Table 2. Duration of illness >3 d, and asexual parasite densities less than 10 000/ml were related to the presence of gametocytemia at enrolment. None of age, gender or fever at presentation was independent risk factor for gametocyte carriage (Table 2).

However, during low transmission seasons, gender, duration of illness >3 d, and asexual parasite densities less than 5000/ml were the independent factors associated with gametocytemia at enrolment (Table 3).

Clinical features and response to chloroquine

Of 335 children that were treated with chloroquine during the study, 165 were placed in the HTS and 165 in the LTS. The clinical features at presentation and parasitological parameters of these children

are summarized in Table 4. The clinical features were similar, although those enrolled in the LTS were significantly younger ($p=0.03$), had significantly lower presenting temperature ($p=0.03$) and lower geometric mean parasite density ($p=0.001$). Though, the fever clearance times were similar in the HTS and LTS, the parasite clearance times were significantly different ($p=0.003$). The therapeutic responses (Table 4) were similar in the two seasons. Analysis of the treatment failures showed that of the 71 that had resistance response in the HTS, 60, 6 and 5 children had RI, RII, and RIII respectively, similarly in the LTS, 52 had RI, 10 had RII and 11 had RIII responses. RIII response occur more in the LTS than HTS but the difference was not significant ($p=0.1$).

TABLE 1
Summary of demographic and other characteristics of the 1031 children enrolled in the study

Variables	Value [mean ± SD (range)]
Age (years)	5.6 ± 2.9 (0.5–12.0)
M:F	493:555
Weight (kg)	16.4 ± 4.8 (5.0–27)
Presenting body temperature (°C)	38.6 ± 1.2 (35.7–42.0)
Duration of illness (d)	3.2 ± 1.7 (1–21)
Asexual parasite density (per µl):	
Geometric mean	34063
Range	2090–2341000
No. >250000	100

Gametocytemia during treatment with chloroquine and follow up

Gametocytemia was found in 27 out of 168 and 28 out of 165 during the HTS and LTS, respectively, at enrolment. There was no difference in the geometric mean gametocyte densities (24, range 12–1344/µl, vs 26, range 6–150/µl, $p=0.3$). Gametocytemia increased significantly in densities by day 7 and 14 in children treated in the HTS when compared to the gametocyte densities obtained on these days in those treated during LTS following chloroquine treatment (Table 5). However, the cumulative gametocyte carriage by day 7 and 14 were significantly higher in the children treated with chloroquine during the LTS ($p=0.015$ and $p=0.03$) than those treated during the HTS.

Discussion

The primary purpose of the present study was to evaluate the effect of seasons in the low and high transmission period characteristic of malaria infection in Nigerian children, on gametocyte carriage, the response to oral chloroquine and gametocyte carriage following treatment. Gametocyte carriage rates may vary widely and depend on several factors. In this study, observed prevalence of malaria infection was significantly higher in the high transmission season (67.2%) than in the LTS (32.8%), but the gametocyte carriage rate was slightly higher in the latter. Such seasonal effect had been observed earlier in the same area[16]. Prompt visit to clinic and early treatment of the infection during HTS compared to

TABLE 2
Risk factors for P. falciparum gametocytemia at enrolment during the high transmission season

	Total No. of children	No. of children with gametocytemia	Crude OR (95% CI)	p value	Adjusted OR (95% CI)	p value
Age (y)						
>5	346	35	1	–	–	–
<5	347	38	1.09 (0.6–1.8)	0.8	–	–
Gender						
Male	320	36	1	–	–	–
Female	373	37	1.2 (0.7–2.0)	0.4	–	–
Parasitaemia (/µl)						
≥10000	561	51	1	–	1	–
<10000	132	21	1.89 (1.06–3.35)	0.03	1.85 (1.11–3.33)	0.03
Fever*						
Febrile	437	46	1	–	–	–
Afebrile	256	37	0.99 (0.5–1.65)	0.9	–	–
Duration of illness						
>3d	156	25	1	–	1	–
<3d	537	45	0.51 (0.31–0.9)	0.01	0.55 (0.33–1.0)	0.03

OR, odds ratio.
*Fever, axillary temperature >37.5°C.
CI, confidence interval.

TABLE 3
Risk factors for P. falciparum gametocytemia at enrolment during the low transmission seasons

	Total No. of children	No. of children with gametocytes	Crude OR (95% CI)	p value	Adjusted OR (95% CI)	P value
Age (y)						
>5	159	13	1	–	–	–
<5	179	27	0.5 (0.23–1.09)	0.7	–	–
Gender						
Male	159	28	1	–	1	–
Female	179	12	0.34 (0.15–0.72)	0.003	0.3 (0.1–0.6)	0.002
Parasitemia (/μL)						
<5000	23	8	1	–	1	–
>5000	315	32	0.21 (0.08–0.63)	0.001	0.22 (0.08–0.64)	0.005
Fever*						
Febrile	98	9	1	–	–	–
Afebrile	240	31	0.68 (0.3–1.54)	0.4	–	–
Duration of illness						
<4 d	299	30	1	–	1	–
>4 d	39	10	3.1 (1.2–7.3)	0.01	3.1 (1.2–7.3)	0.014

OR, odds ratio.
* Fever, axillary temperature >37.5°C.
CI, confidence interval.

slow response of infected individuals during LTS may be contributory. People in this setting appear to suspect malaria infection more in the rainy season once symptomatic or pyrexic. It is noteworthy that asexual parasitemia at enrolment was markedly higher in the HTS than in the LTS. The reason(s) for this is not clear from the present study. A similar observation of low parasite rate during the low transmission period had been earlier reported for the area.[16,17] It may be that the features of asexual *P. falciparum* infectivity or clinical presentation vary with season or respond to changes in the environment in such a way to favour its parasitism and propagation.

A critical evaluation of the risk factors for carriage of the sexual forms may provide some clues in respect of the above observation. In the present study, two and three independent factors were associated with gametocyte carriage in the HTS and LTS respectively. Why would male gender be a risk factor for gametocyte carriage in LTS and not in the HTS remains unclear. Testosterone and corticosteroids had been reported stimulate *P. falciparum* gametocytogenesis *in vitro*.[18] Could there be seasonal variation in the levels of sex hormones in the prepubertal male and female? This finding would require further investigation in African children.

The duration of illness longer than 4 days and reduced parasitemia found as risk factors for gametocyte carriage in the LTS contrary to the shorter duration of illness and two fold parasite density in the HTS suggest that there is delayed presentation of symptoms or possibly low degree of virulence in the circulating asexual parasites during the LTS. Smalley, *et al*.[12] had observed that longer established *P. falciparum* infections are likely to produce gametocytes. It is likely therefore that longer duration of illness before presentation in the LTS may allow sufficient time for the progression of committed asexual parasite to gametocytes.

The effect of antimalarial drugs in sexual differentiation in *P. falciparum* is still not fully understood. Certain antimalarial drugs, for example chloroquine and Pyrimethamine – sulphadoxine, have been reported contribute to gametocytogenesis *in vitro*[11] or gametocyte generation or release *in vivo*.[12,13,14] It is remarkable to note that the children in the cohorts treated with chloroquine in this study during LTS were significantly younger, had lower presenting temperature and low parasite density compared to those treated with chloroquine during the HTS. Although fever clearance times were similar, the parasite clearance times were significantly different in the two transmission seasons. The children treated during the LTS had delayed clearance of their asexual forms suggesting differing parasite behaviour and dynamics during transmission seasons. Thus use of chloroquine in children in the study area in the HTS appeared more favourable and important to reduce circulating parasite load. Despite similar therapeutic outcome and resistance rates in the two transmission periods, early resistance of RII and RIII occur in more children during the LTS.

Surprisingly, the post treatment gametocytemia and gametocyte carriage differ significantly in the two seasons compared to pretreatment gametocytemia

TABLE 4

Comparison of clinical parameters of 333 children with acute falciparum malaria at presentation and their therapeutic response following treatment with chloroquine during high and low transmission seasons

	HTS	LTS	p values
Number of patients	168	165	
Age (years):			
Mean ± SD	5.6 ± 2.8	4.9 ± 3.0	0.03
Range	0.7–13.0	0.6–12.0	
Weight (kg):			
Mean ± SD	16.0 ± 5.6	15.1 ± 5.6	0.16
Range	6.5–33.0	6.5–31.0	
Duration of symptoms (days):			
Mean ± SD	3.3 ± 1.8	3.1 ± 1.5	0.14
Range	1.0–14.0	1.0–8.0	
Body temperature (°C):			
Mean ± SD	38.5 ± 1.2	38.1 ± 1.1	0.03
Range	36.1–42.0	36.5–40.6	
Parasitemia (/µl)			
GMPD (asexual)	36748	27961	0.001
Range	2090–1560000	2116–565333	
FCT (d)			
Mean ± SD	1.5 ± 0.8	1.5 ± 0.8	0.99
Range	1–4	1–5	
PCT (d)			
Mean ± SD	2.7 ± 0.9	3.0 ± 0.9	0.003
Range	1–6	1–5	
Response			
No. Cured	97	92	0.7
No. with RI	60	52	0.4
No. with RII	6	10	0.4
No. with RIII	5	11	0.1

GMPD, geometric mean parasite density; PRR, parasite reduction ratio; FCT, fever clearance time; PCT, parasite clearance time. RI = parasitemia disappears but reappears within 7 to 14 days; RII = decrease of parasitemia but no complete clearance from peripheral blood; RIII = no pronounced decrease or increase in parasitemia at 48 h after treatment; d = day.

and gametocyte carriage that were similar. In the HTS, post treatment gametocyte intensity was high but significantly fewer children were carriers compared with low gametocyte intensity and high carriage rate in the LTS. This antimalarial drug chemotherapy may impose stress on the parasite, response to which could result in increased gametocyte production.[32,35] The higher sexual parasite density in the HTS may in addition support increased parasite burden on mosquito and probability of mosquito infection,[33,36,37] thus creating heavy burden of malaria and high transmission in the area.

TABLE 5

Comparison of gametocyte intensity at presentation and following treatment in 333 children with acute falciparum malaria during high and low transmission seasons

	HTS	LTS	p values
No. patients	168	165	
Parasitemia (per µl) on day 0			
GMPD (gametocytes)	[illegible] (n = 27)	26 (n = 28)	0.29
Range	12–134	6–150	
Parasitemia (per µl) on day 7			
GMPD (gametocytes)	[illegible] (n = 29)	27 (n = 48)	0.04
Range	12–1476	6–264	
Parasitemia (per µl) on day 14			
GMPD (gametocytes)	29 (n = 20)	18 (n = 35)	0.02
Range	12–144	6–108	

GMPD, geometric mean parasite density.

The increase resistance to chloroquine, which still remained most common, readily available, cheap and first line antimalarial drug in the study area, may be contributory to differences in the post treatment gametocytic generation or release and carriage in children. Patients with slow response to treatment are likely to carry gametocytes than those that responded rapidly.[38] Furthermore, high carriage rate in the LTS post treatment with chloroquine may also suggest a compensatory mechanism to ensure furtherance of transmission at almost same potential as in HTS relative to available transmission aids. This may find relevance in our understanding of how the parasite ensures transmission despite chemotherapy of the infection. More studies would be needed to elucidate parasite response and behaviour to other antimalarial drugs during low and high transmission seasons.

Overall a strategy that avoids the identified risk factors for gametocyte carriage in the two transmission seasons and controlled use of antimalarial drugs may reduce gametocyte prevalence and contribute to a reduction in malaria transmission.

References

1. Carter R, Miller LH. Evidence for environmental modulation of gametocytogenesis in *Plasmodium falciparum* in continuous culture. Bull WHO 1979; 57 (Suppl 1): 37–52.
2. Smalley ME, Brown J. *Plasmodium falciparum* gametocytogenesis stimulated by lymphocytes and serum

from infected Gambian children. Trans Roy Soc Trop Med Hyg 1981; 75: 316–17.
3. Ono T, Nakai T, Nakabayashi T. Induction of gametocytogenesis in *Plasmodium falciparum* by culture supernatant of hybridoma cells producing *anti-P. falciparum* antibody. Biken J 1986; 29: 77–81.
4. Mons B. Induction of sexual differentiation in malaria. Parasitol Today 1985; 1: 87–9.
5. Schneweis S, Maier WA, Seitz HM. Haemolysis of infected erythrocytes—a trigger for formation of Plasmodium gametocytes. Parasitol Res 1991; 77: 458–60.
6. Sinden RE. Gametocyte and sexual development. In: Sherman, Irwin W (ed.), Malaria: Parasite Biology, Pathogenesis and Protection. ASM Press, Washington, DC 1998.
7. Beckling AGJ, Taylor LH, Carlton J M-R, Read AF. Adaptive changes in *Plasmodium* transmission strategies following chloroquine chemotherapy. Proc Roy Soc Lond B 1997; 264: 553–59.
8. Robert V, Molez J-F, Trape J-F. Short report: gametocytes, chloroquine pressure, and the relative parasite survival advantage of resistant strains of falciparum malaria in West Africa. Am J Trop Med Hyg 1996; 55: 350–51.
9. Robert V, Awono-Ambene HP, Le Hesran J-Y, Trape J-F. Gametocytemia and infectivity to mosquitoes of patients with uncomplicated *Plasmodium falciparum* malaria attacks treated with chloroquine or sulfadoxine-pyrimethamine. Am J Trop Med Hyg 2000; 62: 210–16.
10. Sutherland CJ, Alloueche A, Curtis J, Drakeley CJ, Ord R, Duraisingh M, Greenwood BM, Warhurst DC, Targett GAT. Gambian children successfully treated with chloroquine can harbor and transmit *Plasmodium falciparum* gametocytes carrying resistant genes. Am J Trop Med Hyg 2002; 67: 575–85.
11. Hogh B, Gamage-Mendis A, Butcher GA, Thompson R, Begtrup K, Mendis C, Enosse SM, Dgedge M, Barreto J, Eling W, Sinden RE. The differing impact of chloroquine and pyrimethamine/sulfadoxine upon the infectivity of malaria species to mosquito vector. Am J Trop Med Hyg 1998; 58: 176–80.
12. Sowunmi A, Fateye BA. Asymptomatic recrudescent, chloroquine-resistant, *Plasmodium falciparum* infections in Nigerian children: clinical and parasitological characteristics and implications for transmission of drug-resistant infections. Ann Trop Med Parasitol 2003; 97: 469–79.
13. Sowunmi A, Fateye BA. *Plasmodium falciparum* gametocytemia in Nigerian children: before, during and after treatment with antimalarial drugs. Trop Med Int Health 2003; 8: 783–92.
14. Rosenberg R, Andre RG, Somchit L. Highly efficient dry season transmission of malaria in Thailand. Trans Roy Soc Trop Med Hyg 1990; 84: 22–8.
15. McElroy PD, Beier JC, Oster CN, Beadle C, Sherwood JA, Oloo AJ, Hoffman SL. Predicting outcome of malaria: correlation between rate of exposure to infected mosquitoes and level of *Plasmodium falciparum* parasitemia. Am J Trop Med Hyg 1994; 51: 523–32.
16. Molineaux LG. The Garki Project. World Health Organization, Geneva, 1980.
17. Nacher M, Carrara VI, Ashley E, McGready R, Hutagalung R, Nguen JV, Thwai KL, Looareesuwan S, Nosten F. Seasonal variation in hyperparasitemia and gametocyte carriage in patients with *Plasmodium falciparum* malaria on the Thai-Burmese border. Trans Roy Soc Trop Med Hyg 2004; 98: 322–28.
18. Salako LA, Ajayi FO, Sowunmi A, Walker O. Malaria in Nigeria: a revisit. Ann Trop Med Parasitol 1990; 84: 435–45.
19. Sowunmi A, Oduola AMJ, Ogundahunsi OAT, Salako LA. Enhancement of the antimalarial effect of chloroquine by chlorpheniramine *in vivo*. Trop Med Int Health 1998; 3: 177–83.
20. Sowunmi A. A randomized comparison of chloroquine, amodiaquine and their combination with pyrimethamine-sulfadoxine in the treatment of acute, uncomplicated, *Plasmodium falciparum* malaria in children. Ann Trop Med Parasitol 2002; 96: 227–38.
21. Sowunmi A. A randomized comparison of chloroquine and chloroquine plus ketotifen, in the treatment of acute, uncomplicated, *Plasmodium falciparum* malaria in children. Ann Trop Med Parasitol 2003; 97: 103–17.
22. World Health Organization. Severe falciparum malaria. Trans Roy Soc Trop Med Hyg 2000; 94 (Suppl. 1): S1–5.
23. Shaper AG, Lewis P. Genetic neutropenia in people of African origin. Lancet 1971; ii1021–23.
24. Ezeilo GC. Neutropenia in Africans. Trop Geog Med 1971; 23: 264–67.
25. Sowunmi A, Akindele JA, Balogun MA. Leukocyte counts in falciparum malaria in African children from an endemic area. Afr J Med Med Sci 1995; 24: 145–49.
26. World Health Organization. Chemotherapy of Malaria and Resistance to Antimalarials. Technical Report Series No. 529. Geneva: WHO, 1973.
27. Epi Info Version 6. A Word Processing Data Base and Statistics Program for Public Health on IBM-compatible Microcomputers. Atlanta, GA: Centers for Disease Control and Prevention; 1994.
28. SPSS. SPSS for Windows Release 10.0.1 (Standard Version). SPSS Inc, Chicago, IL, USA; 1999.
29. Sowunmi A. Body temperature and malaria parasitemia in rural African children. East Afr Med J 1995; 72: 40–3.
30. Maswowe SM, Peters W, Warhurst DC. Corticosteroid stimulation of the growth of *Plasmodium falciparum* gametocytes *in vitro*. Ann Trop Med Parasitol 1985; 79: 607–16.
31. Lingnau A, Margos G, Maier WA, Seitz HM. The effects of hormones on the gametocytogenesis of *Plasmodium falciparum in vitro*. Appl Parasitol 1993; 34: 153–60.
32. Smalley ME, Brown J, Basset NM. The rate of production of *Plasmodium falciparum* gametocytes during natural infections. Trans Roy Soc Trop Med Hyg 1981; 75: 318–19.
33. Buckling A, Ranford-Cartwright LC, Miles A, Read AF. Chloroquine increases *Plasmodium*

falciparum gametocytogenesis in vivo. Parasitology 1999; 118: 339–46.
34. Bucher GA. Antimalarial drugs and the mosquito transmission of *Plasmodium*. Int J Parasitol 1997; 27: 975–87.
35. Smalley ME. *Plasmodium falciparum* gametocytes: the effect of chloroquine on their development in man. Trans Roy Soc Trop Med Hyg 1977; 71: 526–29.
36. Carter R, Graves PM. Gametocytes. In: Wernsdorfer, W.H. & McGregor I. (eds). Malaria: Principles and Practice of Malariology. Vol 1. Churchill Livingstone, Edinburgh 1988; pp. 253–306.
37. Taylor LH, Read AF. Why so few transmission stages? Reproductive restraint by malaria parasite. Parasitol Today 1997; 13: 135–40.
38. Price R, Nosten F, Simpson JA, Luxemburger C, Phaipun L, ter Kuile F, van Vugt M, Chongsuphajaisiddhi T, White NJ. Risk factors for gametocyte carriage in uncomplicated falciparum malaria. Am J Trop Med Hyg 1999; 60: 1019–23.

Eur J Clin Microbiol Infect Dis (2006) 25: 126–128
DOI 10.1007/s10096-006-0083-5

BRIEF REPORT

A. A. Adedeji · B. A. Fateye · A. O. J. Amoo · A. Sowunmi

Response to chloroquine treatment in children with or without gametocytes during uncomplicated *Plasmodium falciparum* malaria

Published online: 16 February 2006

Chemotherapy still remains the most widely used approach to combat malaria infection. However, chloroquine has increasingly been failing to clear parasites in patients in endemic areas, and failure rates as high as 40 and 80% have been reported for West African and East African patients, respectively [1, 2]. This development has necessitated the development of an alternative antimalarial drug therapy. While awaiting the emergence of an alternative to chloroquine, however, efforts need to be geared towards minimizing the morbidity and mortality that may result from the continued use of chloroquine in endemic areas. One strategy that may prove useful for extending the period during which this antimalarial agent remains effective is to consider parasite host-related characteristics, such as gametocyte carriage, and the clinical response of patients to chloroquine. Gametocyte generation, host carriage, and infectivity of mosquitoes are crucial for the successful transmission of malaria infection and may contribute to the maintenance and spread of chloroquine resistance in endemic areas [1, 3]. Thus, the present study was conducted to evaluate the role played by gametocytes at the time of treatment with chloroquine and during the follow-up period on clinical outcome and resistance patterns in children with acute uncomplicated falciparum malaria.

A. A. Adedeji · B. A. Fateye · A. Sowunmi
Department of Pharmacology & Therapeutics and Institute for Medical Research and Training, University of Ibadan, Ibadan, Nigeria

A. A. Adedeji (✉)
Department of Pharmacology, Obafemi Awolowo College of Health Sciences, Olabisi Onabanjo University, Sagamu, Nigeria
e-mail: ahmedade1@yahoo.co.uk
Tel.: +234-8055135819
Fax: +234-22411843

A. O. J. Amoo
Department of Medical Microbiology and Parasitology, Obafemi Awolowo College of Health Sciences, Olabisi Onabanjo University, Sagamu, Nigeria

This study is part of an extensive, long-term study on the efficacy of antimalarial drugs carried out in Ibadan, Nigeria, from July 1996 to March 2003 [4–6]. A total of 142 children with acute uncomplicated *P. falciparum* malaria were enrolled consecutively into two groups; the first group included 71 children who had gametocytes at enrollment and/or during follow-up, and the second group included 71 patients who did not have gametocytes either at enrollment or at any point during treatment. All patients were treated with chloroquine (25 mg/kg of body weight given over a 3-day period: 10 mg/kg on days 0 and 1 and 5 mg/kg on day 2). For enrollment into the study, each child had to meet the following criteria: (a) age <13 years; (b) diagnosis of *P. falciparum* parasitemia with >1,000 asexual forms/μl; (c) negative results of urine tests (Dill–Glazko and lignin) for antimalarial drugs; and (d) no concomitant illness or evidence of severe malaria. Written informed consent was obtained from the parents or guardians of each child prior to enrollment. The study was approved by the local ethics committee.

After receiving a detailed clinical and parasitological assessment and drug administration at presentation, each child underwent clinical and parasitological examination daily on days 1–7 and on day 14. At each assessment, fingerprick blood samples were collected and used to make thick and thin smears for estimating the level of parasitemia. Gametocytemia was quantified on days 0, 3, 5, 7 and 14 using the thick blood smears prepared on those days [1]. Classifications of response to drug treatment were determined according to the criteria outlined by the World Health Organization [7]. Parasite clearance was defined as the amount of time between the start of drug administration and the absence of detectable parasitemia, which was maintained for at least 48 h. Fever clearance was defined as the amount of time from the start of drug administration until the core temperature fell to ≤37.4°C and remained so for 48 h. Data were analyzed using version 6 of the Epi-Info software (Centers for Disease Control and Prevention, Atlanta, GA, USA), and differences giving p values of <0.05 were taken as significant.

The two groups of children had similar clinical characteristics, parasite clearance times (2.9±1.1 vs. 3.0±0.8; $p=0.9$) and fever clearance times (1.6±0.9 vs. 1.4±0.7; $p=0.6$). Of the 71 children with gametocytemia during chloroquine treatment, 43 had gametocytes at presentation. These children were younger and had lower levels of parasitemia at presentation compared with 43 children from the group without gametocytes, who were selected for comparison based on consecutive enrollment (Table 1). The therapeutic response to chloroquine treatment also differed in these two groups. Children who had gametocytes at presentation had significantly shorter times to parasite and fever clearance compared with those who did not have gametocytes at presentation or during treatment (2.7±0.9 vs. 3.1±0.9; $p=0.03$ and 1.2±0.5 vs. 1.6±0.9; $p=0.01$, respectively).

An interesting finding of the study was the significant difference in fever and parasite clearance times following treatment with chloroquine in children with gametocytes compared to those without gametocytes. The reason for this difference could not be elucidated in the present study; however, it has previously been reported that children who present with gametocytes are probably carrying trophozoites that are likely to be committed to gametocyte production [8]. Unfortunately, little is known about the behavior of trophozoites committed to gametocyte production in the presence of antimalarial drugs. We are also unable to explain why the asexual forms of the parasites in our cohort of children with gametocytes at presentation appeared less virulent and were cleared from peripheral blood earlier than in the group of patients without gametocytes. While it has been reported previously that chloroquine treatment may impose considerable stress and greatly reduce the number of parasites [9], this requires further investigation.

The results of this study indicate the presence or absence of gametocytes at presentation modulates the therapeutic response of children to chloroquine significantly. Since the children without gametocytes at presentation responded to chloroquine treatment with significantly higher cure rates, it seems there was comparatively significant resistance to the drug in those children with gametocytes at enrollment. It is clear that chloroquine therapy may be more beneficial in children with malaria who lack gametocytes at the time of treatment initiation. In children with gametocytes, a combination of gametocidal drugs plus chloroquine or chloroquine in combination with an antimalarial agent active against all parasitic stages, like artemether, may be advantageous. Once a child presents with gametocytes in the peripheral blood, alternative antimalarial agents superior to chloroquine may be administered. However, more studies are needed to evaluate the effect that the presence or absence

Table 1 Comparison of clinical parameters and responses to chloroquine treatment in 86 children with acute falciparum malaria who either had or did not have gametocytes at presentation

Parameter	With gametocytemia	Without gametocytemia	p value
Number of patients	43	43	
Age (y)			
Mean±SD	5.6±3.0	6.9±2.8	0.04
Range	0.7–12.0	0.6–13.0	
Weight (kg)			
Mean±SD	16.5±6.4	18.8±6.2	0.09
Range	7.0–30.0	8.5–28.0	
Duration of symptoms (days)			
Mean±SD	3.5±2.3	3.2±1.4	0.46
Range	1.0–14.0	1.0–7.0	
Body temperature (°C)			
Mean±SD	38.4±1.2	38.5±1.2	0.69
Range	36.5–40.6	36.5–40.6	
Parasitemia (per µl)			
GMPD (actual)	13,588	21,716	0.05
Range	209–262,426	681–236,866	
FCT (days)			
Mean±SD	1.2±0.5	1.6±0.9	0.01
Range	1–3	1–4	
PCT (days)			
Mean±SD	2.7±0.9	3.1±0.9	0.03
Range	1–6	1–3	
Day 14 response			
Cured (%)	20 (46.5)	33 (76.7)	0.001
RS	20	7	
RI	3	0	
RII	0	3	
RIII			

GMPD geometric mean parasite density, FCT fever clearance time, PCT parasite clearance time, RI [illegible] within 7 to 14 days, RII decrease of parasitemia but no complete clearance from peripheral blood and [illegible] or [illegible] in [illegible] 48 h after [illegible]

of gametocytes has on the clinical response of infected children to available antimalarial drugs. These further studies should be conducted in an endemic area and could contribute to efforts towards controlling this infection.

Acknowledgements We are grateful to our clinic nurse, Mrs. M. Amao for her assistance with the study. AS is the recipient of a WHO/TDR Career Development Award.

References

1. Sowunmi A, Fateye BA (2003) *Plasmodium falciparum* gametocytaemia in Nigerian children: before, during and after treatment with antimalarial drugs. Trop Med Intern Hlth 8:783–792
2. Wolday D, Kibreab T, Bukenya D, Hodes R (1995) Sensitivity of *Plasmodium falciparum* in vivo to chloroquine and pyrimethamine-sulfadoxine in Rwandan patients in refugee camp in Zaire. Trans R Soc Trop Med Hyg 89:654–656
3. Sinden RE (1983) Sexual development of malaria parasites. Adv Parasitol 22:153–216
4. Sowunmi A, Oduola AMJ, Ogundahunsi OAT, Salako LA (1998) Enhancement of the antimalarial effect of chloroquine by chlorpheniramine in vivo. Trop Med Intern Hlth 3:177–183
5. Sowunmi A (2002) A randomized comparison of chloroquine, amodiaquine and their combination with pyrimethamine–sulfadoxine in the treatment of acute, uncomplicated, *Plasmodium falciparum* malaria in children. Ann Trop Med Parasitol 96:227–238
6. Sowunmi A (2003) A randomized comparison of chloroquine and chloroquine plus ketotifen, in the treatment of acute, uncomplicated, *Plasmodium falciparum* malaria in children. Ann Trop Med Parasitol 97:103–117
7. World Health Organization (1973) Chemotherapy of malaria and resistance to antimalarials. Technical Report Series, No. 529.WHO, Geneva
8. Bruce MC, Alano P, Duthie S, Carter R (1990) Commitment of malaria parasite *Plasmodium falciparum* to sexual and asexual development. Parasitol 100:191–200
9. Buckling A, Taylor LH, Carlton JM-R, Read AF (1997) Adaptive changes in *Plasmodium* transmission strategies following chloroquine chemotherapy. Proc R Soc Lond B 264:553–559

Tropical Medicine and International Health

VOLUME 9 NO 5 PP 606–614 MAY 2004

Open randomized study of pyrimethamine–sulphadoxine vs. pyrimethamine–sulphadoxine plus probenecid for the treatment of uncomplicated *Plasmodium falciparum* malaria in children

A. Sowunmi, F. A. Fehintola, A. A. Adedeji, G. O. Gbotosho, C. O. Falade, E. Tambo, B. A. Fateye, T. C. Happi and A. M. J. Oduola

Department of Pharmacology & Therapeutics and Institute for Medical Research and Training, University of Ibadan, Ibadan, Nigeria

Summary

BACKGROUND Increasing drug resistance in *Plasmodium falciparum* has necessitated renewed search for cheap, effective alternatives to commonly available antimalarials, chloroquine and pyrimethamine–sulphadoxine, for the treatment of malaria in Africa. Probenecid, an inhibitor of organic anion transporters and multidrug-resistance-associated proteins, can chemosensitize *P. falciparum* to pyrimethamine and sulphadoxine in vitro, but the clinical significance is unclear. We assessed the safety, treatment efficacy, and effects on gametocyte carriage of adding probenecid to pyrimethamine–sulphadoxine.

METHODS We evaluated 151 children aged 12 years or younger who had uncomplicated *P. falciparum* malaria. Patients were randomly assigned pyrimethamine–sulphadoxine (25 mg/kg of the sulphadoxine component) or pyrimethamine–sulphadoxine as above plus probenecid 20–25 mg/kg of bodyweight in two divided doses daily for 3 days. The primary endpoints were parasitological cure rates on days 14 and 28.

RESULTS Both regimens were well tolerated; no child was withdrawn because of drug intolerance. Fever (1.9 ± 1.1 vs. 2.4 ± 1.2 days, $P = 0.02$) and parasite clearance (1.3 ± 0.9 vs. 2.7 ± 1.1 days, $P = 0.04$) were significantly shorter, and the parasitological cure rate on day 14 (96.2% vs. 83.5%, $P = 0.02$) but not day 28 (79.4% vs. 72.6%, $P = 0.4$), was significantly higher in children treated with pyrimethamine–sulphadoxine–probenecid than in those treated with pyrimethamine–sulphadoxine. Gametocyte carriage was similar with both treatment regimens.

CONCLUSIONS The combination of pyrimethamine–sulphadoxine, and probenecid, at a relatively moderate dose, improved treatment efficacy but had no effect on gametocyte carriage. The pyrimethamine–sulphadoxine–probenecid combination merits further evaluation as a potential treatment for use in Nigeria.

Keywords: probenecid, pyrimethamine–sulphadoxine, malaria, children, Nigeria

Introduction

Drug resistance in *Plasmodium falciparum* to chloroquine is a major public health problem in much of sub-Saharan Africa, accounting for recent increases in malaria-related morbidity and mortality (Trape *et al.* 1998; Trape 2001), gametocyte carriage, and enhanced transmission of drug-resistant malaria in Africa (Robert *et al.* 1996, 2000; Sutherland *et al.* 2002; Drakeley *et al.* 2004; Happi *et al.* 2003; Sowunmi & Fateye 2003a,b).

As an alternative to chloroquine, pyrimethamine–sulphadoxine is widely used in sub-Saharan Africa, but resistance is rapidly emerging (Sibley *et al.* 2001). It is associated with point mutations in dihydrofolate reductase and dihydropteroate synthetase genes of the parasite (Plowe *et al.* 1997; Wang *et al.* 1997; Duraisingh *et al.* 1999), and confers survival and propagation advantages on the parasite in the population (Sowunmi & Fateye 2003b).

These developments have led to the renewed search for effective alternatives to both chloroquine and pyrimethamine–sulphadoxine, and to the use of both drugs in combination with each other, or in combination with other antimalarials with modes of action different from those of chloroquine and pyrimethamine–sulphadoxine, with the

aims of slowing the progression of resistance to these drugs and prolonging their lifespan (von Seidlein *et al.* 2000; Basco *et al.* 2002; Sowunmi 2002; Drakeley *et al.* 2004; Gasasira *et al.* 2003). It has also led to the use of chloroquine in combination with resistance modulators, e.g. chlorpheniramine (Sowunmi *et al.* 1997).

Experience with chloroquine plus chlorpheniramine for treating chloroquine-resistant infections comes from southwest Nigeria where the prevalence of chloroquine-resistant infection is 35–40% (Sowunmi *et al.* 1998a,b,c; Sowunmi 2003). A recent study has shown that probenecid, an inhibitor of organic anion transporters and multidrug-resistance-associated proteins can chemosensitize *P. falciparum* to pyrimethamine, sulphadoxine or chloroquine *in vitro* (Nzila *et al.* 2003), but the clinical significance is unclear. To date no study has examined, clinically, the usefulness of probenecid in combination with pyrimethamine–sulphadoxine for the treatment of malaria in African children. Such a study is essential for a number of reasons; it is possible that the combination, given in appropriate doses, may improve treatment efficacy. Malaria transmission may be reduced if probenecid modulates the gametocytocyte-releasing effect of pyrimethamine–sulphadoxine. It can help alter the management of paediatric cases of malaria.

Here we report the safety, antimalarial treatment efficacy, and effect on gametocyte carriage of pyrimethamine–sulphadoxine–probenecid and pyrimethamine–sulphadoxine alone in children aged 12 years or younger with acute, symptomatic, uncomplicated *P. falciparum* malaria.

Materials and methods

Study area

The study was carried out in Ibadan, southwest Nigeria from July to September 2003. In this area of hyperendemic malaria, transmission occurs all year round but is more intense during the rainy season, April to October. In the area, it is difficult, clinically, to distinguish recrudescence from re-infection 14 days after commencing antimalarial treatment, and usually antimalarial efficacy tests have been conducted for 14 rather than the customary 28 days (Ekanem *et al.* 1987; Salako *et al.* 1990). Chloroquine resistance was reported in the area in the 1980s (Ekanem 1985; Salako & Adeloumni 1987) and pyrimethamine–sulphadoxine resistance in the 1990s (Sowunmi *et al.* 1993, 1998a; Falade *et al.* 1997). Presently, chloroquine resistance reaches approximately 35–40% (Sowunmi 2003) and, pyrimethamine–sulphadoxine resistance approximately 25% in the under 5-year olds (A. Sowunmi & B. A. Fateye, unpublished data).

Patients, treatment and follow-up

Patients were eligible to join the study if they were aged 12 years or younger, had symptoms compatible with acute uncomplicated malaria, with pure *P. falciparum* parasitaemia >2000 asexual forms/μl, a temperature >37.4 °C or recent pyrexial antecedent, absence of other concomitant illness, no history of antimalarial use in the 2 weeks preceding presentation, negative urine tests for antimalarial drugs (Dill-Glazko and Lanier), and written informed consent from parents or guardians. Patients with severe malaria (WHO 2000), severe malnutrition, serious underlying disease (renal, cardiac, or hepatic), and known allergy to study drugs were excluded from the study. Ethical clearance for the study was provided by the Ethics Committee of Oyo State Ministry of Health, Ibadan, Nigeria. The disease history was recorded by asking patients or their parents when the present symptomatic period had started, and was followed by a full physical examination.

Enrolled patients were randomly assigned pyrimethamine–sulphadoxine 25 mg/kg of bodyweight of the sulphadoxine component at presentation (days 0) as pyrimethamine–sulphadoxine as above plus probenecid (Batch 2011, Industria Farmaceutica Nova Argentina, Milano, Italy); 20–25 mg/kg of bodyweight in two divided doses daily for 3 days (days 0, 1 and 2). The randomization was computer-generated and treatment codes were sealed in individual envelopes. Once enrolled, the study drugs were administered by a physician. Patient evaluation and follow-up after drug administration was performed by another physician blinded to the drug treatment. All drugs were given orally; except the second daily dose of probenecid, all drugs were administered in the clinic, and all patients waited for at least 3 h after drug administration to ensure that the drug was not vomited. If it was, the patient was excluded from the study. If necessary, patients were provided with antipyretics (paracetamol tablets, 10–15 mg/kg every 8 h for 24–48 h). The study nurse obtained thick and thin blood films from each child as soon as they came to the clinic. The slides were carefully labelled with the patients' codes and were air dried before being stained.

Follow up with clinical and parasitological evaluation was performed every day for 7 days (days 1–7) and then on days 14, 21 and 28. Thick and thin blood films prepared from a finger prick were Giemsa stained and were examined by light microscopy under an oil-immersion lens, at 1000× magnification, by two independent assessors who were blinded to the treatment of the patient. Parasitaemia (asexual or sexual forms) in thick films was estimated by counting asexual or sexual forms relative to 1000 leucocytes or 500 asexual or sexual forms, whichever occurred

fast. From this figure, the parasite density was calculated assuming a leucocyte count of 6000/μl of blood.

Routine haematological (haematocrit) and biochemical tests (concentrations of alanine aminotransferase, aspartate aminotransferase, bilirubin, and creatinine) were performed in 54 randomly selected children, pre-treatment and on day 14 after treatment. Blood was spotted on filter papers on days 0, 3, 7, 14, 21 and 28, in all patients, and at the time of treatment failures for parasite genotyping. Parasite genotyping will be reported elsewhere.

Response to drug treatment was assessed using WHO (1973) criteria, as follows: S, sensitive, clearance of parasitaemia without recurrence; RI (mild resistance), parasitaemia disappear but reappears within 7–14 days; RII (moderate resistance), decrease of parasitaemia but no complete clearance from peripheral blood; RIII (severe resistance), no pronounced decrease or increase in parasitaemia at 48 h after treatment. In those with sensitive or RI response, parasite clearance time was defined as the time elapsing between drug administration and absence of detectable parasitaemia for at least 48 h. Fever clearance time was defined as the time from drug administration until the core temperature fell to or below 37.4 °C and remained so for 48 h. Cure rates were defined as the percentages of patients whose asexual parasitaemia cleared from peripheral blood and who were free of patent asexual parasitaemia on days 14, 21 and 28 of follow-up.

Re-treatment of drug treatment failures

In patients who failed treatment (within 14 days), the codes were broken, and if the patient was initially treated with pyrimethamine–sulphadoxine, she or he was re-treated with pyrimethamine–sulphadoxine–probenecid and followed up for another 14–28 days. Those failing initial treatment with pyrimethamine–sulphadoxine–probenecid were re-treated with oral quinine 10 mg/kg over 3 days and followed up for another 14–28 days. Patients were re-treated whenever they became symptomatic (usually 14–21 days after initial enrollment). Patients with profound clinical (hyperpyrexia, oral fluid intolerance) and parasitological deterioration during follow-up were treated with artemether (3.6 mg/kg over 5 days) and were regarded as treatment failure.

Study size and statistical analysis

Sample size was calculated so that the study would be able to detect a difference of 22% in the parasitological failure rate between pyrimethamine–sulphadoxine–probenecid and pyrimethamine–sulphadoxine groups, with 95% power at a 5% significance level (it was assumed that 75% of those given pyrimethamine–sulphadoxine, based on the current cure rate in the under 5-year olds, and 97% of those given pyrimethamine–sulphadoxine–probenecid would be cured on first treatment). At least 71 children were needed in each treatment arm. Data were analysed using version 6 of the Epi-Info software (Anonymous 1994). Variables considered in the analysis were related to the densities of *P. falciparum* gametocytes and trophozoites. Proportions were compared by calculating chi-squared value with Yates' correction or by Fisher exact or by Mantel–Haenszel tests. Normally distributed, continuous data were compared by Student's *t*-tests and analysis of variance (ANOVA). Data not conforming to a normal distribution were compared by the Mann–Whitney *U* tests and the Kruskal–Wallis tests (or by Wilcoxon ranked sum test). All tests of significance, except where specifically indicated, were two tailed. *P*-values of <0.05 were indicated significant differences. Data were edited serially using the patients codes and were only analysed at the end of the study.

Results

Patients' characteristics

One hundred and fifty-three children were enrolled, 79 were treated with pyrimethamine–sulphadoxine–probenecid and 74 with pyrimethamine–sulphadoxine. Two children, one from each of the treatment arms, were lost to follow-up after day 7 because of potential re-location. These children were excluded from data analysis. Figure 1 shows the trial profile. Overall results are for 151 children. The demographic and clinical characteristics of patients at enrollment are shown in Table 1. These characteristics were similar in the two treatment arms, but the duration of illness at presentation was significantly longer in those treated with pyrimethamine–sulphadoxine–probenecid ($P = 0.04$).

Fever and parasite clearance, and gametocyte carriage

One hundred and seven children were febrile at enrollment, 57 in the pyrimethamine–sulphadoxine–probenecid and 50 in the pyrimethamine–sulphadoxine group. By day 2, fever cleared in 42 and 26 children, respectively. There was a significant difference in the proportion of patients whose fever cleared by day 2 [$\chi^2 = 4.5$, $P = 0.03$, odd's ratio (OR) = 0.47, 95% confidence interval (CI) = 0.23–0.96)]. Overall, fever clearance was significantly quicker in those treated with pyrimethamine–sulphadoxine–probenecid (1.9 ± 1.1 vs. 2.4 ± 1.2 days, $P = 0.02$) (Table 2).

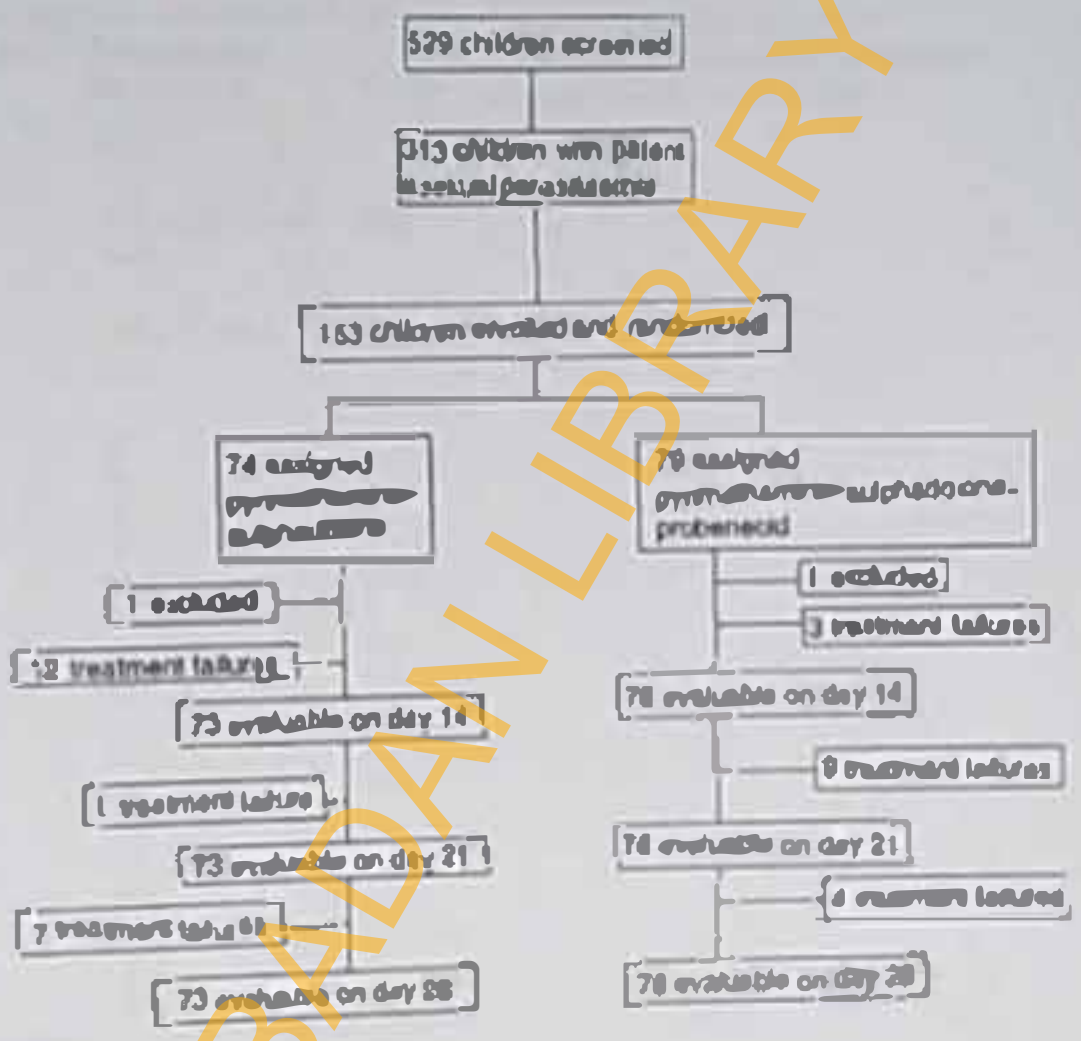


Figure 1 Trial profile.

Table 1 Demographic and clinical characteristics of patients at enrolment

	Pyrimethamine-sulphadoxine-probenecid	Pyrimethamine-sulphadoxine
No. of patients	79	74
Male/female	41/37	38/36
Age (years)		
Mean ± SD	6.3 ± 2.9	5.9 ± 2.9
Range	1.5–12	0.8–11.1
< 1/5 years	20	25
Weight (kg)		
Mean ± SD	17.8 ± 6.1	17.2 ± 5.6
Range	9–35	8–30
Duration of illness (days)		
Mean ± SD	3.5 ± 1.7	3.0 ± 1.3
Range	1–10	1–9
Temperature (°C)		
Mean ± SD	38.1 ± 1.0	38.4 ± 1.2
Range	35.6–40.1	36.1–40.1
Parasite count (/µl)		
Geometric mean	44 792	57 745
Range	2010–1 331 000	2020–1 251 000
Haemoglobin (g/dl)		
Mean ± SD	11.6 ± 1.5	11.1 ± 1.4
Range	8.6–15	8.2–14
< 8 g/dl	0	1

Compared with pyrimethamine-sulphadoxine, pyrimethamine-sulphadoxine-probenecid substantially accelerated the clearance of parasitaemia. By day 2, 53 and 37 children in the pyrimethamine-sulphadoxine-probenecid and pyrimethamine-sulphadoxine treatment arms, respectively, had their parasitaemia cleared. The difference in this proportion was significant ($\chi^2 = 3.98$, $P = 0.04$, OR = 2.06, 95% CI = 1.01–4.22). Overall parasite clearance was significantly quicker in those treated with pyrimethamine-sulphadoxine-probenecid (2.3 ± 0.9 vs. 2.7 ± 1.1 days, $P = 0.04$) (Table 2). The cure rate on day 14 (96.2% vs. 83.5%, $\chi^2 = 3.3$, $P = 0.07$, OR = 4.92, 95% CI = 1.24–28.0) but not day 28 (79.4% vs. 72.6%, $\chi^2 = 0.6$, $P = 0.4$, OR = 1.46, 95% CI = 0.64–3.35), was significantly higher in children treated with pyrimethamine-sulphadoxine-probenecid than in those treated with pyrimethamine-sulphadoxine. Response to both treatment regimens was not related to age: one child and two children from the 2.9 and 4.9 < 5 and 25-year olds, respectively, treated with pyrimethamine-sulphadoxine-probenecid failed treatment by day 14 ($P = 1.0$, by Fisher exact test, OR = 0.34, 95% CI = 0.01–16.8). Similarly, four and eight children from the 25 and 49 < 5- and 25-year olds, respectively, treated with pyrimethamine-sulphadoxine failed treatment by

Table 2 Therapeutic responses to pyrimethamine–sulphadoxine–probenecid or pyrimethamine–sulphadoxine

	Pyrimethamine–sulphadoxine–probenecid	Pyrimethamine–sulphadoxine	P-value
No. of patients	74	73	–
Fever clearance time (days)			
Mean ± SD	1.9 ± 1.1 (n = 67)	2.4 ± 1.2 (n = 60)	0.02
Range	1–5	1–7	
Parasite clearance time (days)			
Mean ± SD	2.3 ± 0.9 (n = 76)	2.7 ± 1.1 (n = 71)	0.04
Range	1–5	1–6	
Day 14 response			
No. cured	71	61	
No. RI	1	10	
No. RII	2	1	
No. RIII	0	1	
Cure rate (%)	96.2	83.5	0.01
Day 28 response			
No. cured	66	60	
No. RI (cumulative)	10	11	
No. RII	2	1	
No. RIII	0	1	
Cure rate (%)	84.6	82.2	0.7
Day 28 response			
No. cured	62	53	
No. RI (cumulative)	14	18	
No. RII	2	1	
No. RIII	0	1	
Cure rate (%)	79.4	71.6	0.4

day 14 ($P = 1.0$, by Fisher exact test, OR = 0.98, 95% CI = 0.19–4.18).

Gametocyte carriage in those who did not have gametocytaemia at enrolment ($n = 73$ and 72 in the pyrimethamine–sulphadoxine–probenecid and pyrimethamine–sulphadoxine, respectively) was similar on days 7 (20/73 (43.8%) vs. 31/72 (43%), $\chi^2 = 0.01$, $P = 0.9$, OR = 1.3, 95% CI = 0.51–2.10) and 14 (16/73 (21.9%) vs. 21/72 (29.1%), $\chi^2 = 0.66$, $P = 0.4$, OR = 0.68, 95% CI = 0.30–1.54) with both regimens.

Response to pyrimethamine–sulphadoxine–probenecid of children with pyrimethamine–sulphadoxine-treatment failures

Seven of 12 children who had recrudescence or re-infection of parasitaemia within 14 days of initial treatment with pyrimethamine–sulphadoxine were retreated with pyrimethamine–sulphadoxine–probenecid. The therapeutic response of these children followed up to day 28 is presented in Table 3. Parasitaemia and fever cleared within 2–4 days of treatment with pyrimethamine–sulphadoxine–probenecid. The child with RIII

response to pyrimethamine–sulphadoxine during initial treatment had an RI response following re-treatment with pyrimethamine–sulphadoxine–probenecid. The cure rates on days 14 and 28 were 86% and 72%, respectively. None of the three children who failed treatment with pyrimethamine–sulphadoxine–probenecid on or before day 14 (Table 2) and who were subsequently re-treated with amodiaquine failed treatment during a 28-day follow-up period. In these children fever and parasitaemia cleared within 2–3 days of initiation of amodiaquine therapy.

Adverse events

Pyrimethamine–sulphadoxine–probenecid and pyrimethamine–sulphadoxine were well tolerated; no child was withdrawn because of drug intolerance. Symptoms reported within the first week and during follow-up were similar (Table 4). However, vomiting was more frequently reported by those treated with pyrimethamine–sulphadoxine. None of the seven children who failed initial treatment with pyrimethamine–sulphadoxine and were re-treated with pyrimethamine–sulphadoxine–probenecid reported adverse symptoms.

Table 3 Clinical and parasitological parameters of the seven children with *Plasmodium falciparum* malaria who had reappearance of or no clearance of parasitaemia following initial treatment with pyrimethamine–sulphadoxine and were subsequently treated with pyrimethamine–sulphadoxine–probenecid

	Pyrimethamine–sulphadoxine	Pyrimethamine–sulphadoxine–probenecid	P-value
No. of patients	7	7	–
Age (years)			
Mean ± SD	6.8 ± 2.6		
Range	3.3–11.5		
Weight (kg)			
Mean ± SD	19.9 ± 3.4	20.1 ± 3.8	0.5
Range	13–26	15–27.5	
Temperature (°C)			
Mean ± SD	38.5 ± 1.3	37.8 ± 1.4	0.1
Range	37.0–40.3	36.0–39.1	
Parasite count (/µl)			
Geometric mean	47 835	8136	0.01
Range	2020–155 500	714–37 621	
Fever clearance time (days)			
Mean ± SD	2.1 ± 1.3	1.3 ± 0.4	0.35
Range	1–4	1–3	
Parasite clearance time (days)			
Mean ± SD	3.6 ± 1.0	2.8 ± 0.9	0.26
Range	2–5	3–4	
Day 14 response			0.001
No. cured	0	6	
No. RI	6	1	
No. RII	1	0	
No. RIII	0	0	
Cure rate (%)	0	86	

Table 4 Adverse drug reactions reported during the study

	Pyrimethamine–sulphadoxine–probenecid	Pyrimethamine–
No. of children enrolled	78	73
[illegible]	0	0
Vomiting	1	1
Abdominal pain	5	1
Diarrhoea	0	1
Anorexia	0	4*
Drowsiness	0	0
Cough	4	7
Headache	3	3
% weight gain (21.0 [illegible])	38 (n = 66)	32 (n = 60)

* Significant statistical difference, $P = 0.05$

Haematological and biochemical parameters

[illegible] 25% at enrolment [illegible] eight and [illegible] children in pyrimethamine–sulphadoxine–probenecid and pyrimethamine–sulphadoxine groups, [illegible] day 7 in four and [illegible] children respectively, [illegible] biochemical and other [illegible] before and after treatment [illegible] was present in 10 and 12 children in pyrimethamine–sulphadoxine–probenecid and pyrimethamine–sulphadoxine groups, respectively, at enrollment, but was not seen on day 14 in any child.

Discussion

Given the increasing prevalence and intensities of resistant infections to pyrimethamine–sulphadoxine in much of sub-Saharan Africa (Falade et al. 1997; Sowunmi et al.

1998a; van Dillen et al. 1999; Omar et al. 2001; Sibley et al. 2001) and the tendency for the drug to increase gametocyte carriage after its use for the treatment of malaria (Robert et al. 2000; von Seidlein et al. 2001; Sowunmi & Fateye 2003a,b), there is a need to review chemotherapeutic strategies based on the use of this drug alone. The results of the present study indicate that probenecid, an inhibitor of organic anion transporters and multiresistance-associated proteins (Borst et al. 2000), and a chemosensitizer of P. falciparum in vitro to antifolate agents (Nzila et al. 2003), enhances the antimalarial effect of pyrimethamine–sulphadoxine in vivo in children with uncomplicated falciparum infections. This is the first report of the enhancement of the antimalarial activity of an antifolate agent by probenecid in humans.

The relatively accelerated clearance of fever and parasitaemia produced a cure rate of 96% by day 14 after treatment with pyrimethamine–sulphadoxine–probenecid. Interestingly, only one of the three children who failed treatment was ≤5 years old. Evaluation of the acetylation status of the failures would have been helpful. Longer duration of illness (>2–3 days) is associated with increased risk of gametocyte carriage in uncomplicated falciparum malaria (Price et al. 1999). However, despite a longer duration of illness before presentation in the pyrimethamine–sulphadoxine–probenecid treated children, gametocyte carriage before and following treatment was similar to those of pyrimethamine–sulphadoxine treated children. The similar gametocyte carriage following treatment with both regimens indicates that the use of pyrimethamine–sulphadoxine–probenecid may not overtly discourage the transmission of gametocytes arising from drug-sensitive or resistant infections.

Virtually all of the pyrimethamine–sulphadoxine treatment failures were cured of their infections when they were re-treated with pyrimethamine–[illegible]. Overall, this indicates a beneficial effect of probenecid. However, this beneficial effect may also be due to additional increases in drug levels arising from repeated administration of pyrimethamine–sulphadoxine. Although no untoward effect was observed following re-treatment, caution is required with this step, as it may increase the chances of adverse drug reactions to pyrimethamine–sulphadoxine.

The drugs used were well tolerated. The most frequently reported adverse reactions were of gastrointestinal origin, and most were indistinguishable from the symptoms of [illegible] malaria, and the drugs evaluated [illegible]. It is possible that the significantly reduced reporting of [illegible] by those treated with pyrimethamine–sulphadoxine–probenecid was related to the accelerated clearance of fever and parasitaemia. Both probenecid and sulphadoxine can also induce haemolysis in glucose 6 phosphate dehydrogenase (G6PD)-deficient subjects, but no child, following treatment, reported features suggestive of drug-induced haemolytic anaemia.

It remains unclear exactly how probenecid enhanced the antimalarial effect of pyrimethamine–sulphadoxine in the cohort of children studied. Probenecid can reduce folate uptake by P. falciparum in vitro (Nzila et al. 2003), in addition to increasing plasma sulphonamides concentrations by reducing renal tubular secretion of the latter. Both of these actions are independent of parasite sensitivity status to pyrimethamine–sulphadoxine. It is possible that following treatment with pyrimethamine–sulphadoxine–probenecid, sulphadoxine concentrations were significantly higher than in those treated with pyrimethamine–sulphadoxine alone, but drug levels were not measured. Probenecid can also reverse resistance in cancer cells to methotrexate (Hooijberg et al. 1999) and resistance in P. falciparum to chloroquine in vitro (Nzila et al. 2003), by inhibiting the multi-drug resistance associated proteins. Inhibition of the multi-drug resistance associated proteins is an unlikely mechanism of the enhancement of the antimalarial effect of pyrimethamine–sulphadoxine by probenecid as resistance to pyrimethamine–sulphadoxine is associated with mutations in the dihydropteroate synthetase and dihydrofolate reductase, and not mutations in the pfmdr1 gene of the parasite (Duraisingh et al. 1997; Wang et al. 1997; Diourté et al. 1999).

There are justifications for our dosing regimen: the relatively moderate dose was based on the dose used to retard tubular secretion of penicillin in children – a convenient starting point as the drug has not been previously co-administered with pyrimethamine–sulphadoxine for the treatment of malaria in children; the 3-day dosing regimen is practicable, and compliance is more likely than if it were for longer periods. Certainly pharmacokinetic and pharmacodynamic studies are required before optimal dosing regimens can be achieved. There are potential clinical applications of our findings. If at moderate doses probenecid enhances the antimalarial efficacy of pyrimethamine–sulphadoxine, it follows that as resistance increases to pyrimethamine–sulphadoxine, higher doses of probenecid may be effective, as it is possible that enhancement may be dose related.

Acknowledgements

The study was supported by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. We are grateful to our clinic nurse, Mrs Moji Aman. AS is a recipient of a WHO/TDR Career Development Award.

References

Anonymous (1994) Epi Info Version 6. A Word Processing Data Base and Statistics Program for Public Health on IBM-compatible Microcomputers. Centers for Disease Control and Prevention, Atlanta, GA.

Basco LK, Same-Ekobo A, Ngane VF et al. (2002) Therapeutic efficacy of sulfadoxine–pyrimethamine, amodiaquine and the sulfadoxine–pyrimethamine–amodiaquine combination against uncomplicated Plasmodium falciparum malaria in young children in Cameroon. Bulletin of the World Health Organization 80, 538–545.

Borst P, Evers R, Kool M & Wijnholds J (2000) A family of drug transporters: the multidrug resistance-associated proteins. Journal of the National Cancer Institute 92, 1295–1302.

van Dillen J, Custers M, Wensink A et al. (1999) A comparison of amodiaquine and sulfadoxine–pyrimethamine as first-line treatment of falciparum malaria in Kenya. Transactions of the Royal Society of Tropical Medicine and Hygiene 93, 185–188.

Diourte Y, Djimde A, Doumbo OK et al. (1999) Pyrimethamine–sulfadoxine efficacy and selection for mutations in Plasmodium falciparum dihydrofolate reductase and dihydropteroate synthase in Mali. American Journal of Tropical Medicine and Hygiene 60, 475–478.

Drakeley CJ, Jawara M, Targett GAT et al. (2004) Addition of artesunate to chloroquine for the treatment of Plasmodium falciparum malaria in Gambian children causes a significant but short-lived reduction in infectiousness for mosquitoes. Tropical Medicine and International Health 9, 53–61.

Duraisingh MT, Drakeley CJ, Muller O et al. (1997) Evidence for selection for the tyrosine-86 allele of the pfmdr 1 gene of Plasmodium falciparum by chloroquine and amodiaquine. Parasitology 114, 205–211.

Ekanem OJ (1985) Plasmodium falciparum infection not responding to chloroquine. Transactions of the Royal Society of Tropical Medicine and Hygiene 79, 141–142.

Ekanem OJ, Weisfeld JS, Salako LA et al. (1990) Sensitivity of Plasmodium falciparum to chloroquine and sulfadoxine–pyrimethamine in Nigerian children. Bulletin of the World Health Organization 68, 45–52.

Falade CO, Salako LA, Sowunmi A, Oduola AMJ & Larcier P (1997) Comparative efficacy of halofantrine, chloroquine and sulfadoxine–pyrimethamine for treatment of acute uncomplicated falciparum malaria in Nigerian children. Transactions of the Royal Society of Tropical Medicine and Hygiene 91, 58–62.

Gasasira AF, Dorsey G, Nzarubara B et al. (2003) Comparative efficacy of aminoquinoline–antifolate combinations for the treatment of uncomplicated falciparum malaria in Kampala, Uganda. American Journal of Tropical Medicine and Hygiene 68, 127–132.

Happi CT, Thomas SM, Gbotosho GO et al. (2003) Point mutations in the pfcrt and pfmdr-1 genes of Plasmodium falciparum and clinical response to chloroquine, among malaria patients from Nigeria. Annals of Tropical Medicine and Parasitology 97, 439–451.

Hooijberg JH, Broxterman HJ, Kool M et al. (1999) Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2. Cancer Research 59, 2532–2535.

Nzila A, Mberu E, Bray P et al. (2003) Chemosensitization of Plasmodium falciparum by probenecid in vitro. Antimicrobial Agents and Chemotherapy 47, 2108–2112.

Omar SA, Adagu IS, Gump DW, Ndaru NP & Warhurst DC (2001) Plasmodium falciparum in Kenya: high prevalence of drug-resistance-associated polymorphisms in hospital admissions with severe malaria in an epidemic area. Annals of Tropical Medicine and Parasitology 95, 661–669.

Plowe CV, Cortese JF, Djimde A et al. (1997) Mutations in Plasmodium falciparum dihydrofolate reductase and dihydropteroate synthase and epidemiologic patterns of pyrimethamine–sulfadoxine use and resistance. Journal of Infectious Diseases 176, 1590–1596.

Price R, Nosten F, Simpson JA et al. (1999) Risk factors for gametocyte carriage in uncomplicated falciparum malaria. American Journal of Tropical Medicine and Hygiene 60, 1019–1023.

Robert V, Molez JF & Trape JF (1996) Short report: Gametocytes, chloroquine pressure, and the relative parasite survival advantage of resistant strains of falciparum malaria in West Africa. American Journal of Tropical Medicine and Hygiene 55, 350–351.

Robert V, Awono-Ambene HP, Le Hesran JY & Trape JF (2000) Gametocytemia and infectivity to mosquitoes of patients with uncomplicated Plasmodium falciparum malaria attacks treated with chloroquine or sulfadoxine plus pyrimethamine. American Journal of Tropical Medicine and Hygiene 62, 210–216.

Salako LA & Aderounmu AF (1987) In vitro chloroquine and mefloquine-resistant Plasmodium falciparum in Nigeria. Lancet i, 572–573.

Salako LA, Ajayi FO, Sowunmi A & Walker O (1990) Malaria in Nigeria: a revisit. Annals of Tropical Medicine and Parasitology 84, 435–445.

von Seidlein L, Milligan P, Pinder M et al. (2000) Efficacy of artesunate plus pyrimethamine–sulphadoxine for uncomplicated malaria in Gambian children. Lancet 355, 352–357.

von Seidlein L, Drakeley C, Greenwood B, Walraven G & Targett G (2001) Risk factors for gametocyte carriage in Gambian children. American Journal of Tropical Medicine and Hygiene 65, 523–526.

Sibley CH, Hyde JE, Sims PF et al. (2001) Pyrimethamine–sulfadoxine resistance in Plasmodium falciparum: what next? Trends in Parasitology 17, 582–588.

Sowunmi A (2002) A randomized comparison of chloroquine, amodiaquine and their combination with pyrimethamine–sulfadoxine in the treatment of acute, uncomplicated, Plasmodium falciparum malaria in children. Annals of Tropical Medicine and Parasitology 96, 227–238.

Sowunmi A (2003) A randomized comparison of chloroquine and chloroquine plus ketotifen in the treatment of acute, uncomplicated, Plasmodium falciparum malaria in children. Annals of Tropical Medicine and Parasitology 97, 103–117.

Sowunmi A & Fateye BA (2003a) Asymptomatic, recrudescent, chloroquine-resistant *Plasmodium falciparum* infections in Nigerian children: clinical and parasitological characteristics and implications for transmission of drug resistant infections. *Annals of Tropical Medicine and Parasitology* 97, 469–479.

Sowunmi A & Fateye BA (2003b) *Plasmodium falciparum* gametocytaemia in Nigerian children: before, during and after treatment with antimalarial drugs. *Tropical Medicine and International Health* 8, 783–792.

Sowunmi A, Oduola AMJ, Salako LA *et al.* (1993) Intramuscular sulphadoxine–pyrimethamine in chloroquine-resistant *Plasmodium falciparum* malaria during pregnancy. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 84, 672.

Sowunmi A, Oduola AMJ, Ogundahunsi O *et al.* (1997) Enhanced efficacy of chloroquine/chlorpheniramine combination in acute uncomplicated falciparum malaria in children. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 91, 63–67.

Sowunmi A, Oduola AMJ, Ogundahunsi OAT & Salako LA (1995a) Comparative efficacy of chloroquine plus chlorpheniramine and pyrimethamine–sulphadoxine in acute uncomplicated falciparum malaria in Nigerian children. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 89, 77–81.

Sowunmi A, Oduola AMJ, Ogundahunsi OAT & Salako LA (1995b) Enhancement of the antimalarial effect of chloroquine by chlorpheniramine in vivo. *Tropical Medicine and International Health* 1, 177–183.

Sowunmi A, Fehintola FA, Ogundahunsi OAT & Oduola AMJ (1998a) Comparative efficacy of chloroquine plus chlorpheniramine and halofantrine in acute uncomplicated falciparum malaria in Nigerian children. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 92, 441–445.

Sutherland CJ, Alloueche A, Curtis J *et al.* (2002) Gambian children successfully treated with chloroquine can harbour and transmit *Plasmodium falciparum* gametocytes carrying resistance genes. *American Journal of Tropical Medicine and Hygiene* 67, 374–381.

Trape JF (2001) The public health impact of chloroquine resistance in Africa. *American Journal of Tropical Medicine and Hygiene* 64 (Suppl 1–2), 12–17.

Trape J F, Pison G, Preziosi M P *et al.* (1998) Impact of chloroquine resistance on malaria mortality. *Life Sciences* 321, 689–697.

Wang P, Lee CS, Bayoumi R *et al.* (1997) Resistance to antifolates in *Plasmodium falciparum* monitored by sequence analysis of dihydropteroate synthetase and dihydrofolate reductase alleles in a large number of field samples of diverse origin. *Molecular and Biochemical Parasitology* 89, 161–177.

World Health Organization (WHO) (1973) *Chemotherapy of Malaria and Resistance to Antimalarials.* WHO Technical Report Series No. 529, Geneva.

World Health Organization (2000) Severe falciparum malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 94 (Suppl 1).

Authors

A. Sowunmi (corresponding author), F. A. Fehintola, A. A. Adedeji, G. O. Gbotosho, C. O. Falade, E. Tambo, B. A. Fateye, T. C. Happi and A. M. J. Oduola, Department of Clinical Pharmacology, University College Hospital, Ibadan, Nigeria.
Tel.: +234-2 2412101; Fax: +234-2-2411843; E-mail: malariaiha@alpha.linkserve.com

Annals of Tropical Medicine & Parasitology, Vol. 98, No. 8, 873–878 (2004)

SHORT COMMUNICATION

Comparative effects of pyrimethamine–sulfadoxine, with and without probenecid, on *Plasmodium falciparum* gametocytes in children with acute, uncomplicated malaria

The increasing spread of *Plasmodium falciparum* resistant to pyrimethamine–sulfadoxine (PS), the first- or second-line treatment of malaria in many endemic countries in Africa (Sibley *et al.*, 2001), has led to a renewed search for cheap, effective alternatives to PS, and to renewed efforts to prolong the clinical utility of this drug combination in Africa (Nzila *et al.*, 2003). The treatment of acute, *P. falciparum* malaria with PS is often associated with drug-induced increases in the frequency of gametocyte carriage and the level of gametocytaemia (Sowunmi and Fateye, 2003a, b). When PS is used in combination with other antimalarial drugs, particularly with amodiaquine or the artemisinin derivatives, however, these unwelcome changes may be considerably reduced (Sowunmi, 2002; Sowunmi and Fateye, 2003a).

Probenecid, an inhibitor of organic anion transporters and multi-resistance-associated proteins, can chemosensitize *P. falciparum* to pyrimethamine and sulfadoxine, both in vitro (Nzila *et al.*, 2003) and, at least in Nigerian children treated with PS, in vivo (Sowunmi *et al.*, 2004). The effects of probenecid on the frequency of gametocyte carriage, level of gametocytaemia, and temporal changes in gametocyte sex ratios, when added to PS for the treatment of malaria in children, are, however, unknown. The aims of the present study were (1) to determine the effects, on the frequency of gametocyte carriage and the level of gametocytaemia, of the addition of probenecid to PS (PSP) and (2) to follow the temporal changes in gametocytaemia and gametocyte sex ratios in children treated with PSP.

The study took place in Ibadan, a hyperendemic area for malaria in south-western Nigeria, in July–September 2003. Overall, 151 children presenting with acute, uncomplicated, *P. falciparum* malaria were randomized to receive a single treatment with PS alone — the PS was given orally at presentation (day 0) as 25 mg of the sulfadoxine component/kg — or the same dose of PS plus probenecid (20–25 mg/kg.day given orally, in two divided doses, on each of days 0–2). The study protocol was approved by the local ethics committee. To be enrolled on the study, a child had to be aged ≤12 years, have a pure *P. falciparum* parasitaemia of >2000 asexual forms/µl blood, give a negative result in (Dill–Clarke and ligam) urine test for antimalarial drugs, have no concomitant illness or evidence of severe malaria, and have the written informed consent of his or her parents or guardians.

After detailed clinical and parasitological assessment and drug administration at presentation, each child was checked clinically and parasitologically on each of days 1–7 and 14. Fingerprick samples of blood, collected on days 0–7 and 14, were used to make thin and thick smears so that the levels of parasitaemia could be estimated. Gametocytaemia was quantified on days 0, 3, 5, 7 and 14, using the thick blood smears prepared on those days (Sowunmi and Fateye, 2003b). If the level of gametocytaemia was at least 10 sexual forms/µl, the gametocytes were typed as described by Carter and Graves (1988)

DOI: 10.1179/000349804225021244

and Robert *et al.* (1996). The time elapsing from first drug treatment until a sex ratio of 1 was achieved (SR1) was calculated for each patient, from a plot of sex ratio *v.* time, by computer extrapolation. The data from a patient were excluded, from the exploration of the disposition kinetics of gametocytaemia, if gametocyte sex ratios had not been estimated for that patient at least three times after SR1.

Gametocyte kinetic parameters were estimated, from the levels of the micro- and macro-gametocytaemias, by a non-compartmental method, generally as previously described (Sowunmi and Fateye, 2003*b*). Data were analysed using version 6 of the Epi-Info software (Anon., 1994), and differences giving *P* values of ≤ 0.05 were taken as significant.

Of the 159 children enrolled, 73 (39 treated with PSP and 34 with PS) were found gametocytaemic at least once during the study. Although the enrolment characteristics for the PSP and PS groups were similar, mean (S.D.) fever-clearance times [1.9 (0.8) days, with a range of 1–4, *v.* 2.5 (1.0) days, with a range of 1–7; $P=0.009$] and parasite-clearance times [2.2 (0.8) days, with a range of 1–4, *v.* 2.7 (1.0), with a range of 1–5; $P=0.001$] were significantly faster, and the frequency of parasitological cure on day 14 (37 of 39 *v.* 26 of 34 children; $P=0.02$) was significantly higher in those treated with PSP than in those given PS.

The frequency of gametocyte carriage was significantly higher on each of days 7 and 14 than on day 0, both in the PSP-treated patients — five children on day 0 *v.* 32 on day 7 ($P=0.000001$) and 16 children on day 14 ($P=0.01$) — and the PS-treated — one child on day 0 *v.* 31 on day 7 ($P=0.000001$) and 22 on day 14 ($P=0.000003$). Similarly, the levels of gametocytaemia were significantly higher on each of days 3, 5, 7 and 14 than on day 0, both in the PSP-treated children ($P=0.007$) and the PS-treated ($P=0.0001$). On days 0, 3, 5, 7 and 14, the geometric mean levels of gametocytaemia (gametocytes/μl) in the PSP-treated gametocytaemics, for example, were 17 (range = 12–365; $N=5$), 32 (range = 24–36; $N=3$), 33 (range = 24–48; $N=9$), 63 (range = 12–960; $N=32$) and 44 (range = 12–216; $N=16$), respectively. The corresponding values for the PS-treated subjects were 12 ($N=1$), 50 (range = 36–72; $N=3$), 67 (range = 48–84; $N=5$), 41 (range = 12–687; $N=31$) and 30 (range = 12–84; $N=22$). The mean level of gametocytaemia was, however, significantly higher on day 5 in the PS-treated subjects than in the PSP-treated ($P=0.004$). Two of the children treated with PSP and one of those given PS were found to be gametocyte carriers every time they were checked.

Sex could be determined for > 90% of all gametocytes. At presentation the overall sex ratio was male-biased — with a mean of 59% (range = 30%–100%; 95% confidence interval (CI) = 26%–92%) of all gametocytes sexed then being identified as male — and there was no significant correlation between the proportion of gametocytes that were male and the level of asexual parasitaemia ($r=0.7$; $P=0.17$), core temperature ($r=0.2$; $P=0.8$), or level of gametocytaemia ($r=0.02$; $P=0.98$). The temporal changes observed in the gametocyte sex ratios were similar for the PSP- and PS-treated children (Fig.). In both treatment groups there was a progressive increase in the proportions of gametocytes that were male such that > 80% of the gametocytes were male by day 7. In the three children (one given PSP and two given PS) who had a female-biased sex ratio on presentation, SR1 was reached by day 5. Of the children treated with PSP, five of the 32 gametocytaemic on day 7 and one of the 16 gametocytaemic on day 14 had female-biased gametocytaemias at those times. Among the children treated with PS, one of the 31 gametocytaemic on day 7 but none of the 22 gametocytaemic on day 14 had a female-biased ratio at those times. In the children treated with PSP, the proportions of gametocytes that were male on days 7 and

14 were significantly higher than the proportion on day 0 (with *P*-values of 0.00003 and 0.00001, respectively). Similarly, in those treated with PS, the proportions of gametocytes that were male on days 7 and 14 were significantly higher than the proportion on day 0 (with a *P*-value of 0.000001 for each comparison; see Figure).

In the PSP-treated children, the level of microgametocytaemia in the peripheral blood was similar to that of macrogametocytaemia between days 0-5 (see Table) but male gametocytes became significantly more numerous than the female by day 7. In those treated with PS, the level of microgametocytaemia slightly exceeded that of macrogametocytaemia between days 0-5. By day 7, however, the mean intensity of the microgametocytaemias was significantly higher than that of the macrogametocytaemias.

As, at each time-point investigated, the sex ratios and levels of gametocytaemia for the PSP-treated children were similar to those treated with PS (with the exception of the intensities of the day-5 gametocytaemias; see Figure and Table), the data from both treatment groups were pooled for the analysis of the disposition kinetics. For the male gametocytes, the mean (S.E.) 'area under the curve' of the plot of gametocyte density *v.* time (AUC) — calculated from SRI and expressed in units of asexual forms/μl.h — was significantly higher [17,619 (3831), with a range of 5040–40,017 and a CI of 8561–26,678; $N=8$] than that for the female gametocytes [4728 (691), with a range 3034–6250 and a CI of 2530–6927; $N=4$; $P=0.017$]. The mean (S.E.) half-life ($t_{\frac{1}{2}}$) of the male gametocytes [265.6 (59.4) h, with a range of 66.3–624.0 and a CI of 125.1–406.1 h] was also significantly longer than that of the female gametocytes [74.3 (12.8) h, with a range of 37.1–96.0 and a CI of 33.5–115.1 h; $P=0.05$]. Although the mean (S.E.) clearance of the female gametocytes, expressed in units of μl/kg.h [0.0002 (0.00008), with a range of 0.00002–0.0004 and a CI of −0.00007–0.00018] was 2.5-fold higher than that of the male gametocytes [0.00008 (0.00003), with a range of 0.00002–0.00015 and a CI of 0.000009–0.00016], this difference was not statistically significant ($P=0.14$).

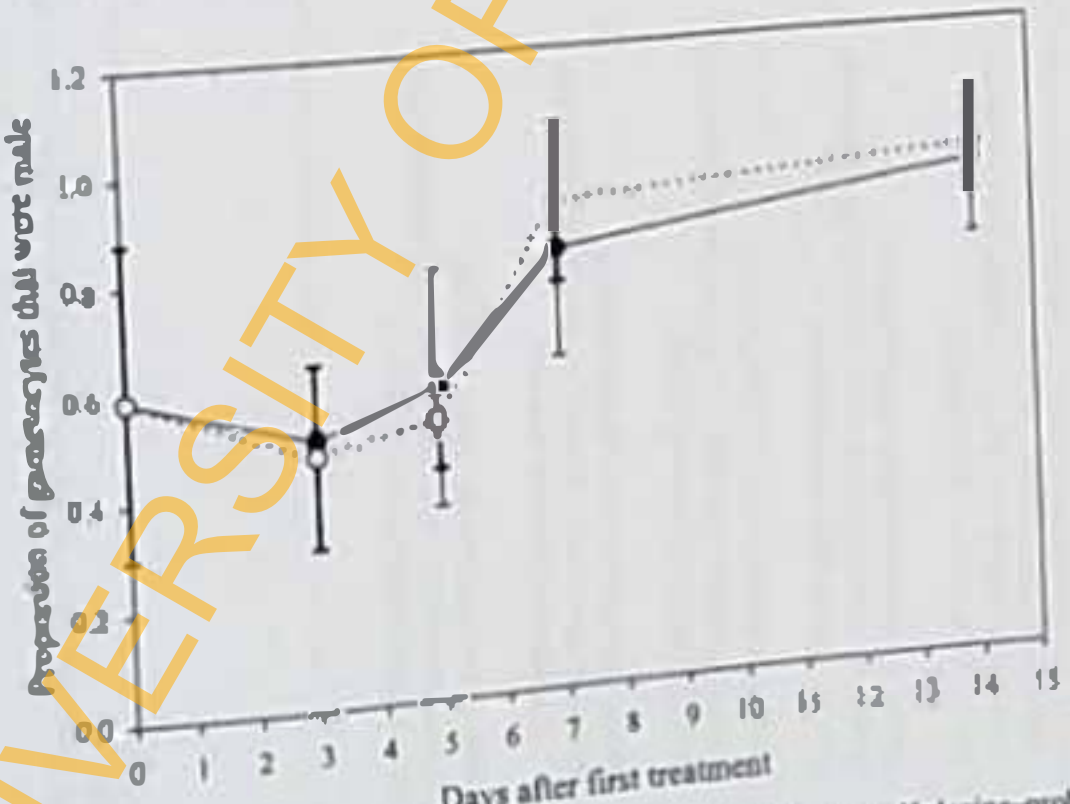


FIG. Changes in the sex ratio of gametocytes before and after pyrimethamine–sulfadoxine–probenecid (●) or pyrimethamine–sulfadoxine (○) treatment of acute, *Plasmodium falciparum* malaria in children. The vertical lines indicate S.E.

TABLE. *Prevalences and intensities of micro- and macro-gametocytaemias in 73 malarious children treated with pyrimethamine-sulfadoxine (PSP) or pyrimethamine-sulfadoxine (PS)*

	PSP			PS		
	Male gametocytes	Female gametocytes	P^*	Male gametocytes	Female gametocytes	P^*
DAY 0						
No. of children with gametocytaemia	3	1		1	1	
Geometric mean level of gametocytaemia (gametocytes/μl)	15	12	–	12	12	–
Range of gametocytaemias (gametocytes/μl)	12–24	–		–		
DAY 3						
No. of children with gametocytaemia	3	3		3	3	
Geometric mean level of gametocytaemia (gametocytes/μl)	17	17	1.0	17	24	0.65
Range of gametocytaemias (gametocytes/μl)	12–24	12–24		12–24	12–48	
DAY 5						
No. of children with gametocytaemia	9	6		3	3	
Geometric mean level of gametocytaemia (gametocytes/μl)	21	27	0.83	30	91	0.1
Range of gametocytaemias (gametocytes/μl)	12–24	12–36		24–48	36–60	
DAY 7						
No. of children with gametocytaemia	22	7		29	17	
Geometric mean level of gametocytaemia (gametocytes/μl)	115	28	0.02	98	43	0.01
Range of gametocytaemias (gametocytes/μl)	60–852	12–108		24–504	12–313	
DAY 14						
No. of children with gametocytaemia	20	3		16	4	
Geometric mean level of gametocytaemia (gametocytes/μl)	64	29	0.18	30	12	0.18
Range of gametocytaemias (gametocytes/μl)	24–180	24–36		12–96	12–12	

*Calculated by the Wilcoxon signed-rank test.

Overall, PSP appeared to be therapeutically superior to PS, in clearing asexual parasitaemia and fever. Treatment with PSP or PS was, however, generally associated with increases in the frequency of gametocyte carriage and the levels of gametocytaemia. The between-treatment difference in the level of gametocytaemia on day 5 is perhaps attributable to the relatively rapid clearance of asexual parasites induced by the addition of probenecid to PS.

The observation of a predominance of male gametocytes over the female, in both treatment groups at most times during follow-up (Fig.), is in contrast to the general, early predominance of female gametocytes observed, in the same study area, by Sowunmi and Fateye (2003b). It may be related to the relatively low level of gametocytaemia seen at presentation in the present study — a male bias when there are few gametocytes in the peripheral blood being a form of fertility insurance (Gardner *et al.*, 2003). In addition, the children investigated in the present study could have been exposed to other sex-ratio-modifying factors prior to presentation (Paul *et al.*, 2002; Robert *et al.*, 2003). Such factors may explain why, in the present study, SR1 was reached in some of the children before, at or shortly after presentation.

Despite the increasing intensities of the gametocytaemias, there were significant increases in the percentages of gametocytes that were male following treatment with either PS or PSP (Fig.). Although, in a longitudinal follow-up of gametocyte carriers in a village in Senegal, Robert *et al.* (2003) detected a density-dependent relationship with sex ratios, they reported that peaks of gametocytaemia were sometimes associated with very low male:female sex ratios. It therefore appears that PS, acting singly or in concert with other factors, substantially increases the percentage of gametocytes that are male. When added to PS, probenecid appears to have no effect on gametocyte sex ratios (Fig.).

The present observations support the notion that male gametocytes persist longer in the peripheral circulation than female gametocytes, or are longer-lived (Ponnudurai *et al.*, 1986; Reece *et al.*, 2003; Sowunmi and Fateye, 2003b, c). The addition of probenecid to PS had no significant impact on the disposition of male or female gametocytes in the peripheral blood. Given that intensities of gametocytaemia and the male bias in gametocyte sex ratios correlate positively with gametocyte infectivity to mosquitoes feeding on humans (Tchuinkam *et al.*, 1993; Robert *et al.*, 1996), it would appear that the addition of probenecid to treatment with PS is unlikely to decrease (or increase) the transmission of malaria in the population, whether the gametocytes involved arise from PS-sensitive or -resistant infections.

ACKNOWLEDGEMENTS. The study received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, partly via a WHO/TDR Career Development Grant awarded to A.S. The authors thank their clinic nurse, M. Amao, for her assistance with running the study.

A. Sowunmi
A. A. Adedeji
B. A. Fateye
F. A. Fehintola
Department of Pharmacology and Therapeutics,
and Institute for Medical Research and Training,
University of Ibadan, Ibadan, Nigeria

Received 25 November 2003, Revised 2 September 2004,
Accepted 6 September 2004

Reprint requests to: A. Sowunmi, Department of Clinical Pharmacology, University College Hospital, Ibadan, Nigeria.
E-mail: malaria.ibe@alpha.linkserve.com; fax: +234 2 2411843.

REFERENCES

Anon. (1994). *Epi Info Version 6. A Word Processing Data Base and Statistics Program for Public Health on IBM-compatible Microcomputers*. Atlanta, GA: Centers for Disease Control and Prevention.

Carter, R. & Graves, P. M. (1988). In *Malaria: Principles and Practice of Malariology*, Vol. 1, eds Wernsdorfer, W. H. & McGregor, I. pp. 253–305. Edinburgh, U.K.: Churchill Livingstone.

Gardner, A., Reece, S. E. & West, S. A. (2003). *Journal of Theoretical Biology*, 223, 515–521.

Nzila, A., Mberu, E., Bray, P., Kokwaro, G., Winstanley, P., Marsh, K. & Ward, S. (2003). *Antimicrobial Agents and Chemotherapy*, 47, 2108–2112.

Paul, R. E. L., Brey, P. T. & Robert, V. (2002). *Trends in Parasitology*, 18, 32–38.

Ponnudurai, T., Lensen, A. H. W., Meis, J. F. G. M. & Meuwissen, J. H. E. T. (1986). *Parasitology*, 93, 263–274.

Reece, S. E., Duncan, A. B., West, S. A. & Read, A. F. (2003). *Parasitology*, 127, 419–426.

Robert, V., Read, A. F., Essong, J., Tchuinkam, T., Mulder, B., Verhave, J. P. & Carnevale, P. (1996). *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 90, 621–624.

Robert, V., Sokhna, C. S., Rogier, C., Ariey, F. & Trape, J.-F. (2003). *Parasitology*, 127, 1–8.

Sibley, C. H., Hyde, J. E., Sims, P. G. P., Plowe, C. V., Kublin, J. G., Mberu, E. K., Cowman, A. F., Winstanley, P. A., Watkins, W. M. & Nzila, A. M. N. (2001). *Trends in Parasitology*, 17, 582–588.

Sowunmi, A. (2002). *Annals of Tropical Medicine and Parasitology*, 96, 227–238.

Sowunmi, A. & Fateye, B. A. (2003a). *Tropical Medicine and International Health*, 8, 783–792.

Sowunmi, A. & Fateye, B. A. (2003b). *Annals of Tropical Medicine and Parasitology*, 97, 671–682.

Sowunmi, A. & Fateye, B. A. (2003c). *Parasitology*, 126, 363–369.

Sowunmi, A., Fehintola, F. A., Adedeji, A. A., Gbotosho, G. O., Falade, C. O., Tambo, E., Fateye, B. A., Happi, T. C. & Oduola, A. M. J. (2004). *Tropical Medicine and International Health*, 9, 606–614.

Tchuinkam, T., Mulder, B., Dechering, K., Stoffels, H., Verhave, J.-P., Cot, M., Carnevale, P., Meuwissen, J. H. E. T. & Robert, V. (1993). *Tropical Medicine and Parasitology*, 44, 271–276.

Effects of pyrimethamine-sulphadoxine, chloroquine plus chlorpheniramine, and amodiaquine plus pyrimethamine-sulphadoxine on gametocytes during and after treatment of acute, uncomplicated malaria in children

A Sowunmi/+, MA Adedeji, GO Gbotosho, BA Faleye, TC Happi

Department of Pharmacology & Therapeutics and Postgraduate Institute for Medical Research and Training, University of Ibadan, Ibadan, Nigeria

The effects of pyrimethamine-sulphadoxine (PS), chloroquine plus chlorpheniramine, a HI receptor antagonist that reverses chloroquine resistance in Plasmodium falciparum in vitro and in vivo (CQCP), and amodiaquine plus pyrimethamine-sulphadoxine (AQPS) on gametocyte production were evaluated in 137 children with acute, symptomatic, uncomplicated falciparum malaria who were treated with these drugs. PS was significantly less effective than CQCP or AQPS in clearing asexual parasitaemia or other symptoms of malaria. Gametocyte carriage on days 3, 7, and 14 were significantly higher in those treated with PS. The ratio of the density (per µl blood) of peripheral young gametocyte (PYG), that is, stage II and V, or later of circulating generation of gametocytes, now to 1 by day 7 of (PMG), that is, stage II and V, or later of circulating generation of gametocytes, now to 1 by day 7 of treatment in those treated with PS, but remained consistently below 1 in the other treatment groups. PYG-PMG density ratio increased significantly from day 0-14 in those treated with PS and CQCP ($\chi^2 = 76$, $P = 0.00000$) and $\chi^2 = 42.2$, $P = 0.00001$, respectively) but decreased significantly in those treated with AQPS ($\chi^2 = 53.2$, $P = 0.00000$). Both PS-sensitive and -resistant infections generated PYG (11 of 29 vs 13 of 20, $\chi^2 = 0.04$, $P = 0.93$) but PYG was present only in those with resistant response to CQCP. Combination of PS with amodiaquine (AQ, that is, AQPS) resulted in less production of PYG but in this setting PYG was not indicative of response to AQPS. These data indicate that PS enhanced production or release of young gametocytes when used alone but generated less young gametocytes when used in combination with AQ. PYG can be used as an indicator of response to CQCP but not PS or PS-based combination drugs.

Key words: malaria - gametocytaemia - pyrimethamine - sulphadoxine - chloroquine - chlorpheniramine - amodiaquine - children - Nigeria

As resistance to chloroquine (CQ) increases in extent and severity, alternative regimens available to control programmes in developing endemic countries including pyrimethamine-sulphadoxine (PS), amodiaquine (AQ) (Olliaro et al. 1996, Brasseur et al. 1999, Sowunmi et al. 2001) or combination of AQ with PS (AQPS) (Sowunmi 2002) or other suitable combinations have become increasingly used in the treatment of CQ-resistant falciparum infections. These alternatives have varying effects on clearance of asexual parasitaemias or sexual forms of *Plasmodium falciparum*. For example, PS may (Puta & Manyando 1997) or may not (Hogh et al. 1995) enhance gametocyte carriage during treatment of acute falciparum infections. Although the presence of gametocytes in peripheral blood after antimalarial treatment is no proof of viability, their generation is required for the transmission of the infection from the vertebrate to the anopheline host. In order to improve the management of paediatric cases of malaria and reduce transmission in our area of study, the effects of these drugs on gametocyte production needs urgent assessment. In addition, it is not clear whether the enhancement or non-enhancement of gametocyte production by PS will be influenced by its use in combination with other antimalarial drugs. It is noteworthy that antifolates are ineffective in the treatment of uncomplicated falciparum malaria in South America, for example, in Brazil (Fontes et al. 2002).

Resistance to CQ in *P. falciparum* can be reversed by chlorpheniramine (CP) in vitro and in vivo (Sowunmi et al. 1997, 1998a, b, c). We have recently shown that, the presence in peripheral blood of very young gametocytes (PYG) 72 h after commencing CQ may be used as indicator of response to CQ (Sowunmi et al. 2003). However, it is unclear whether the addition of CP to CQ will alter the use of PYG as an indicator of response to CQ or indeed as an indicator of failure of reversal of CQ resistance in vivo by CP. Although the combination of CQ with CP will not be employed by control programmes in Africa in the very near future, it is still essential to study PYG and peripheral mature gametocyte (PMG) generation during treatment with CQCP in the event that this or other similar combination becomes available.

Financial support: the UNDP/World Bank/ WHO Special Programme for TDR.

+Corresponding author and WHO/TDR Career Development grant. E-mail: malaria.ibad@alpha.linkserve.com/akinsowunmi@hotmail.com

Received 16 January 2006
Accepted 21 November 2006

In order to address these issues, we have evaluated gametocyte generation during treatment of falciparum malaria in children with PS, CQCP and AQPS. The main aims of our study were (i) to evaluate the effects of PS, CQCP and AQPS on gametocyte generation during treatment with these drugs, (ii) to determine whether or not the addition of PS to AQ will influence the generation of gametocytaemia by PS, and (iii) to evaluate PYG as an indicator of response to PS, CQCP or AQPS.

PATIENTS AND METHODS

Study site - The study site, Ibadan, is a hyperendemic area for malaria in Southwestern Nigeria (Salako et al. 1990). In the area, it is difficult to distinguish, clinically, re-infection from recrudescence after day-14 of treatment because of intense transmission. Antimalarial drugs have therefore generally, until recently, been evaluated on the basis of data recorded up to day 14, rather than the customary day 28 (Ekanem et al. 1990, Sowunmi & Salako 1992).

Patients - The study took place at the University College Hospital in Ibadan, Nigeria. Overall, 166 children who presented with acute, symptomatic, uncomplicated *P. falciparum* malaria were enrolled in the study between September 1999 and September 2001.

The study was designed to elicit a 20% difference in cure rates between AQPS/CQCP on one hand and PS on the other hand with 80% power and at 95% level of confidence. The minimum number of patients required for each treatment arm is 45. In general, to be enrolled, the children had to be aged 0.5-10 years, and have symptoms compatible with acute, falciparum malaria (with fever or history of fever in the 24-48 h preceding presentation) and a pure *P. falciparum* parasitaemia of > 2000 asexual forms/µl blood. Those who had taken antimalarial drugs in the 2 weeks preceding presentation, provided a urine sample found positive for 4-aminoquinolines or sulphonamides (by the Dill-Glazko and Ehrlich tests, respectively), or who had a concomitant illness, such as sickle-cell anaemia, or severe or complicated malaria (Warrell et al. 1990, WHO 2000) were excluded. The informed consent of a parent or guardian was obtained for each child included in the study. A child was withdrawn from the study if she/he developed concomitant illness during the follow-up period, or if his/her parent or guardian requested it. The study received ethical approval from the local ethics committee.

Before enrolment in the study, a medical history of each child was obtained from an accompanying parent/guardian and each was physically examined. Body weight and oral or rectal temperature were recorded, and thick and thin films were prepared from finger-prick blood samples. These smears were Giemsa-stained for parasite identification and quantification of any peripheral parasitaemia.

Drug treatment - Children were randomly allotted to one of 3 treatment groups. One group received PS at presentation (day 0) at a dose 25 mg/kg of the sulphonamide component. Each tablet of PS contained 500 mg of sulphadoxine and 25 mg pyrimethamine. The other groups received chloroquine base, 30 mg/kg of body weight over 3 days (days 0-2) plus chlorpheniramine maleate, 6 mg at presentation followed by 4 mg every 8 h for 7 days (days 0-6) if the child was aged < 5 years, or 8 mg at presentation followed by 6 mg every 8 h if the child was ≥ 5 years; or a single dose of PS at presentation plus AQ 30 mg/kg over 3 days (days 0-2). All drugs were given by a physician orally and each child was observed for at least 3 h after each such supervised drug treatment, in order to ensure that the drug was not vomited. If it was, the child was excluded from the study. Additional management of some children included the administration of an antipyretic (e.g. 10-15 mg paracetamol/kg, every 8 h for 24 h) and fanning and tepid sponging when necessary.

Evaluation of response - Clinical observations were recorded daily for 8 days (days 0-7) and then on day 14. Thick and thin blood films, for quantification of parasitaemia, were prepared at the same times. At each follow-up, the guardians or parents (and, when possible, the children) were actively questioned, using a standard questionnaire, and the children were examined for the presence of adverse reactions to drugs.

Giemsa-stained blood films were examined by light microscopy under an oil-immersion objective, at × 1000 magnification, by two independent assessors who did not know the drug treatment of the patients. Parasitaemia in thick films was estimated by counting asexual parasites relative to 1000 leukocytes, or 500 asexual forms, whichever occurred first. From this figure, the parasite density was calculated assuming a leukocyte count of 6000/µl blood. Young gametocytes (stage I-III) and mature gametocytes (stage IV and V) (Sinden 1998) were also counted in thick blood films against 1000 leukocytes on days 0, 3, 4, 5, 6, 7, and 14. The responses to drug treatment were classified according to World Health Organization (1973) criteria. Treatment was considered a failure if the day-3 parasitaemia was > 25% of the day 0 value, if parasitaemia did not clear by day 7, or if parasitaemia cleared before day 7 but re-appeared before day 28. The parasite clearance time (PCT) was defined as the time elapsing from drug administration until there was no patent parasitaemia. The fever clearance time (FCT) was defined as the time from drug administration until the oral or rectal temperature fell to ≤ 37.4°C and remained so for at least 72 h. (This definition was necessary because of the routine use of paracetamol during the first 36 h of treatment in some children).

Cure rates were defined as the proportions of patients who remained free of parasitaemia on day 14 of follow-up.

Re-treatment of drug treatment failures - All treatment failures were re-treated with AQPS on day 14 provided they were not symptomatic before this time. Patients with profound clinical (hyperpyrexia, oral fluid intolerance) and parasitological deterioration during follow-up were treated with artemether, 9.6 mg/kg of body weight over five days and were regarded as treatment failures.

Statistical analysis - Data were analyzed using version 6 of the Epi-Info software (Anon 1994). Proportions were compared by calculating χ^2 with Yates' correction or by Fisher exact tests. Normally distributed, continuous data were compared by Student's t tests and analysis of variance (ANOVA). Data not conforming to a normal distribution were compared by the Mann-Whitney U-tests and the Kruskal-Wallis tests (or by Wilcoxon rank sum test). The values presented below are generally means and standard deviations (sd). P-values of < 0.05 were taken to indicate significant differences.

RESULTS

Clinical and parasitological characteristics at enrolment and therapeutic responses - A total of 166 children was enrolled in the study. 51, 52, and 63 children were enrolled in the PS, COCP, and AQPS groups, respectively. Of these, 49, 48, and 60 children in the PS, COCP, and AQPS groups, respectively completed the mandatory 14-day follow up period and were analysed. The clinical and parasitological characteristics at enrolment were similar in all groups (Table I). The therapeutic responses to drug treatment are also summarized in Table I. AQPS was significantly more effective than COCP or PS in clearing fever and parasitaemia and with a significantly higher cure rate on day 14. Direct comparison of PS and COCP showed that fever (2.2 ± 1.1 vs 1.6 ± 0.8 day; P = 0.008) but not parasite clearance in those with sensitive response (2.7 ± 1.1 vs 2.5 ± 0.8 day; P = 0.33) was significantly faster with COCP than with PS. The cure rate on day 14 was also significantly higher with COCP than with PS (80.8 vs 59.2%; P = 0.03).

Gametocytaemia during follow-up - The prevalence and intensity of gametocytaemia before, during and after treatment are summarized in Table II. Gametocyte carriage on days 3, 7, and 14 or days 3, 7, and 14 combined were significantly higher in the PS group than in the other treatment groups. However, the geometric mean gametocyte densities (GMGD) were similar in all the treatment groups.

The median survival time for peripheral young gametocytes (PYG) in PS, COCP, and AQPS treatment groups were 3.5, 1.5, and 1.5, respectively. There was a significant difference in the overall comparison of the survival experience using Wilcoxon (Gehan) statistics (χ^2 = 14.7, P = 0.0006). The ratios of the densities (per µl blood) of peripheral young gametocytes (PYG) to peripheral mature gametocytes are summarized in Table

TABLE I

Clinical and parasitological parameters of the children enrolled in the study

	PS (n = 49)	COCP (n = 48)	AQPS (n = 60)	P value
Age (years)				
mean ± sd	5.1 ± 2.1	6.0 ± 2.3	5.5 ± 2.5	0.32
range	0.6-10	2.0-10	1.2-10	
Weight (kg)				
mean ± sd	15.4 ± 3.6	16.8 ± 5.2	15.5 ± 4.7	0.32
range	6.5-26	8.1-35	6-26	
Duration of illness (d)				
mean ± sd	3.1 ± 1.4	3.6 ± 2.4	2.8 ± 1.3	0.06
range	1-7	2-14	1-8	
Parasitaemia (per µl)				
mean ± sd	35,535 ± 13,...	38,636 ± 12,...	38,133 ± 13,...	0.88
range	35,000-...	36,200-...	36,000-...	
Parasitaemia (per µl)				
geometric mean	37,858	39,348	38,082	0.56
range	3,310-375,...	5,111-219,...	6,...	
Fever clearance time (days)				
mean ± sd	2.2 ± 1.1	1.6 ± 0.8	1.2 ± 0.9	0 (...
range	1-5	1-4	1-5	
Parasite clearance time (d)				
mean ± sd	2.7 ± 1.1	2.5 ± 0.8	2.2 ± 0.7	0.012
range	1-6	1-4	1-4	
No. of children				
cured	29	39	60	0 (...
D1	10	7	-	
D2	7	1	-	
D3	3	-	-	

95% CI = 95% confidence interval; PS = Fansidar; COCP = chloroquine plus ...; AQPS = amodiaquine plus pyrimethamine-sulphadoxine.

III. The ratios were consistently below 1 in the CQCP and AQPS groups up till day 7. However, in the PS group, this ratio rose progressively to 1 on day 7 indicating continuing production (or generation or mobilization) of young gametocytes. PYG-PMG density ratio increased significantly from day 0-14 in those treated with PS and CQCP ($\chi^2 = 76$, P = 0.000001 and $\chi^2 = 42.2$, P = 0.00001, respectively) but decreased significantly in those treated with AQPS ($\chi^2 = 53.2$, P = 0.0000011).

Relationship between PYG and responses to drug treatment. - None of the children successfully treated with CQCP had PYG during the follow-up. In children who had sensitive response to PS treatment (n = 29), PYG was present on days 0, 3, 5, 7, and 14 in 5, 12, 13, 13, and 7 children, respectively. Similarly in those successfully treated with AQPS (n = 60), PYG was present on days 0, 3, 5, 7, and 14 in 2, 2, 3, 3, and 0 patients, respectively. The PYG rates were significantly higher in those treated with PS than in those treated with AQPS at all times during follow up (P ≤ 0.006 in all comparisons).

Post Hoc Turkey HSD test for repeated measure of the effect of PYG generation over the 14 day follow up showed significant differences in the comparisons of PS vs AQPS and PS vs CQCP (P = 0.0001 and 0.0001 respectively). There was no significant difference in the comparison of PYG generated by those treated with AQPS and CQPS (P = 0.08).

Relationship between PYG and outcomes of treatment in the children treated with PS and CQCP. - The relationship between treatment outcomes and presence of PYG in children treated with PS and CQCP are shown in Tables IV and V. PYG rates were similar in those with sensitive or resistant responses to PS (18 of 29 vs 13 of 20, $\chi^2 = 0.04$, P = 0.93) and the rates were similar from days 0-14. In contrast, PYG was seen only in those with resistant response to CQCP. In those without gametocytaemia at presentation, but who subsequently developed PYG 72 h after commencement of CQCP, the presence of PYG was associated with treatment failure on or before day 14 (Table V).

TABLE II

Gametocytaemias before, during and after the treatment, with pyrimethamine-sulphadoxine (PS), chloroquine plus chlorpheniramine (CQCP) or amodiaquine plus pyrimethamine-sulphadoxine (AQPS) of *Plasmodium falciparum* infections in children

	PS (n = 49)	CQCP (n = 48)	AQPS (n = 60)	P value
Day 0 gametocytaemia	36	22	40	0.49
Geometric mean (/μl)				
Mean ± S.E.	87 ± 52.1	26 ± 5.3	74 ± 43.3	
Range	12-444	12-36	12-288	
Day 3 gametocytaemia	36	44	40	0.92
Geometric mean (/μl)				
Mean ± S.E.	100 ± 43.5	127 ± 83.5	86 ± 54.1	
Range	12-[illegible]	12-612	12-408	
Day 5 gametocytaemia	50	61	41	0.74
Geometric mean (/μl)				
Mean ± S.E.	243 ± 112.9	118 ± 64.3	141 ± 121.0	
Range	12-[illegible]	12-315	12-504	
Day 7 gametocytaemia	125	39	36	0.38
Geometric mean (/μl)				
Mean ± S.E.	399 ± 141.2	136 ± 73.6	98 ± 74.1	
Range	12-3520	12-690	12-468	
Day 14 gametocytaemia	83	34	19	0.21
Geometric mean (/μl)				
Mean ± S.E.	139 ± 29	60 ± 30.1	24 ± 12	
Range	12-480	12-168	12-48	
No. of gametocyte carriers				0.42
Day 0	9	5	8	0 [illegible]
Day 3	23	6	7	0 [illegible]
Day 7	26	9	6	0 [illegible]
Day 14	30	4	5	0 [illegible]
Day 0, 7, 14	32	11	3	0 [illegible]
Day 7, 14	27	11	3	

Wilcoxon (Gehan) for survival analysis ($\chi^2 = 14.7$, P = 0.00066)

TABLE III

Prevalence and intensities of peripheral young gametocytes and peripheral mature gametocytes in children treated with pyrimethamine-sulphadoxine (PS), chloroquine plus chlorpheniramine (CQCP) or amodiaquine plus pyrimethamine-sulphadoxine (AQPS)

Day	PS		CQCP		AQPS	
	GMPYGD (/µl)	GMPMGD (/µl)	GMPYGD (/µl)	GMPMGD (/µl)	GMPYGD (/µl)	GMPMGD (/µl)
0	21 (5)[a] 12-36[b] [illegible]	65 (4) 12-400 1.1	12 (4) 12-12 1	36 (1) - 1	42 (2) 36-72 1	51 (2) 12-216 1.3
3	28 (20) 12-372 1	57 (9) 12-552 2.0	53 (4) 12-144 1	106 (3) 24-460 1.9	27 (2) 12-60 -	340 (1) - -
5	37 (21) 12-640 1	54 (17) 12-1840 1.3	46 (6) 12-348 1	58 (4) 12-180 1.1	21 (1) 12-[illegible] 1	39 (5) 12-420 1.7
7	79 (24) 12-1920 1	89 (21) 12-2210 1.1	42 (7) 13-216 1	97 (4) 12-[illegible] 2.3	28 (3) 12-156 1	87 (2) 24-112 1.1
14	50 (14) 12-240 1	78 (12) 12-444 1.6	21 (4) 12-120 1	33 (3) 12-[illegible] 1.7	- - -	19 (2) 12-48

GMPYGD: geometric mean peripheral young gametocyte density; GMPMGD: geometric mean peripheral mature gametocyte density; [illegible] GMPYGD [illegible] GMPMGD [illegible] or values in parentheses represent [illegible] of children with gametocytaemia [illegible]
a: no peripheral young gametocytes was observed

TABLE IV

Prevalence of peripheral young gametocytaemia (PYG) in the children with [illegible] following treatment with oral pyrimethamine-sulphadoxine (PS) or chloroquine plus chlorpheniramine (CQCP)

No. of children with PYG on day	PS-sensitive (n = 29)	PS-resistant (n = 20)	P value	CQCP-sensitive (n = 38)	CQCP-resistant (n = 10)	P value
0	5	4	0.51	-	3	-
3	12	8	0.96	-	4	0.001
5	13	8	0.85	-	6	0.0002
7	13	11	0.38	-	7	0.00002
14	7	7	0.14	-	4	0.001
3, 7 and/or 14	28	13	0.65	-	6	0.00002
7 and/or 14	13	13	0.11	-	7	0.0000001

TABLE V

Peripheral young gametocytaemia (PYG) on or after day 3 in children treated with oral pyrimethamine-sulphadoxine (PS) or chloroquine plus chlorpheniramine (CQCP) and who did not have gametocytaemia [illegible]

No. of children with PYG on day	PS-sensitive (n = 29)	PS-resistant (n = 20)	P value	CQCP-sensitive (n = 38)	CQCP-resistant (n = 10)	P value
3	5	4	0.64	-	2	0.008
4	6	6	0.72	-	1	0.06
5	4	7	0.91	-	1	0.08
6	10	8	0.69	-	2	0.009
7	13	10	0.65	-	2	0.005
14	7	9	0.64	-		

DISCUSSION

The ideal antimalarial drugs or drug combinations for the treatment of falciparum malaria should not only promptly clear parasitaemia, fever or other symptoms of malaria, but should also prevent the generation of gametocytes from asexual forms during treatment. In the present study, PS was significantly less effective than CQCP or AQPS in clearing parasitaemia or fever in children with acute falciparum infections. This is not surprising since progressive decline in sensitivity of *P. falciparum* to PS has been reported from the area of study from the late 1990s (Falade et al. 1997, Sowunmi et al. 1998a). The decline in sensitivity of the parasite to PS has also occurred in many areas of Africa (Sibley et al. 2001).

In addition to their effects on the sexual forms, gametocyte carriage may be influenced to a considerable extent by the sensitivity of the asexual parasites to the drugs used for the treatment of infections. For example, as resistance of the asexual parasites to the 4-aminoquinolines, CQ, and AQ, increases, gametocyte carriage also increases (Strickland et al. 1986, Hogh et al. 1995). In these studies, gametocyte carriage rates 28 days after PS treatment were significantly less than those of CQ and AQ since PS was more effective than the 4-aminoquinolines on asexual parasites in the settings of these studies. However, increased carriage may also be related to decreased sensitivity to PS in certain circumstances (Sowunmi et al. 1998a, Tjitra et al. 2002). In our cohort of children, gametocyte carriage was significantly higher at all times after treatment with PS than in the other treatment groups. In addition, PYG rates were similar in both PS- sensitive and -resistant infections supporting a known fact that PS enhanced generation or release of gametocytes during treatment of acute falciparum infections (Puta & Mayando 1997). However, GMGD were similar in all the treatment groups.

Many antimalarial drugs appear to reduce gametocytaemia by clearing the asexual stage infections. This clearance, if exceptionally rapid, may reduce transmissibility particularly in areas of low transmission. For example, the artemisinin derivatives have reduced transmissibility in some parts of Thailand by this process (Price et al. 1996).

In order to determine the influence of treatment with antimalarial drugs on gametocyte production and densities, we have quantified both young and mature gametocytes and expressed these as ratios. The ratios of PYG to PMG were consistently below 1 up to day 7 in those treated with CQCP and AQPS, but rose to 1 by day 7 in those treated with PS irrespective of the sensitivity of the asexual parasite to PS. This showed continuing and enhanced production or, preferential mobilization of gametocytes by PS irrespective of the sensitivity of the asexual parasites to PS. This process of continuing or preferential mobilization of young gametocytes by PS may explain why gametocytes persist longer in some patients treated with PS. This is plausible because the young gametocytes must grow and run the normal time-course of survival of the normal mature gametocytes.

Given that gametocyte density may correlate with mosquito infectivity and therefore transmission success (Tchuinkam et al. 1993, Drakeley et al. 1999), the effects of PS on gametocytes carriage and mobilization have implications for malaria control programmes with respect to the use of this drug. Recent WHO recommendations (WHO 2001a, b) have focused on the use of combination antimalarial therapy (CT), particularly artemisinin-based combination therapy (ACT). Although several control programmes in Africa have switched to CT, some programmes use PS-based combination, for example, AQPS (Sowunmi 2002). The modulating effect of AQ on enhanced production of PYG by PS may provide supporting argument for the use of combination therapy. However, the reduced generation of PYG by PS despite its combination with other drugs suggests that generation of gametocyte is an inherent property of antifolate antimalarials (Sowunmi et al. 2005, Hamel et al. 2005).

In a recent study, we have shown that the detection of PYG 72 h after the start of CQ therapy may be used as an indicator of response to this drug (Sowunmi & Fateye 2002). Our results show that PYG may also be used as an indicator of response to CQCP. Failure of the enhancement of the antimalarial efficacy of CQ by CP *in vivo* was associated with the presence in peripheral blood of young gametocytes. However, PYG was not an indicator of response to PS, since both PS-sensitive and resistant infections generated PYG. In addition, the presence of PS in combination with AQ also generated PYG and was clearly not an indicator of response to AQPS since the cure rate in this group was 100%.

The limitation of the present study is the fewer number of gametocyte carriers in the AQPS and CQCP groups following treatment. Therefore caution is required with the interpretation of the data from these two groups.

REFERENCES

Anon 1994. Epi Info Version 6. A Word Processing Data Base and Statistics Program for Public Health on IBM-compatible Microcomputers. Centers for Disease Control and Prevention, Atlanta, GA.

Brasseur P, Guiguemde R, Diallo S, Guiyedi V, Kombila M, Ringwald P, Olliaro P 1999. Amodiaquine remains effective for the treating uncomplicated malaria in West and Central Africa. *Trans R Soc Trop Med Hyg* 93: 645-650.

Drakeley CJ, Secka I, Correa S, Greenwood BM, Targett GA 1999. Host haematological factors influencing the transmission of *Plasmodium falciparum* gametocytes to *Anopheles gambiae s.s.* mosquitoes. *Trop Med Inter Hlth* 4: 131-138.

Ekanem OJ, Weisfeld JS, Salako LA, Nahlen BL, Ezedinachi ENU, Walker O, Breman JG, Laoye OJ, Hedberg K 1990. Sensitivity of *Plasmodium falciparum* to chloroquine and sulphadoxine-pyrimethamine in Nigerian children. *Bull WHO* 68: 45-52.

Falade CO, Salako LA, Sowunmi A, Odaola AMJ, Larcier P 1997. Comparative efficacy of halofantrine, chloroquine and sulphadoxine-pyrimethamine in the treatment of acute uncomplicated falciparum malaria in Nigerian children. *Trans R Soc Trop Med Hyg* 91: 58-62.

Fanes CJ, Ribeiro LC, Pang LW 2002. Proguanil plus sulfamethoxazole in the treatment of acute uncomplicated Plasmodium falciparum malaria. *Southeast Asian J Trop Med Public Health 33*: 685-688.

Hamel MJ, Holtz T, Mkandala C, Kaimila N, Chizani N, Bloland P, Kublin J, Kazembe P, Steketee R 2005. Efficacy of trimethoprim-sulfamethoxazole compared with sulfadoxine-pyrimethamine plus erythromycin for the treatment of uncomplicated malaria in children with integrated management of childhood illness dual classification of malaria and pneumonia. *Am J Trop Med Hyg 73*: 609-615.

Hogh B, Thompson R, Hetzel C, Kruse NA, Jones I, Dgedge M, Barreto J, Sinden RE 1995. Specific and nonspecific responses to Plasmodium falciparum blood-stage parasites and observations on the gametocytaemia in school children living in a malaria-endemic area of Mozambique. *Am J Trop Med Hyg 52*: 50-59.

Olliaro P, Nevill C, Le Bras J, Ringwald P, Mussano P, Garner P 1996. Systematic review of amodiaquine treatment in uncomplicated malaria. *Lancet 348*: 1196-1201.

Price RN, Nosten F, Luxemburger C, ter Kuile FO, Paiphun L, Chongsuphajaisiddhi T, White NJ 1996. Effects of artemisinin derivatives on malaria transmissibility. *Lancet 347*: 1654-1658.

Puta C, Manyando C 1997. Enhanced gametocyte production in Fansidar treated *Plasmodium falciparum* malaria patients: implications for malaria transmission control programmes. *Trop Med Inter Hlth 2*: 227-229.

Salako LA, Ajayi FO, Sowunmi A, Walker O 1990. Malaria in Nigeria: a revisit. *Ann Trop Med Parasitol 84*: 435-445.

Sibley CH, Hyde JE, Sims PFG, Plowe CV, Kublin JG, Mberu EK, Cowman AF, Winstanley PA, Watkins WM, Nzila AM 2001. Pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum*: what next. *Trends Parasitol 17*: 582-588.

Sinden RE 1998. Gametocytes and sexual development. In IW Sherman, *Malaria: Parasite Biology, Pathogenesis and Protection*, ASM Press, Washington DC, p. 25-48.

Sowunmi A 2002. A randomized comparison of chloroquine, amodiaquine and their combination in the treatment of acute uncomplicated falciparum malaria in children. *Ann Trop Med Parasitol 96*: 227-238.

Sowunmi A, Fateye BA, Happi CT, Gbotosho GO, Oduola AMJ 2003. *Plasmodium falciparum* gametocytaemia in Nigerian children: peripheral immature gametocytaemia as an indicator of a poor response to chloroquine treatment, and its relationship to molecular determinants of chloroquine resistance. *Ann Trop Med Parasitol 97*: 453-468.

Sowunmi A, Salako LA 1992. Evaluation of the relative efficacy of various antimalarial drugs in Nigerian children under five years of age suffering from acute uncomplicated falciparum malaria. *Ann Trop Med Parasitol 86*: 1-8.

Sowunmi A, Ayede AI, Falade AG, Ndikum VN, Sowunmi CO, Adedeji AA, Falade CO, Happi TC, Oduola AMJ 2001. A randomized comparison of chloroquine and amodiaquine in the treatment of acute, uncomplicated, *Plasmodium falciparum* malaria in children. *Ann Trop Med Parasitol 95*: 549-558.

Sowunmi A, Fateye BA, Adedeji AA, Fehintola FA, Bamgboye AE, Babalola CP, Happi TC, Gbotosho GO 2005. Effects of antifolates- cotrimoxazole and pyrimethamine sulfadoxine- on gametocytes in children with acute, symptomatic, uncomplicated, *Plasmodium falciparum* malaria. *Mem Inst Oswaldo Cruz 100*: 451-455.

Sowunmi A, Oduola AMJ, Ogundahunsi OAT, Salako LA 1998a. Comparative efficacy of chloroquine plus chlorpheniramine and pyrimethamine-sulphadoxine in acute uncomplicated falciparum malaria in Nigerian children. *Trans R Soc Trop Med Hyg 92*: 77-81.

Sowunmi A, Fehintola FA, Ogundahunsi OAT, Oduola AMJ 1998b. Comparative efficacy of chloroquine plus chlorpheniramine and halofantrine in acute uncomplicated falciparum malaria in Nigerian children. *Trans R Soc Trop Med Hyg 92*: 441-445.

Sowunmi A, Oduola AMJ, Ogundahunsi OAT, Salako LA 1998c. Enhancement of the antimalarial effect of chloroquine by chlorpheniramine in vivo. *Trop Med Inter Hlth 3*: 177-183.

Sowunmi A, Oduola AMJ, Ogundahunsi OAT, Falade CO, Gbotosho GO, Salako LA 1997. Enhanced efficacy of chloroquine-chlorpheniramine combination in acute uncomplicated falciparum malaria in children. *Trans R Soc Trop Med Hyg 91*: 63-67.

Strickland GT, Fox E, Sarwar M, Khaliq AA, Macdonald M 1986. Effects of chloroquine, amodiaquine and pyrimethamine-sulphadoxine on *Plasmodium falciparum* gametocytaemia. *Am J Trop Med Hyg 35*: 259-262.

Tchuinkam T, Mulder B, Dechering K, Stoffels H, Verhave JP, Cot M, Carnevale P, Meuwissen JH, Robert V 1993. Experimental infections of *Anopheles gambiae* with *Plasmodium falciparum* of naturally infected gametocyte carriers in Cameroon: factors influencing the infectivity to mosquitoes. *Trop Med Parasitol 44*: 271-276.

Tjitra E, Suprianto S, Anstey NM 2002. Higher gametocyte prevalence following failure of treatment of *Plasmodium falciparum* malaria with sulfadoxine-pyrimethamine and the combination of chloroquine plus sulfadoxine-pyrimethamine: implications for progression of anti-folate resistance. *Trans R Soc Trop Med Hyg 96*: 434-437.

Warrell DA, Molyneux ME, Beales PF 1990. Severe and complicated malaria. *Trans R Soc Trop Med Hyg 80* (Suppl.): 1-50.

WHO-World Health Organization 1973. *Chemotherapy of Malaria and Resistance to Antimalarials*, Technical Report Series No. 529, Geneva.

WHO-World Health Organization 2000. Severe falciparum malaria. *Trans R Soc Trop Med Hyg 94* (Suppl.1).

WHO-World Health Organization 2001a. *The Use of Antimalarial Drugs. Report of a WHO Informal Consultation*, Document WHO/CDS/RBM/2001.33, Geneva.

WHO-World Health Organization 2001b. *Antimalarial Drug Combination Therapy. Report of a WHO Technical Consultation*. Document WHO/CDS/RBM/2001.35, Geneva.

Effects of antifolates – co-trimoxazole and pyrimethamine-sulfadoxine – on gametocytes in children with acute, symptomatic, uncomplicated, *Plasmodium falciparum* malaria

A Sowunmi/+, BA Fateye, AA Adedeji, FA Fehintola, AE Bamgboye, CP Babalola*, TC Happi, GO Gbotosho

Department of Pharmacology & Therapeutics and Institute for Medical Research and Training, *Department of Pharmaceutical Chemistry, University of Ibadan, Ibadan, Nigeria

Antimalarial drugs including the antifolate, pyrimethamine-sulfadoxine (PS), can modulate the prevalence and intensities of gametocytaemia following treatment of acute malaria infections. They may also directly influence the transmission and spread of drug insensitivity. Little is known of the effects of co-trimoxazole (Co-T), another antifolate antimalarial, on gametocytes in children with acute malaria infections. We compared the effects of Co-T and PS on the prevalence and intensities of gametocytaemia and gametocyte sex ratios in 102 children aged 0.5-12 years presenting with acute and uncomplicated falciparum malaria. Compared to pre-treatment, both drugs significantly reduced gametocyte carriage post-initiation of treatment. However, gametocyte carriage was significantly lower on day 14 in those treated with Co-T than PS. Significant increase in gametocytaemia with time occurred in PS – but not Co-T-treated children. Kaplan-Meier survival curve of the cumulative probability of remaining gametocyte-free in children who were agametocytaemic at enrolment showed that by day 7 of follow up, children treated with PS had a significantly higher propensity to have developed gametocytes than in Co-T-treated children (log rank statistic = 13, df = 1, P = 0.02). Gametocyte sex ratio changes were similar following treatment with both drugs. PS and Co-T treatment of acute malaria infections in children from this endemic area is associated with significant increases in prevalence and intensities of gametocytaemia but these effects are more marked in those treated with PS than Co-T.

Key words: co-trimoxazole - pyrimethamine-sulfadoxine - malaria - gametocytaemia - sex ratio - children - Nigeria

The antifolate antimalarial, pyrimethamine-sulfadoxine (PS), has become increasingly used as first line treatment of falciparum malaria in several African countries because of increasing resistance in *Plasmodium falciparum* to chloroquine. In spite of frequent use and of in vitro and in vivo studies (Hogh et al. 1998, Sowunmi & Fateye 2003a), its effects on gametocytes in children with falciparum infections remain incompletely understood.

With increasing use, resistance in *P. falciparum* to PS is increasing (Sibley et al. 2001) probably as a consequence of long half-lives of its components. It has recently been suggested that, co-trimoxazole (Co-T), an antifolate antimalarial with relatively short half-lives of its components compared to PS, may be used as alternative to the latter for the treatment of uncomplicated falciparum infections in children because it is as efficacious as PS (Omar et al. 2001, Fehintola et al. 2004). It is assumed that the relatively short half-life of Co-T may, when compared with PS, reduce the chances of engendering resistance in *P. falciparum* to this drug and may provide additional advantage with transmission of drug resistant infections over PS. It is noteworthy that antifolate antimalarials are not effective in the treatment of uncomplicated falciparum malaria in South America, for example, Brazil (Fontes et al. 2002).

However, while the effects on PS on gametocytes and gametocyte sex ratios (GSR) are known (GSR may influence infectivity to mosquitoes and transmission – see Robert et al. 1996, Sowunmi et al. 2003 a,b), the effects of Co-T on gametocytes are relatively unknown in African children with falciparum malaria. We hypothesized that PS and Co-T have similar effects on gametocyte prevalence, density, and sex ratio, and possess similar effects on gametocyte survival in children treated with these drugs. We tested this hypothesis in a group of children with acute symptomatic uncomplicated *P. falciparum* malaria who were randomized to and who received PS and Co-T for the treatment of their infections.

PATIENTS AND METHODS

Patients - Between June and August 1999, a randomized trial of Co-T and PS for the treatment of uncomplicated falciparum malaria was conducted in 102 children at the University College Hospital in Ibadan, a hyperendemic area for malaria in Southwestern Nigeria (Salako et al. 1990). Ethical clearance for the study was provided by the local ethics committee. In general, to be enrolled, the children had to be aged 0.5-12 years, and have symptoms compatible with acute, falciparum malaria (with fever or history of fever in the 24-48 h preceding presentation) and a pure *P. falciparum* parasitaemia of >2000 asexual forms/µl blood.

Financial support: the UNDP/World Bank/WHO Special Programme for TDR. AS was supported by a WHO/TDR Career Development Grant.
*Corresponding author. E-mail: malaria.iba@alpha.linkserve.com/akinsowunmi@hotmail.com
Received 24 January 2005
Accepted 19 May 2005

Those who had taken antimalarial drugs in the two weeks preceding presentation, provided a urine sample found positive for four aminoquinolines or sulphonamides (by the Dill-Glazko and lignin tests, respectively), or who had a concomitant illness, such as sickle-cell anaemia or severe or complicated malaria (WHO 2000) were excluded. The informed consent of a parent or guardian was obtained for each child included in the study. A child was withdrawn from the study if she/he developed concomitant illness during the follow-up period, or if his/her parent or guardian requested it. Thick and thin blood films from all patients who participated in the study were examined for the presence and density of asexual and sexual parasites at enrolment and start of treatment (day 0), and at follow-up at days 1-7, and then on day 14. Co-T was given as 20 mg/kg of the sulfamethoxazole component twice daily for five days (day 0-4); PS was given as the 25 mg/kg of the sulfadoxine component at presentation (day 0). All drugs were administered orally.

Assessment of parasitaemia and gametocyte sex ratio - Thick and thin blood films prepared from a finger prick were Giemsa-stained and were examined by light microscopy under an oil-immersion objective, at × 1000 magnification, by two independent assessors who did not know the drug treatment of the patients. Parasitaemia in thick films was estimated by counting asexual parasites relative to 1000 leukocytes, or 500 asexual forms, whichever occurred first. From this figure, the parasite density was calculated assuming an average leukocyte count of 6000/µl of blood (Shaper & Lewis 1971, Ezeilo 1971, Sowunmi et al. 1995). Gametocytes were also counted in thick films against 1000 leukocytes assuming an average leukocyte count of 6000/µl of blood at enrolment (day 0) and on days 3, 5, 7, and 14. Gametocytes were sexed, in each blood film, if gametocytaemia was ≥ 12 sexual forms/µl. Gametocyte sex determination was based on the following criteria (Carter & Graves 1988, Robert et al. 1996): males are smaller than females, the nucleus is bigger in males than females; the ends of the cells are round in males and angular in females; the cytoplasm stains pale purple in males and deep blue in females; and the granules of malaria pigment are centrally located in females and more widely scattered in males. GSR was defined as the proportion of gametocytes in peripheral blood that were microgametocytes (Pickering et al. 2000, West et al. 2001).

Statistical analysis - Data were analysed using version 6 of the Epi-Info software (Anon 1994), and the statistical program SPSS for Windows version 10.01 (SPSS Inc. 1999). Proportions were compared by calculating χ^2 with Yates' correction or Fisher exact test. Normally distributed, continuous data were compared by Student's t-tests and analysis of variance (ANOVA). Data not conforming to a normal distribution were compared by the Mann-Whitney U-test and the Kruskal-Wallis test (or by Wilcoxon rank sum test). Kaplan-Meier analysis was used to estimate the cumulative probability of remaining free of gametocytes during follow-up for all cases of malaria combined and for those cases that were free of gametocytaemia at enrolment. Differences in survival time were assessed by inspection of Kaplan-Meier curves and pairwise log-rank tests. P-values of ≤ 0.05 were taken to indicate significant differences.

RESULTS

Demographic characteristics and therapeutic responses - A total of 104 children were enrolled into the study. Two children, one in each treatment group, were excluded from the study due to parental relocation. These children were cleared of their peripheral parasitaemia at the time of exclusion. The demographic characteristics of the children enrolled in the study and the therapeutic responses to the treatment given are summarized in Table I. These were similar in the two treatment groups. However, parasite clearance was significantly shorter in those treated with Co-T than PS.

Prevalence of gametocytaemia - The prevalence of gametocytaemia before and after treatment with PS, and before, during and after treatment with Co-T is shown in Table II. Gametocyte carriage was similar on days 0-7 in both treatment groups and it peaked at day 7 in both the PS and Co-T groups. Gametocyte carriage was significantly lower on day 14 in those treated with Co-T than PS ($\chi^2 = 5.6$, P = 0.018). Eleven and 19 children treated with Co-T and PS, respectively were gametocyte carriers on both days 7 and 14. The difference between these proportions was significant ($\chi^2 = 4.0$, P = 0.046).

In general, compared to pre-treatment, both drugs significantly increased gametocyte carriage post-initiation of treatment ($\chi^2 = 20.9$, P = 0.003 for Co-T and $\chi^2 = 28.4$, P = 0.0001 for PS, see Table II). In children without patent gametocytaemia at enrolment, there was a greater propensity to be gametocyte-positive by day 7 with a significantly greater proportion of children treated with PS having gametocytes by day 14 of follow up compared with Co-T (63.6% vs 34.3%, $\chi^2 = 5.9$, P = 0.016) (Table II).

Gametocytaemia - Gametocytaemia before and after treatment with PS, and before, during and after treatment with Co-T is shown in Table II. Gametocytaemia was similar throughout the duration of the study in both Co-T and PS-treated children with peak gametocytaemia occurring in both treatment groups on day 7. Peak gametocytaemia (on day 7) was significantly higher than day 3 gametocytaemia in both treatment groups (t = 0.066, P = 0.015 for Co-T; t = 0.08, P = 0.017, by Wilcoxon sign rank test for paired data). Gametocytaemias occurring on days 3-14 were not compared with pre-treatment gametocytaemia because of the small number of patients in both groups. However, multiple comparison of gametocytaemia using Friedman test showed that there was significant increase in gametocytaemia with time on days 3, 5, 7, and 14 in those treated with PS (P = 0.011). In comparison, there was no significant increase in gametocytaemia with time on days 3, 5, 7, and 14 in those treated with Co-T (P = 0.29).

The Kaplan-Meier survival curve of the cumulative probability of remaining gametocyte-free in children who were agametocytaemic at enrolment is shown in Fig. 1. By day 7 of follow up, children treated with PS had a significantly higher propensity to have developed game-

TABLE I

Summary of clinical characteristics at enrolment and therapeutic responses in patients with acute falciparum malaria treated with co-trimoxazole (Co-T) or pyrimethamine-sulphadoxine (PS)

Parameter	Co-T (n = 53)	PS (n = 49)	P value
Age (y)			
mean ± sd	6.3 ± 2.9	6.3 ± 2.8	0.9
range	1.5 - 12.0	0.8 - 10.5	
Weight (kg)			
mean ± sd	18.2 ± 6.4	17.6 ± 5.2	0.6
range	7.5 - 31.5	7.0 - 28.0	
Temperature (°C)			
mean ± sd	38.1 ± 1.3	38.4 ± 1.4	0.2
range	35.7 - 40.9	35.9 - 41.0	
Parasite density (/µl)			
geometric mean	36543	34983	0.29
range	2200-349636	2552-652800	
Gametocyte density (/µl)			
geometric mean	15 (n = 3)	17 (n = 2)	0.8
range	12 - 24	12 - 24	
PCT (d)			
mean ± sd	2.5 ± 0.9 (n = 50)	3.2 ± 1.2 (n = 44)	0.002
range	1-5	1-6	
FCT (d)			
mean ± sd	2.0 ± 1.0	2.3 ± 1.3	0.20
range	1 - 4	1 - 6	
Day 14 response [a]			
No of subjects			0.88
Cured (%)	47 (88.7)	43 (87.7)	
RI	6	5	
RII	0	0	
RIII	0	1	

[a] using WHO (1973) criteria. FCT: fever clearance time. PCT: parasite clearance time. sd: standard deviation. All comparisons were two-tailed.

TABLE II

Intensity and prevalence of *Plasmodium falciparum* gametocytaemia following treatment of uncomplicated malaria with co-trimoxazole (Co-T) or pyrimethamine-sulphadoxine (PS) of 102 malarious children

	Co-T (n = 53)	PS (n = 49)	P value
Day 0 [a]	18 [12 - 48] [b]	17 [12 - 24]	1.0 [c]
	5/ 53 (9.4%) [d]	2/ 49 (4.1%)	0.44 [d]
Day 3	27 [12 - 120]	27 [12 - 144]	1.0
	13/ 53 (25%)	12/ 49 (24.5%)	1.0
Day 5	33 [12 - 420]	45 [12 - 1872]	0.49
	21/53 (41.2%)	25/45 (52.1%)	0.31
Day 7	42 [12 - 444]	71 [12 - 2916]	0.27
	28/49 (57.1%)	34/46 (73.9%)	0.13
Day 14	33 [12 - 120]	43 [12 -1200]	0.52
	13/37 (35.1%)	23/35 (65.7%)	0.018

[a] gametocyte mean [range]. [b] gametocyte positive no. of patients examined. values in parentheses represent percentage of patients with gametocytaemia. [c] Mann Whitney test. [d] chi-square test. e within group comparison of gametocyte carriage pre-treatment and post-treatment. There was evidence of treatment in Co-T treatment ($\chi^2 = 20.9$, $P = 0.003$), and for PS treatment ($\chi^2 = 28.4$, $P = 0.0001$) arms show significant increase in gametocyte carriage following treatment with both drugs.

cyte than in Co-T-treated children (Log-rank statistic 5.35, df = 1, P = 0.02).

Temporal changes in gametocyte sex ratios - In Co-T-treated children, 7, 28, 104, 134, and 44 gametocytes were counted on days 0, 3, 5, 7, and 14, respectively and approximately 77% of these gametocytes could be sexed. In PS-treated children, 7, 34, 230, 293, and 168 gametocytes were counted on days 0, 3, 5, 7, and 14, respectively and approximately 76% of these gametocytes could be sexed. The data on GSR at enrolment were pooled because of the small number of gametocyte carriers observed pre-treatment (three among Co-T-treated children and two among PS-treated children). Overall, pre-treatment GSR was female-biased, but became male-biased by day 3 in both treatment groups, and remained male-biased till day 14 in both groups (Fig. 2). GSR was similar in the two treatment groups on days 3, 5, 7, and 14 (P = 0.4, 0.7, 0.7, and 0.2, respectively on days 3, 5, 7, and 14.

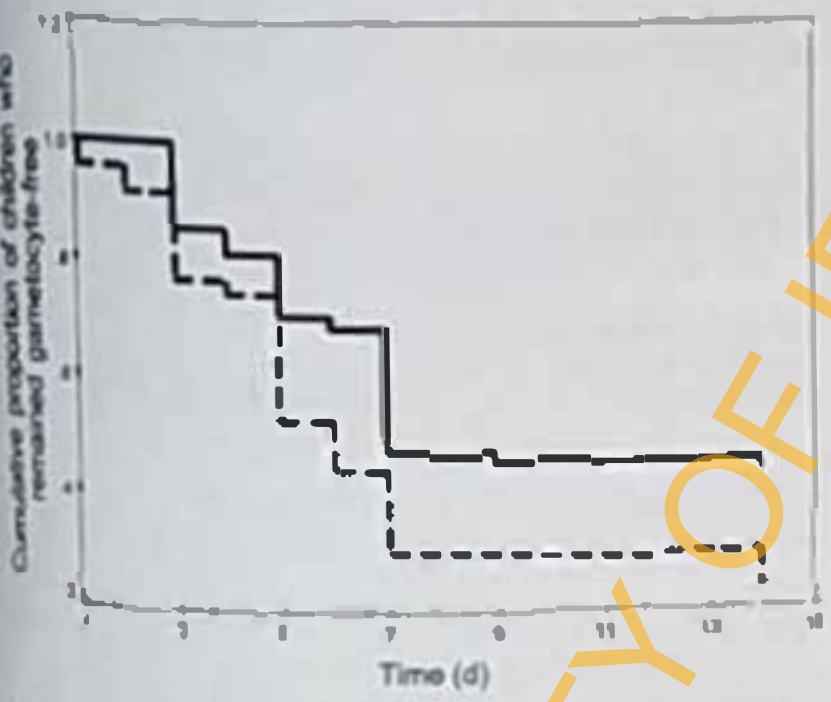


Fig. 1: Kaplan-Meier plot (survival curve) of cumulative probability of remaining gametocyte-free in 95 children who were gametocytaemic at enrolment following treatment with cotrimoxazole (broken line) or pyrimethamine-sulphadoxine (solid line).

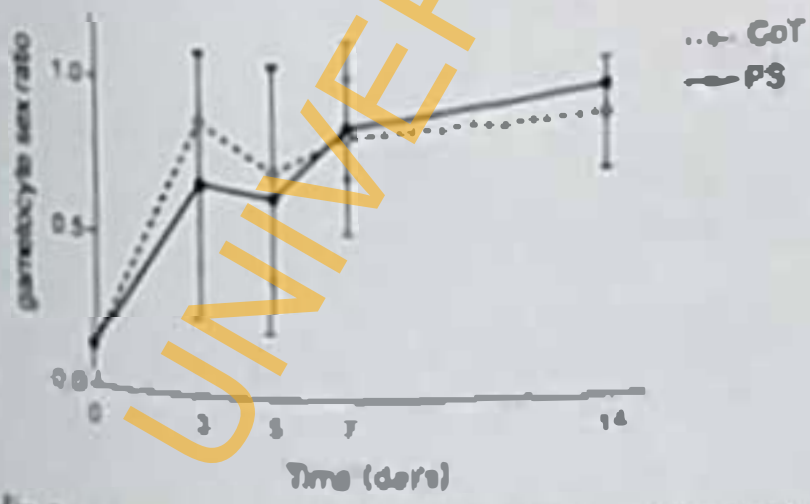


Fig. 2: changes in sex ratio of gametocytes before and after treatment with pyrimethamine-sulfadoxine (PS), and before, during and after treatment with co-trimoxazole (Co-T) in children with acute, uncomplicated, falciparum malaria. The vertical lines indicate standard error.

DISCUSSION

Co-T and PS were both effective in the treatment of uncomplicated falciparum malaria in children from this endemic area of Southwest Nigeria. Apart from a significantly shorter parasite clearance in the Co-T-treated children, none of the outcome measures, clinical or parasitological, differed between the two antifolate drug combinations. The results support those of recent findings from the same area (Fehintola et al. 2004) and are in agreement with those from Kenya (Omar et al. 2001). However, the results are contrary to the suggestion that Co-T is less effective than PS for the treatment of malaria (WHO 1996). In many areas in Africa, for example in Uganda, there has been appreciable decline in the sensitivity of *P. falciparum* to Co-T (Kilian et al. 1998).

The prevalence of gametocytaemia significantly increased following treatment with both drugs but this effect was more marked in those treated with PS than in those treated with Co-T. Sexual development in the malaria parasite and its modulation may be influenced by several factors (Carter & Miller 1979, Mons 1988). It is not clear whether the significantly lower carriage on day 14 in those treated with Co-T was due to fundamental differences in the responses of the asexual parasite populations to switch to gametocyte production following exposure to the two drugs. The components of Co-T have shorter half lives than those of PS and it is possible that this, coupled with individual variation in response, may partly explain the observed difference in gametocyte prevalence between the two drugs.

Although there were no significant differences in gametocyte density in the two treatment groups, the significant increases in gametocyte prevalence with time, the greater proportion of children with patent gametocytaemia on both days 7 and 14 among children treated with PS, and the significantly higher propensity to have developed gametocytes by day 7 in PS compared with Co-T treated children (see Fig. 1) suggest a more marked effects of PS on gametocyte production. These findings with PS is in agreement with our previous observations (Sowunmi & Fateye 2003 a,b). Thus, the significantly reduced effects of Co-T on gametocyte retention may be an advantage for the use of Co-T over PS in endemic setting.

Despite lower gametocyte prevalence and insignificant increase in gametocytaemia with time in Co-T treated children, both Co-T and PS appear to have similar effects on GSR. None of the post-treatment initiation GSR data differed between the two antifolate drug combinations; both drugs favoured gametocyte maleness. It is not clear whether the effects of the drugs on gametocytaemia is fundamentally different from their effects on GSR. Since GSR may be influenced by several factors (West et al. 2002, Gardner et al. 2003), this may impact on the temporal changes in GSR. The male-biased sex ratio after PS treatment is in agreement with our recent findings from the same area (Sowunmi & Fateye 2003b). The gametocyte maleness seen after initiation of treatment with both drugs suggests that antifolates, in general, may favour gametocyte maleness. Since gametocyte infectivity to mosquito

is increased by gametocyte maleness (Roberts et al. 1996) and infectivity correlates with gametocyte density (Tchuinkam et al. 1993, Robert et al. 2000), both Co-T and PS by enhancing gametocyte maleness, gametocyte carriage and gametocytaemia, may markedly enhance malaria transmission whether the treated patients have antifolate sensitive or resistant infections. This a demerit for the use of these drugs alone for the treatment of malaria.

REFERENCES

Anon 1994. *Epi Info Version 6. A Word Processing Data Base and Statistics Program for Public Health on IBM-compatible Microcomputers*, Centers for Disease Control and Prevention, Atlanta, GA.

Carter R, Miller LH 1979. Evidence for environmental modulation of gametocytogenesis in *Plasmodium falciparum* in continuous culture. *Bull WHO 57* (Suppl. 1): 37-52.

Carter R, Graves PM 1988. Gametocytes. In WH Wernsdorfer, I McGregor (eds), *Malaria. Principles and Practice of Malariology*, Vol. 1, Churchill Livingstone, Edinburgh, p. 253-305.

Ezeilo GC 1971. Neutropenia in Africans. *Trop Geogr Med 23*: 264-267.

Fehintola FA, Adedeji AA, Tambo E, Fateye BB, Happi TC, Sowunmi A 2004. Cotrimoxazole in the treatment of acute uncomplicated falciparum malaria in Nigerian children: a controlled clinical trial. *Clin Drug Invest 24*: 149-155.

Fernandes CJ, Ribeiro LC, Pang LW 2002. Proguanil plus sulfamethoxazole in the treatment of uncomplicated *Plasmodium falciparum* malaria. *Southeast Asian J Trop Med Public Health 33*: 685-688.

Gardner A, Reece SE, West SA 2003. Even more extreme fertility insurance and the sex ratios of protozoan blood parasites. *J Theor Biol 223*: 515-521.

Hogh B, Gamage-Mendis A, Butcher GA, Thompson R, Begtrup K, Mendis C, Enosse SM, Dgedge M, Barreto J, Eling W, Sinden RE 1998. The differing impact of chloroquine and pyrimethamine/sulfadoxine upon the infectivity of malaria species to the mosquito vector. *Am J Trop Med Hyg 58*: 176-182.

Kilian AHD, Jelinek T, Prislin I, Kabagambe W, Byamukama G, Mpigika B, Korte R, von Sonnenburg F 1998. Resistance in vivo of *Plasmodium falciparum* to co-trimoxazole in Western Uganda. *Trans R Soc Trop Med Hyg 92*: 197-200.

Mons B 1985. Induction of sexual differentiation in malaria. *Parasitol Today 1*: 87-89.

Omar SA, Bakari A, Owiti A, Adagu IS, Warhurst DC 2001. Co-trimoxazole compared with sulfadoxine-pyrimethamine in the treatment of uncomplicated malaria in Kenyan children. *Trans R Soc Trop Med Hyg 95*: 657-660.

Pickering JA, Read AF, Guerrero S, West SA 2000. Sex ratio and virulence in two species of of lizard malaria parasites. *Evol Ecol Res 2*: 171-184.

Robert V, Awono-Ambene HP, Le Hesran J-Y, Trape J-F 2000. Gametocytaemia and infectivity to mosquitoes of patients with uncomplicated *Plasmodium falciparum* malaria attacks treated with chloroquine or sulfadoxine plus pyrimethamine. *Am J Trop Med Hyg 62*: 210-216.

Robert V, Read AF, Essong J, Tchuinkam T, Mulder B, Verhave JP, Carnevale P 1996. Effect of gametocyte sex ratio on infectivity of *Plasmodium falciparum* to *Anopheles gambiae*. *Trans R Soc Trop Med Hyg 90*: 621-624.

Salako LA, Ajayi FO, Sowunmi A, Walker O 1990. Malaria in Nigeria: a revisit. *Ann Trop Med Parasitol 84*: 435 - 445.

Shaper AG, Lewis P 1971. Genetic neutropenia in people of African origin. *Lancet ii*: 1021-1023.

Sibley CH, Hyde JE, Sims PFG, Plowe CV, Kublin JG, Mberu EK, Cowman AF, Winstanley PA, Watkins WM, Alexis MN 2001. Pyrimethamine-sulphadoxine resistance in *Plasmodium falciparum*: what next? *Trends Parasitol 17*: 582-588.

Sowunmi A, Fateye BA 2003a. *Plasmodium falciparum* gametocytaemia in Nigerian children: before, during and after treatment with antimalarial drugs. *Trop Med Inter Hlth 8*: 783-792.

Sowunmi A, Fateye BA 2003b. Gametocyte sex ratios in children with asymptomatic, recrudescent, pyrimethamine-sulfadoxine-resistant, *Plasmodium falciparum* malaria. *Ann Trop Med Parasitol 97*: 671-682.

Sowunmi A, Akindele JA, Balogun MA 1995. Leukocyte counts in falciparum malaria in African children from an endemic area. *Afr J Med Med Sci 24*: 145-149.

SPSS 1999. SPSS for Windows Release 10.01 (Standard Version). SPSS Inc, Chicago, IL.

Tchuinkam T, Mulder B, Dechering K, Stoffels H, Verhave JP, Cot M, Carnevale P, Meuwissen JEHT, Robert V 1993. Experimental infection of *Anopheles gambiae* with *Plasmodium falciparum* of naturally infected gametocyte carriers in Cameroon: factors influencing the infectivity to mosquitoes. *Trop Med Parasitol 44*: 271-276.

West SA, Reece SE, Read AF 2001. Evolution of sex ratios in malaria and related apicomplexan (protozoan parasites). *Trends Parasitol 17*: 525-531.

West SA, Smith TG, Nee S, Read AF 2002. Fertility insurance and the sex ratios of malaria and related hemosporin blood parasites. *J Parasitol 88*: 258-263.

WHO-World Health Organization 1973. *Chemotherapy of Malaria and Resistance to Antimalarials*, Technical Report Series No. 529, Geneva.

WHO-World Health Organization 1996. Management of uncomplicated malaria and the use of antimalarial drugs for the protection of travellers. Report of an informal consultation. WHO/MAL/96.1075Rev.1 (1997).

WHO-World Health Organization 2000. Severe falciparum malaria. *Trans R Soc Trop Med Hyg 94* (Suppl. 1).