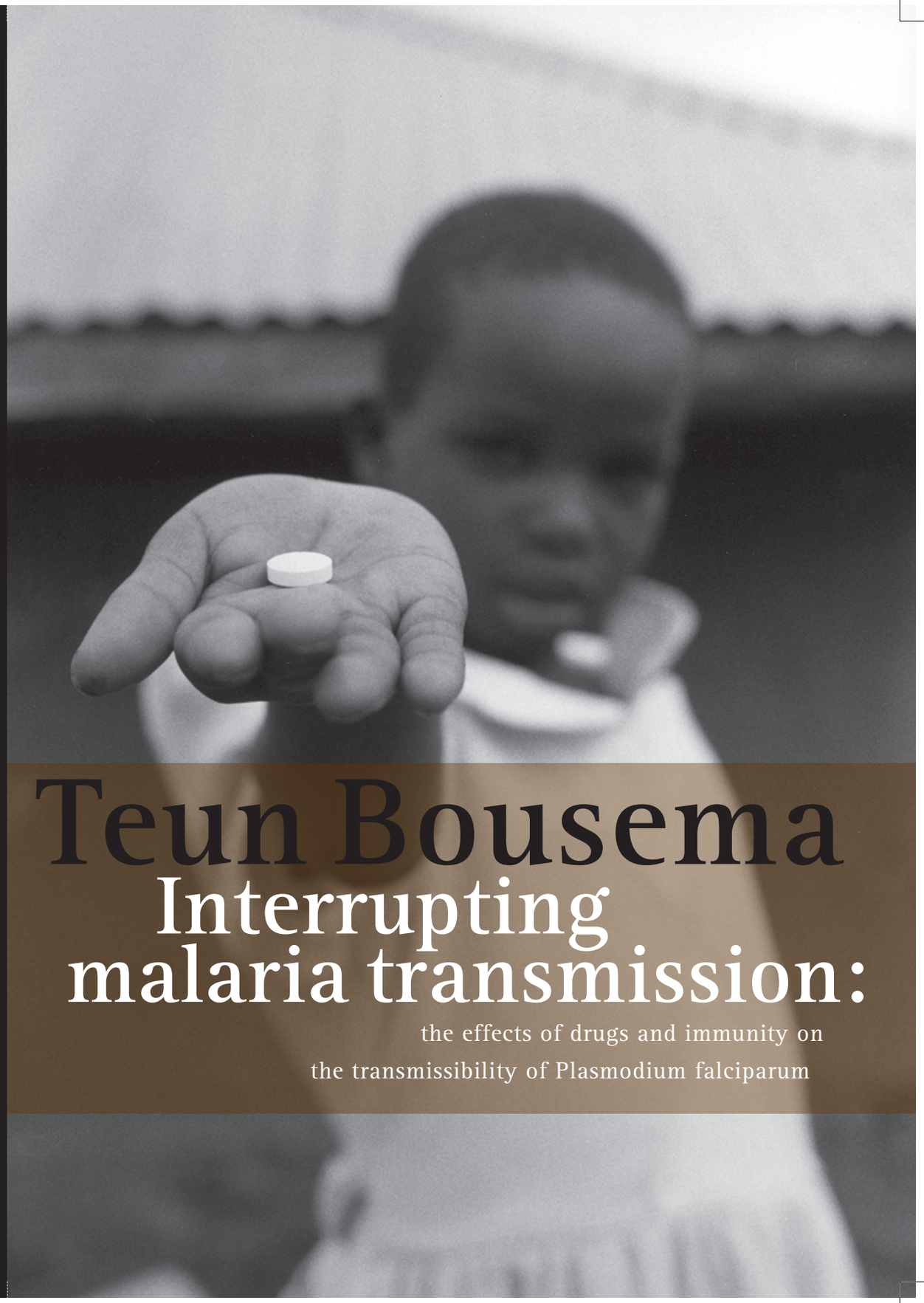
Interrupting malaria transmission: the effects of drugs and immunity on the transmissibility of Plasmodium falciparum **Teun Bousema** 2007



Teun Bousema

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Interrupting malaria transmission: the effects of drugs and immunity on the transmissibility of *Plasmodium falciparum*

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Chapter 1

General introduction

Parts of this chapter have been published in:
The epidemiology of Plasmodium falciparum gametocytes: weapons of mass dispersion[1].

Trends in Parasitology 22,424-30 (2006)

Each year, an estimated 300-500 million clinical cases of malaria occur, resulting in an estimated 1 million deaths [2], 100 000 children with permanent learning problems [3] and a reduction in economic growth of 1.3% in countries with intense malaria transmission [4]. While four malaria species commonly infect humans (*Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*), *P. falciparum* is almost exclusively responsible for malaria associated severe disease and mortality. Around 90% of *P. falciparum* associated deaths occur in sub-Saharan Africa [5] with pregnant women and especially young children as most vulnerable groups. The life cycle of malaria is given in figure 1.1 and shows two distinct phases, an asexual phase where parasites are replicating in the human vertebrate host and a sexual phase where fertilisation of sexual stage parasites takes place inside the mosquito midgut [6,7].

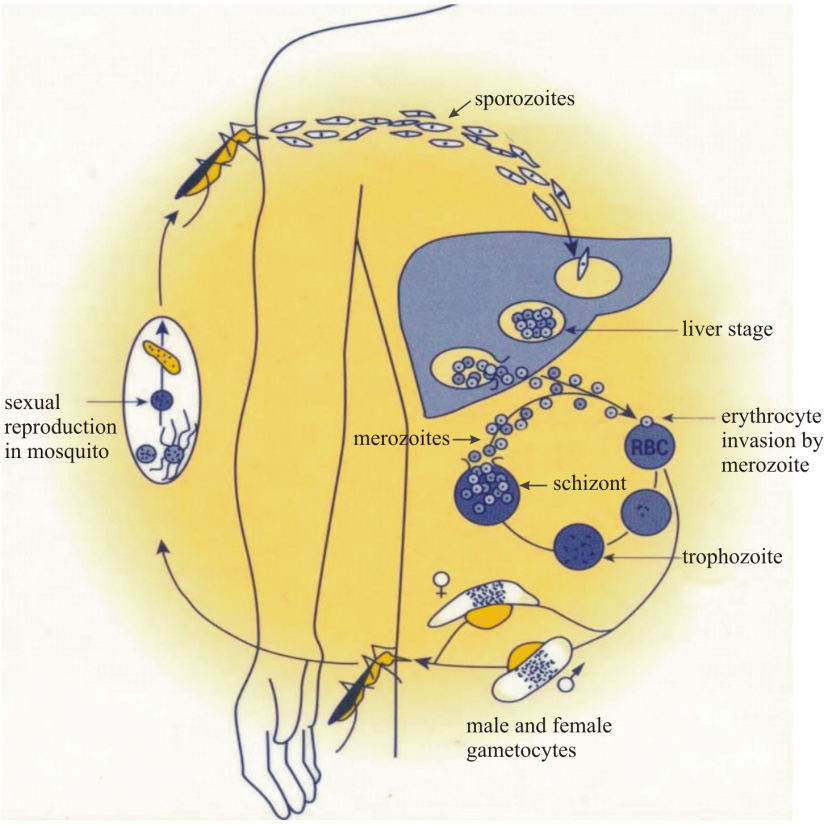

Figure 1.1 The malaria lifecycle. *Plasmodium parasites enter the human body by the injection of infected saliva of Anopheles mosquitoes. Within minutes, these sporozoites enter liver cells where they mature and multiply. After approximately one week, liverschizonts burst and release merozoites that invade red blood cells. Asexual replication of parasites takes place in red blood cells with erythrocytes releasing merozoites 48 hours after infection. A fraction of asexual parasites transforms into sexual stage parasites, gametocytes. Once ingested by mosquitoes that are taking a blood meal, each individual gametocyte forms 1 female macrogamete or 6-8 male*

microgametes. In the mosquito midgut, the fusion of gametes results in the formation of a zygote that in turn develops into a motile ookinete that can penetrate the midgut wall to form oocysts. The oocyst enlarges over time and bursts to release sporozoites. These sporozoites migrate to the mosquito salivary gland, ready to infect a new human being.

While asexual forms are responsible for malaria associated morbidity and mortality, sexual forms assure malaria transmission by infecting *Anopheles* mosquitoes. Mosquito infection depends on the presence of mature sexual stage parasites, gametocytes, in the peripheral blood. Gametocytes are essential for malaria transmission but microscopic examination shows that gametocytes are only observed in the peripheral blood of a proportion of the malaria infected individuals. The trigger of gametocytogenesis has been the source of much debate. Several factors have been related to the appearance of gametocytes including stress factors induced by immune cells [8-11]. However, the role of 'immune stress' is not free of controversy. If immune responses would stimulate the appearance of gametocytes, one would expect gametocytes to be most common in immune individuals. In endemic settings this would implicate that gametocytes are most common in adults. Epidemiological studies on the contrary consistently find higher levels of gametocytes in children [12-16], probably as a result of the age-dependent acquisition of antibodies against asexual stage parasites [17,18] or antibodies affecting both asexual parasites and gametocytes [19].

Studies on gametocytes in naturally infected individuals have almost exclusively focused on patients treated with antimalarial drugs [20-29], leaving many questions about their occurrence in the asymptomatic population unanswered. The low sensitivity of microscopy has certainly contributed to this limited understanding of gametocyte dynamics. Despite their distinctive shape and colour, *P. falciparum* gametocytes (figure 1.2) are particularly prone to being missed during routine examination of slides. The reason for this is that gametocytes typically occur at low densities [6,30], making it tedious to detect gametocytes by microscopy. Gametocyte prevalence increases by 29% when the number of fields of a thick film examined is doubled from to 200 to 400 [31] and by 3 fold (from 7 to 21%) when 1000 fields are screened [32].

The low sensitivity of routine microscopy, typically screening 100 fields of a thick film, has led to the development of molecular tools for gametocyte detection that have tremendously improved the sensitivity of gametocyte detection [33-35]. One of these molecular techniques is the quantitative nucleic acid sequence-based amplification (QT-NASBA) [35,36] that uses three enzymes (AMV-RT, RNase H and T7 RNA polymerase) and two target-specific primers (one of which includes a T7 polymerase/promoter) to amplify parasitic RNA at a temperature of 41°C [37]. The QT-NASBA allows stage-specific parasite quantification in 50μL finger prick blood samples with a lower detection limit of 10 asexual

parasites/mL [38] and 20-100 gametocytes/mL [35]. The QT-NASBA technique has been adapted for use in the field and parasitic ribosomal and messenger RNA was found to be stable for at least 2 months when bound to silica and stored dry at -20°C [37]. The QT-NASBA can be used to determine the prevalence of submicroscopic gametocyte carriage in different populations and the relative importance of submicroscopic gametocytaemia for malaria transmission.

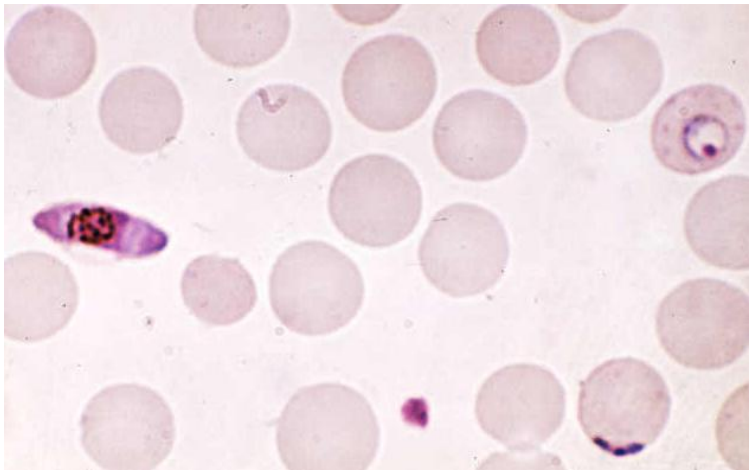

Figure 1.2 Gametocyte detection by microscopy. *A thin blood smear with a gametocyte (left) and two asexual parasites (right)*

Antimalarial drugs and malaria transmission

The most important tool to reduce gametocyte carriage is treatment with antimalarial drugs. Successful treatment limits the lifespan of malaria parasites; the rate at which the parasite biomass is reduced depending on the type of drug [39,40]. Although most antimalarial drugs have been developed to clear asexual parasites, they will indirectly influence the production of gametocytes that are derived from asexual parasites. In this manner successful antimalarial treatment with any drug will reduce the gametocytaemic period of infected individuals [41,42]. In addition, drugs can also have direct effects on gametocyte survival or infectiousness [41,43-45]. These effects are of particular importance for *P. falciparum* since gametocytes of this species have a long lifespan of up to 24 days [41,46]. Drugs that actively reduce gametocyte survival can therefore have a beneficial effect on post-treatment malaria transmission. Nearly all currently available drugs for *P. falciparum* fall into two groups: the quinolines (chloroquine, quinine, primaquine and mefloquine) and the antifolates (sulphadoxine, pyrimethamine, proguanil and dapsone). The direct effect of the most of these antimalarials is limited to asexual stage parasites [47]. The most commonly used antimalarial drugs in sub Saharan Africa, chloroquine (CQ) and sulphadoxine-pyrimethamine

(SP), do not actively clear gametocytes and treatment with these drugs is even followed by an initial increase in gametocyte prevalence and density [20,21,23,24,27,48]. Artemisinin has a mode of action than is not completely understood but is different from the quinolines and antifolates. It is believed to involve the cleavage of a peroxide bridge, a structural feature of artemisinin. This cleavage results in highly reactive radicals that inhibit a variety of parasite molecules, resulting in parasite death [49]. Artemisinin (qinghaosu) is isolated from the plant *Artemisia annua* [50], and artemisinin derivatives have a short half-life [40,51-53] and are the most potent antimalarial compounds available for human use [40,51-55]. Because artemisinin alone is often not completely curative, its preferred use is in combination with longer acting drugs such as SP, CQ or amodiaquine [40,56-58]. Artemisinin based combination therapy (ACT) reduces gametocyte carriage after treatment [23,27,40,59-61] and may thereby reduce malaria transmission [27,59,62]. The efficacy of ACT in reducing submicroscopic gametocyte densities is unknown.

Antimalarial drug resistance and malaria transmission

In the past decades parasite resistance to commonly used antimalarial drugs has increased in sub Saharan Africa [5,57,63-67], contributing to an increase in malaria morbidity and mortality [68,69]. Drug resistance in malaria is defined as the ability of a parasite strain to multiply or to survive in the presence of concentrations of a drug that would normally destroy parasites or prevent their multiplication [70]. The emergence of resistance comes in two phases. The first is the initial genetic event which produces the resistant mutant; the second is the subsequent selection process in which the survival advantage of the mutant parasite leads to a preferential transmission [71]. As a consequence of the preferential transmission, mutant parasite strains can outcompete wildtype parasite strains in the presence of drug pressure [72,73].

Several parasite mutations have been identified that are associated with CQ [73,74] and SP [74,75] treatment failure. SP is a drug combination of sulphadoxine and pyrimethamine that work synergistically by blocking different enzymes needed for folic acid synthesis, which is essential for DNA and RNA synthesis. Sulphadoxine inhibits the malarial enzyme dihydropteroate synthase (DHPS); pyrimethamine inhibits dihydrofolate reductase (DHFR) [76,77]. Mutations in the parasite's dihydrofolate reductase and dihydropteroate synthase genes (*dhfr*, *dhps*) have been related to a reduced efficacy of SP [74,75,78]. Multiple mutations can occur in both genes simultaneously. The predictive value of these mutations for clinical treatment outcome varies for different populations [75,79,80] as a result of geographical variation in parasite populations and differences in human immune responses [79,81].

The transmission of drug resistant parasite strains is crucial for determining the effective shelf-life of antimalarial drugs [27,57,59,82,83]. Drug

resistant parasites may persist after antimalarial drug treatment and, by doing so, have a better chance to produce gametocytes and subsequently infect mosquitoes. For CQ resistant parasites, there have been several reports that drug pressure elicits a higher production of gametocytes [20,21,24]. For SP, that was introduced as firstline antimalarial treatment in east Africa in the last decade [84], the relation between drug resistance and malaria transmission is unclear [29,85].

Infectiousness of gametocytes in membrane feeding assays

The presence of gametocytes in the peripheral blood does not equal the infectiousness of an individual to mosquitoes. This is influenced by a number of factors including gametocyte density and fitness [12,86-89] and human immune factors such as complement, granulocytes and specific antibodies [90-98]. Membrane feeding assays are the designated tools to directly determine the infectiousness to mosquitoes and associated factors. In these assays gametocytes are offered to reared *Anopheles* mosquitoes in an experimental set-up (figure 1.3).

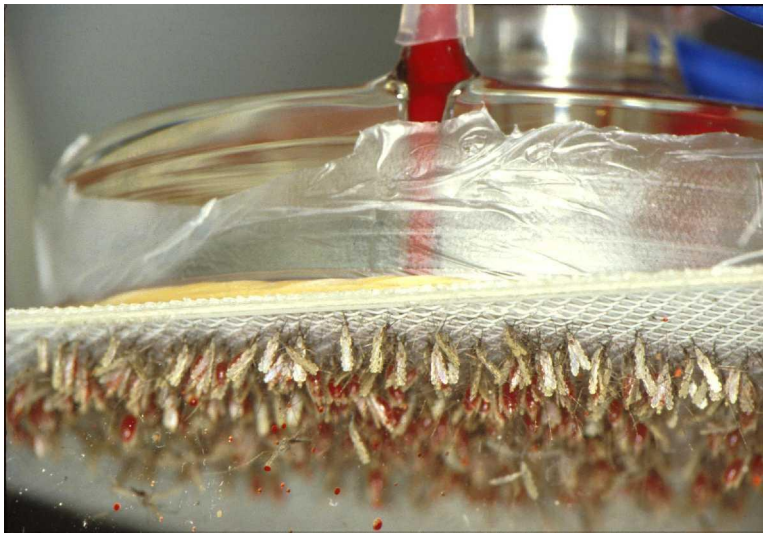

Figure 1.3 Membrane feeding assay. *Mosquitoes taking a blood meal through a parafilm membrane*

Three membrane feeding approaches are commonly used to answer different questions: i) venous blood samples can be offered to mosquitoes directly, without serum replacement or other modifications, to determine the infectiousness of naturally infected individuals; ii) venous blood samples can also be offered to mosquitoes in the presence of (autologous) test serum and control serum [16,87,88] to determine the transmission reducing activity of serum; iii) *in vitro* cultured gametocytes can be offered to mosquitoes in the presence of test

or control serum [99,100]. The latter method is called the standard membrane feeding assay (SMFA) and is the gold standard for determining the transmission reducing capacity of sera. In all membrane feeding assays, mosquito infection is assessed by the detection of oocysts on the mosquito midgut 7-9 days after feeding [20,27,101].

Transmission reducing activity can be determined in the last two approaches by comparing the number of developed oocysts in mosquitoes fed with test versus control serum. Serum from malaria endemic populations may reduce malaria transmission and this activity is associated with antibodies against sexual stage specific parasite antigens [93,95-98]. Two main groups of antigens are potential targets for human sexual stage immune responses: pre-fertilisation and post-fertilisation antigens. Antibodies against pre-fertilisation antigens such as Pfs230 and Pfs48/45 can block malaria transmission by preventing the formation of zygotes in the mosquito midgut [93,95,96,98]. Post-fertilisation antigens are involved in the sporogonic development after zygote formation. Antibodies against Pfs25, the most extensively studied post-fertilisation antigen, can prevent the penetration of ookinetes through the midgut epithelium [102-104]. The development of naturally occurring sexual stage immunity appears to differ from asexual stage immunity [91,105-107], but little is known about the acquisition and longevity of sexual stage antibodies. Most available data is derived from cross-sectional studies in microscopically confirmed gametocyte carriers. Studies in the general population and longitudinal studies are scarce.

Outline of this thesis

This thesis deals with four aspects of malaria transmission: i) the epidemiology of gametocytaemia, ii) the relation between gametocyte density and malaria transmission, iii) the influence of antimalarial drug treatment and treatment failure on gametocytaemia and malaria transmission and iv) human immune responses influencing malaria transmission. Both microscopy and QT-NASBA are used to determine gametocyte carriage and chapters are organised based on detection method: studies using microscopy are followed by studies using QT-NASBA.

i) the epidemiology of gametocytaemia

The objectives are to determine:

- risk factors for microscopic gametocytaemia in asymptomatic individuals (chapter 2)
- the prevalence of (sub-)microscopic gametocytaemia in symptomatic individuals (chapter 5,6)

- the prevalence of (sub-)microscopic gametocytaemia in the general population (chapter 8)

ii) the relation between gametocyte density and malaria transmission

The objectives are to determine:

- the relation between (sub-)microscopic gametocyte density and mosquito infection rates (chapter 7)
- the relevance of submicroscopic gametocytaemia for malaria transmission in symptomatic malaria patients (chapter 6,7)

iii) the influence of antimalarial drug treatment and treatment failure on gametocytaemia and malaria transmission

The objectives are to determine:

- the relation between parasite resistance to sulphadoxine-pyrimethamine and gametocytaemia (chapter 2-4)
- the impact of antimalarial drugs on (sub-)microscopic gametocytaemia (chapter 5,6)
- the impact of antimalarial drugs on post-treatment malaria transmission (chapter 6)

iv) human immune responses influencing malaria transmission

The objectives are to determine:

- the relation between exposure to malaria and sexual stage specific immunity (chapter 9,11,12)
- the relation between age and sexual stage specific immunity (chapter 9-12)
- the longevity of sexual stage specific immunity (chapter 11)

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Chapter 2

Plasmodium falciparum gametocyte carriage in asymptomatic children in western Kenya

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Summary

Studies on *Plasmodium falciparum* gametocyte development and dynamics have almost exclusively focused on patients treated with antimalarial drugs, while the majority of parasite carriers in endemic areas are asymptomatic. This study identified factors that influence gametocytaemia in asymptomatic children in the absence and presence of sulphadoxine –pyrimethamine (SP) antimalarial treatment.

A cohort of 526 children (6 months – 16 years) from western Kenya was screened for asexual parasites and gametocytes and followed weekly up to four weeks. Children with an estimated parasitaemia of ≥1,000 parasites/µl were treated with SP according to national guidelines. Factors associated with gametocyte development and persistence were determined in untreated and SP-treated children with *P. falciparum* mono-infection.

Gametocyte prevalence at enrolment was 33.8% (22/65) in children below five years of age and decreased with age. In the absence of treatment 18.6% of the children developed gametocytaemia during follow-up; in SP-treated children this proportion was 29.8%. Age, high asexual parasite density and gametocyte presence at enrolment were predictive factors for gametocytaemia. The estimated mean duration of gametocytaemia for children below five, children from five to nine and children ten years and above was 9.4, 7.8 and 4.1 days, respectively.

This study shows that a large proportion of asymptomatic untreated children develop gametocytaemia. Gametocytaemia was particularly common in children below five years who harbour gametocytes for a longer period of time. The age-dependent duration of gametocytaemia has not been previously shown and could increase the importance of this age group for the infectious reservoir.

Introduction

The transmission of *Plasmodium falciparum* parasites from humans to mosquitoes requires the presence of infectious gametocytes in the human peripheral blood. The prevalence of gametocytes is therefore commonly used as a parameter of malaria transmission. Any strategy that interferes with gametocyte development or persistence could result in a reduction of malaria transmission. For this purpose, it is important to identify parameters that influence gametocyte development as well as gametocyte dynamics under natural conditions. Although the majority of parasite carriers in endemic countries are asymptomatic [1], studies on gametocyte development and dynamics have almost exclusively focused on patients treated with antimalarial drugs [2-11]. In these studies, gametocytaemia is influenced by the duration of symptoms [3,7], anaemia [4,7], type of antimalarial treatment [2,4,5,9], response to treatment [3,7,10] and the age of infected individuals [6,8,11]. The relation between asexual parasites and gametocytes remains equivocal; a positive relation has been described between asexual parasite density at enrolment and gametocyte prevalence after treatment [6], but this was contradicted by several other studies [2-4].

In this study, we describe the dynamics of gametocytaemia in a cohort of asymptomatic children naturally exposed to malaria in western Kenya. Children with microscopically detectable parasitaemia were observed longitudinally. Those developing parasitaemia at levels requiring antimalarial treatment were treated with the first line drug sulphadoxine- pyrimethamine (SP). We identified factors associated with *P. falciparum* gametocyte production in the absence or presence of SP-treatment. Within the group of gametocyte carriers, we determined factors associated with gametocyte persistence.

Methods

Study area and design

This five week study was conducted in October – November 2001 in Mbita and Lwanda, small rural villages on the shores of Lake Victoria in Suba district, western Kenya. The transmission of *P. falciparum* is variable, depending upon local environmental conditions that support mosquito populations. The entomological inoculation rate (EIR) was recently estimated at six infectious bites per person per month [12]. Whilst variation in EIR may influence the clinical spectrum of malaria and gametocytaemia, it is unlikely to be a factor in this short study. Data were collected as described elsewhere (*Chapter 3*) [13]. Briefly, apparently healthy children (aged six months – sixteen years) were recruited from primary schools and the community. Screening for asexual parasites and gametocytes of *P. falciparum* took place weekly for a period of five weeks. Finger

prick blood samples were collected and thick blood smears were dried, stained with 10% Giemsa and examined microscopically. A slide was considered negative if no asexual parasites were seen after examination of 100 fields. Gametocyte and asexual parasite densities were assessed by counting against 500 and 200 leukocytes, respectively, and converted to numbers of parasites per µL by assuming a standard leukocyte count of 8,000/µL. Children with a positive blood smear with an estimated parasite density higher than 1,000 parasites/µL on the initial or any subsequent visits were treated immediately with pyrimethamine-sulphadoxine (SP) according to national guidelines. Only children who were treated once in the first or second week of the study were included in the analyses to achieve a minimum follow up period of three weeks. In case of treatment failure, alternative treatment was administered under supervision of the Clinical Officer. This resulted in the exclusion from the further study and follow-up.

Children or guardians signed an informed consent form. This study was approved by the ethical committee of the Kenya Medical Research Institute (KEMRI) and by the National Institute of Health (NIH) ethical review board of the United States.

Data analyses

Children who presented on at least three consecutive visits were used for analyses. Those who reported the use of antimalarial drugs in the two weeks prior to enrolment or had a *Plasmodium malariae* mixed infection were excluded. The influence of age on transformed (natural logarithm) parasite densities was analysed in linear regression models. Discrete data were compared using chi-square or Fisher's exact test, trends in binary outcomes using the Cochran-Armitage test for trend. Predictive factors for gametocyte prevalence on days seven or fourteen were tested in non-treated and SP-treated children separately, using multiple regression models with SAS Generalized Estimating Equations (GEE). Presentation parasitaemia was included in the models as a continuous variable and non-parasitaemic children were excluded from the analyses. A random effect was included in the models to allow for correlations within individuals. The Kaplan-Meier estimator was used to determine the proportion of parasitaemic children developing gametocytaemia during the follow-up period. Only for this analysis, gametocyte carriers at enrolment were excluded.

The duration of gametocytaemia in gametocyte carriers was estimated using the method described by Drakeley *et al.* [14], assuming that a single gametocyte positive slide represented gametocytes circulating in the peripheral blood for at least 2.5 days [15]. When children had gametocyte positive blood films on successive weeks, the estimated duration of gametocytaemia was the interval between observations plus 2.5 days.

Results

Gametocyte prevalence at enrolment was 33.8% (22/65) in children below five years of age and was negatively associated with age (table 2.1). Mean gametocyte density at enrolment was 24.5 (IQR 16–32) gametocytes/μL with no difference between age groups, whilst both asexual parasite prevalence and density decreased with age. SP treatment was given to 27.2% (143/526) of the children who presented with asexual parasites on the first or second visit with a geometric mean density of 1,108.0 (IQR 249–4,235) parasites/μL. A group of 121 parasitaemic children exhibited relatively low parasitaemia at presentation (geometric mean density of 203.0 (IQR 80–400) parasites/μL) and was not given treatment throughout the study.

The Kaplan-Meier estimator of time to gametocytaemia for these two groups of parasitaemic children is presented in figure 2.1.

Table 2.1 Parasitological data at enrolment

Age	N	Asexual parasite prevalence, % (n)[¥]	Asexual parasite density, mean (IQR)[†]	Gametocyte prevalence, % (n)[¶]	Gametocyte density, mean (IQR)[‡]
<5 years	65	73.8 (48)	2302.4 (530–14149)	33.8 (22)	25.3 (16–32)
5–9 years	211	50.2 (106)	357.8 (120–1040)	10.4 (22)	27.9 (16–32)
10–16 years	250	30.4 (76)	202.7 (80–440)	5.2 (13)	18.8 (16–23)
Total	526	43.7 (230)	444.8 (120–1220)	10.8 (57)	24.5 (16–32)

IQR = interquartile range [¥] Cochran-Armitage test for age-dependent trend in prevalence of asexual parasites ($Z = -6.75$, $P < 0.001$). [†] Geometric mean of parasite carriers only (parasites/μl). Linear regression for log-transformed asexual parasite density and age groups, $\beta = -1.155$; se = 0.147, $P < 0.001$. [¶] Cochran-Armitage test for age-dependent trend in prevalence of gametocytes ($Z = -5.91$, $P < 0.001$). [‡] Geometric mean of gametocyte carriers only (parasites/μL). Linear regression for log-transformed gametocyte density and age groups, $\beta = -0.123$; se = 0.166, $P = 0.46$.

At the end of the four week follow-up period, the cumulative proportion of gametocytaemia was lower in children not receiving antimalarial treatment than in SP treated children, 18.6% and 29.8%, respectively. In both groups, gametocyte prevalence during the first two weeks of follow-up was independently associated with several factors (table 2.2).

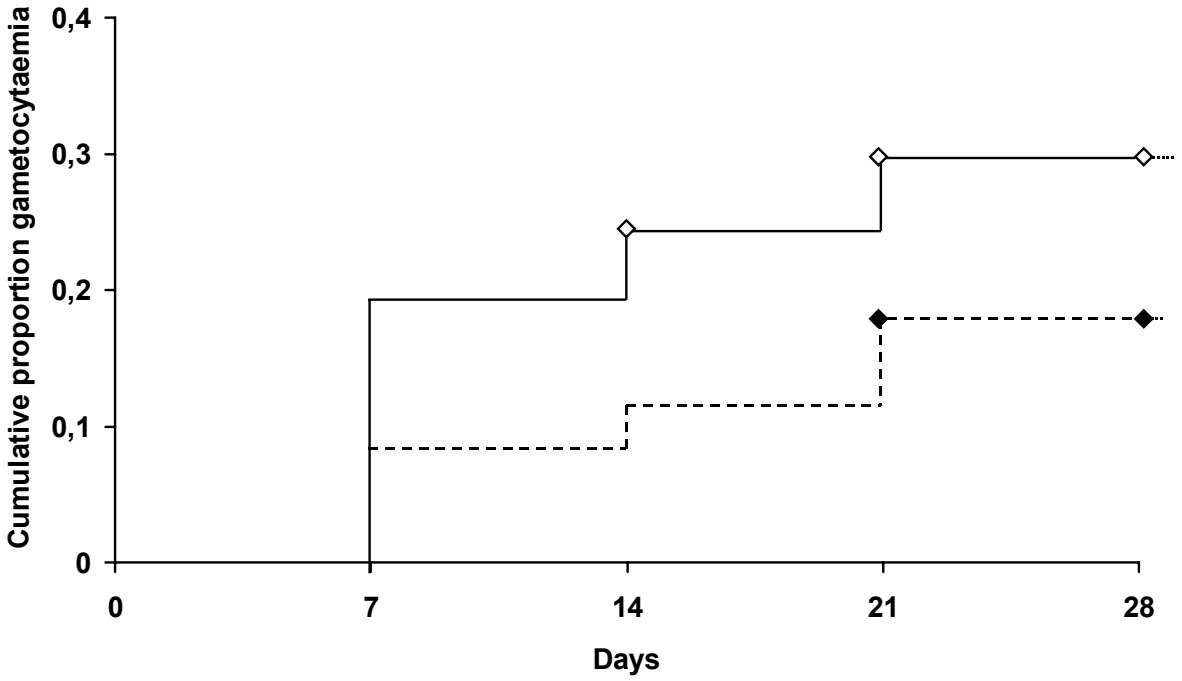

Figure 2.1 The cumulative proportion of gametocytaemia in SP-treated and untreated children. *Kaplan-Meier estimator of the time to gametocytaemia for untreated children (dotted line) and SP-treated children (solid line). Groups differed in asexual parasite density at enrolment. Patients with incomplete follow-up are marked on the curve. Gametocyte carriers on day 0 were excluded, as well as children who reported the use of antimalarial drugs prior to enrolment. Log-rank P = 0.05*

The risk for gametocytaemia during follow-up was positively associated with asexual parasite density at enrolment and negatively with age. Children with detectable gametocytes at enrolment were more likely to show gametocytes during follow-up, after adjustment for age and asexual parasite density at the start of treatment. In children who received SP treatment, the probability of gametocyte prevalence seemed higher on day seven after treatment compared to day fourteen, although this was not statistically significant. R1 resistance did not increase the risk of gametocytes during follow-up while R2/R3 resistance did show such an association, OR 3.40 (95% CI 1.61–7.19). The estimated mean duration of gametocytaemia was 9.4 days (range 2.5 – 23.5) for children below five years of age, 7.8 days (range 2.5 – 23.5) for

children aged five to nine years and 4.1 days (range 2.5–16.5) for children aged ten years and above. The duration of gametocytaemia was negatively associated with age (β = -1.65, se = 0.73; p = 0.02), after adjustment for asexual parasite density and gametocyte density at enrolment. Treatment with SP did not independently influence the duration of gametocytaemia.

Table 2.2 Adjusted odds ratio (OR) of the probability of gametocyte prevalence, using a multivariate random effect logistic model for untreated and SP-treated children separately.

Risk factors for gametocytaemia		Adjusted OR (95% CI)	
		No treatment	Treatment with SP
Day of follow-up	Day 7	1.48 (0.62 – 3.56)[¥]	1.77 (0.93–3.34)[¥]
	Day 14	1 (ref)	1 (ref)
Age	<5 years	6.34 (1.15–34.90)	5.38 (1.91–15.17)
	5–9 years	3.07 (0.98–9.55)[¥]	3.22 (1.19–8.72)
	10–16 years	1 (ref)	1 (ref)
Asexual parasite density at enrolment	Per 100 parasites/µL	1.05 (1.00–1.11)	1.01 (1.00–1.01)
Gametocyte prevalence at enrolment	Present	3.35 (1.22–9.18)	4.12 (2.11–8.02)
	Absent	1 (ref)	1 (ref)
SP treatment outcome	R2/3 resistance	-	3.40 (1.61–7.19)
	RI resistance	-	1.06 (0.45–2.47)[¥]
	Adequate response	-	1 (ref)

Adjusted = adjusted for all other variables in the model, OR = odds ratio, CI = confidence interval, ref = reference group, [¥] = not statistically significant, - = not applicable. A GEE model was used to allow for correlation between observations from the same individuals. Children reporting the use of antimalarial drugs prior to enrolment were excluded from these analyses.

Discussion

This descriptive study on the largely neglected group of asymptomatic children was part of a larger study on SP and gametocytes. A direct comparison between SP-treated and untreated children was not the aim of the current study and would require a different study design. SP-treated and untreated children

differed with regards to treatment and asexual parasite density at enrolment and were therefore analysed separately.

In this study, 18.6% of the asymptomatic non-treated children developed gametocytaemia during a four week follow-up period. Gametocytaemia was particularly common in children <5 years of age, of whom one-third were already carrying gametocytes on enrolment. The high gametocyte prevalence in young children is in line with previous findings [6,11], although the longer duration of gametocytaemia for this age group has not been reported before. The age dependent duration of gametocytaemia could not be explained by asexual parasitaemia, gametocyte density or SP treatment, suggesting a role for age-dependent immune suppression of gametocytaemia. Acquired sexual stage specific immunity has previously been reported to influence gametocyte prevalence [16], but remains poorly defined. Although the exact duration of gametocytaemia could not be determined because of weekly intervals in data-collection, the longer estimated duration of gametocytaemia in the youngest age group combined with their higher gametocyte prevalence could increase the importance of this age group for the infectious reservoir in this area. The importance of children as the infectious reservoir has been stressed elsewhere [17].

Differences in gametocytaemia during follow up between SP-treated and untreated children should be interpreted carefully since they are at least partially explained by differences in asexual parasite density at enrolment. The observed peak in gametocytaemia on day seven after SP treatment has been previously described [3-6] and may include gametocytes circulating prior to treatment since seven days may be short for gametocytogenesis, as was suggested by *in vitro* observations [18]. This study determined predictive factors for gametocytaemia for SP-treated and untreated children independently and found that risk factors were similar in both groups. Age and gametocyte presence at enrolment were previously described as predictors for gametocytaemia in symptomatic treated children [4,6-8,11]. Asexual parasitaemia at enrolment was positively associated with gametocytaemia during follow-up. Other studies [2-4] may not have found such an association because they only included patients with a high parasitaemia, resulting in little variation in asexual parasite density. Parasitological treatment failure was an important predictor of gametocytaemia in the SP-treated group (*Chapter* 3) [13].

This study shows that many asymptomatic children develop gametocytes, even in the absence of treatment. Gametocyte prevalence is especially high in children below five years of age who also appear to harbour gametocytes for a longer period of time. Risk factors for gametocytaemia are similar to those

described in symptomatic children and do not appear to differ between treated and untreated children.

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Chapter 3

Treatment failure of pyrimethamine-sulphadoxine and induction of *P. falciparum* gametocytaemia in children in western Kenya

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Summary

Sub-Saharan Africa faces increasing levels of resistance of *Plasmodium falciparum* parasites to the first-line drug sulphadoxine- pyrimethamine (SP). Successful treatment with SP is reported to induce gametocytes and drug-resistance may further increase gametocytaemia after treatment.

Treatment success, gametocyte prevalence and gametocyte density were determined in 224 asymptomatic children in western Kenya on day 7 after treatment with SP. Treatment failure (R2 or R3 resistance) was observed in 22% (49/224) of the children. The risk to show gametocytes on day 7 after treatment was higher in children with treatment failure compared to children with a sensitive infection, odds ratio 4.1 (95% CI 1.4-11.6) after adjustment for age and trophozoite density at the start of treatment. In addition, gametocyte density was also higher upon SP treatment failure.

These findings are reason for concern since the increased gametocyte prevalence and density after SP treatment failure may increase the spread of SP-resistant parasite strains in the population.

Introduction

Chloroquine (CQ) resistance is a major problem in treating malaria in sub-Saharan Africa and many countries have switched to sulphadoxine-pyrimethamine (SP), which is an affordable alternative [1]. In Kenya, SP was chosen to replace CQ as the first-line anti-malarial drug in treating uncomplicated malaria in 1997 [2]. SP rapidly eliminates *Plasmodium falciparum* parasites and ensures an infection-free period for the host through its long residual time in the body; however this long residual time may enhance the selection of resistant parasite strains [3].

While it has been suggested that the prevalence and density of gametocytes in the population can be reduced following treatment of asexual stages [4,5], other studies report that *P. falciparum* chemotherapy with SP promotes the appearance of gametocytes [6-8]. Parasite resistance against SP is becoming increasingly common [2,9] and may further increase gametocytaemia and transmission. Recrudescent infection is a known risk factor for gametocyte carriage after treatment [10] and increased gametocytaemia and transmission have been clearly described in CQ resistant infections [8,11,12]. While an opposite effect was suggested for SP resistance [13], recent studies indicate that SP treatment failure can also result in a higher gametocyte prevalence but not in a higher gametocyte density [11,14].

In this study, we establish the level of SP treatment failure in a rural area in western Kenya and the prevalence and density of gametocytaemia in SP-sensitive and SP-resistant infections.

Methods

This study was conducted in Mbita and Lwanda, small rural villages on the shores of Lake Victoria in Suba district, western Kenya. The transmission of *P. falciparum* is highly variable and the entomological inoculation rate (EIR) was recently estimated at 6 infectious bites per person per month [15]

A cohort of apparently healthy children was recruited from primary schools within the compound of the ICIPE Mbita Point Field Station and the surrounding community. Screening for asexuals and gametocytes of *P. falciparum* took place weekly for a period of 5 weeks. Finger prick blood samples were collected and thick blood smears were dried, stained with 10% Giemsa and examined microscopically. A slide was considered negative if no asexual parasites were seen after examination of 100 fields. Gametocyte and trophozoite densities were assessed by counting against 500 and 200 leukocytes, respectively. Gametocyte and trophozoite counts were converted to numbers of parasites per μL by assuming a standard leukocyte count of 8000/μL [16]. Children with high blood parasitaemia (≥3000/μL) or with concomitant diseases requiring

hospitalisation were referred to the Mbita Health Centre but were excluded from this study. The inclusion criterion for the study was a positive blood smear with an estimated parasite count between 1000-3000 parasites/μL. Children fulfilling this criterion were referred and accompanied to the Mbita Health Centre for first line anti-malarial treatment with sulphadoxine- pyrimethamine (SP) and follow-up. Drug administration was supervised in close collaboration with the Clinical Officer at the Mbita Health Centre and the administered dosage was 0,5 tablet 500/25 mg SP per 10 kg bodyweight, according to the National Malaria Treatment Guidelines.

Information about previous SP use and the duration of symptoms was recorded in a short questionnaire. Selected children were followed up for 5 weeks to study the dynamics of gametocytaemia. In this study, we determined the effect of SP on gametocyte prevalence and density on day 7 after treatment, comparing sensitive infections and treatment failures. SP sensitive infection was defined as the clearance of asexual parasites by day 7 after treatment. If parasites were still present on day 7, treatment was considered a failure and children were referred to the health centre for appropriate alternative treatment. Alternative treatment was installed under supervision of the Clinical Officer according to the National Malaria Treatment Guidelines. The outcome of this study was success or failure of the initial treatment; children were not monitored after this. In terms of WHO standards, this study compares sensitive and R1 resistant infections with R2 and R3 resistant infections [17]. To study the effects of SP on gametocytaemia, children without any detectable level of gametocytaemia at the start of treatment and without reported use of SP in the two weeks prior to the screening were selected and followed up for 7 days after treatment.

Children or guardians were asked to sign an informed consent form. This study was approved by the ethical committee of the Kenya Medical Research Institute (KEMRI) and by the National Institute of Health (NIH) ethical review board of the United States.

Statistical analysis

Differences between the sensitive and treatment failure group in baseline parameters were tested for statistical significant differences using the non-parametric Wilcoxon-rank sum test in case of continuous variables and the Fisher's exact test in case of dichotomous variables. Geometric means of trophozoite and gametocyte densities were calculated using the geometric mean of Williams [exponential (arithmetic mean (Log_e (x_i + 1)))-1]. Multiple logistic and linear regression models were used to test differences between the sensitive and treatment failure group on day 7 after treatment, allowing adjustment for potential confounders. All variables that altered the regression coefficient for treatment success were entered in the regression model as covariables. The

dependent variable in the logistic regression models was the presence or absence of gametocytes. Crude and adjusted odds ratios (OR) with 95% confidence intervals (95% CI) were presented. The dependent variable in the linear regression model was the log-transformed gametocyte density with a detection level of 16 gametocytes/μL. For non-gametocyte carriers, a gametocyte density of 1 gametocyte/μL was assumed to allow log-transformation. Crude and adjusted relative differences with 95% CI were presented.

Results

A total of 843 children were included in the study, with a mean age of 7.7 years (range 0-16 years). Asexual parasite prevalence at first visit was 49.0% (350/715), gametocyte prevalence 16.8% (120/715). During the study 224 children with estimated asexual parasite counts ≥1000/μL were treated for uncomplicated malaria with SP. A total of 78% (175/224) of the children responded well to the therapy and the remaining 22% (49/224) still had detectable asexual parasites on day 7 after treatment.

The prevalence of gametocytes clearly differed between the sensitive and the treatment failure group (table 3.1).

Table 3.1. Comparison of malariometric indices between SP-treatment success and failure.

	SP-treatment outcome on day 7		
	Success	Failure	P-value
N	120	28	
Age, mean (25-75th percentiles)	8.3 (5.0-12.0)	6.9 (3.3-10.8)	0.09
Sex, male (%)	56 (46.7)	14 (50.0)	0.84
Trophozoite density/μL on day 0, geometric mean (25-75th percentiles)	1,265.6 (520 – 3,240) ¥	2,095.2 (766-5,389) ¥	0.048
Prevalence of gametocytes on d7 (%)	13 (10.8)	10 (35.7)	0.001
Gametocyte density/μL on d7, geometric mean (range)	0.41 (0 – 112) ¥	2.37 (0 - 64) ¥	0.001

¥ *Geometric mean of Williams. Children with gametocytes at enrolment were excluded (n = 76).*

The risk to show gametocytes on day 7 after treatment in children with treatment failure was higher compared to children with a sensitive infection, after adjustment for age and trophozoite density at the start of treatment, OR 4.1 (95% CI 1.4-11.6). Fever at the start of treatment (>37.4°C) did not confound the relation and was not included in the regression model. Next to the

increased gametocyte prevalence, gametocyte density also appeared to be higher upon unsuccessful SP treatment. On average, the gametocyte density was 2.1 (95% CI 1.3-3.3) times higher following SP treatment failure, after adjustment for age and trophozoite density at the start of treatment (table 3.2).

Table 3.2 Comparison of gametocytaemia and gametocyte density on day 7 after treatment between SP-treatment success and failure.

	Treatment outcome on day 7	
	Success	Failure
Risk of gametocytaemia		
Crude OR (95% CI)	1.00 (ref)	4.6 (1.7-12.0)
Adjusted OR (95% CI)	1.00 (ref)	4.1 (1.4-11.6) ¥
Gametocyte density		
Crude relative difference (95% CI)	1.00 (ref)	2.4 (1.5-3.9) †
Adjusted relative difference (95% CI)	1.00 (ref)	2.1 (1.3-3.3) ¥, †

OR = odds ratio, 95% CI = 95% confidence interval. ¥After adjustment for age and trophozoite density at the start of treatment. †For non-gametocyte carriers, a density of 1 gametocyte/μL was assumed to allow log-transformation.

Discussion

The high level of treatment failure (R2 and R3 resistance) of SP that was observed in this study is comparable with SP resistance rates recently found in Kenya, ranging from 9.5% to 34.5% [2,18]. Children with treatment failure were more likely to show gametocytes on day 7 after treatment than children with a sensitive infection.

The increased risk of gametocytaemia and the elevated gametocyte density for treatment failure could not be explained by differences in age, trophozoite density or by the previous use of SP. Duration of parasitaemia and symptoms before treatment could also influence gametocytaemia [8,10,11] but were not known for the majority of children. The latter was probably not relevant in this study, considering the asymptomatic status of the children at enrolment.
The observed increased risk of gametocytaemia following SP treatment failure is in line with recent observations [11,14], the higher gametocyte density was not found in a previous study [11].

Although the exact influence of SP on the process of gametocytogenesis remains unclear, the higher prevalence of gametocytes after SP treatment failure is certainly reason for concern. While the infectiousness of SP resistant gametocytes remains to be established, a strong suggestion for an increased transmission comes from the finding that the elevated gametocyte prevalence

after successful SP treatment in comparison with CQ treatment results in a higher estimated average probability of transmission at a population level [6]. This study clearly suggests that parasite resistance for SP could worsen the situation by further increasing the risk of gametocytaemia and thereby possibly the transmission of SP resistant parasites.

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Chapter 4

Plasmodium falciparum dhfr but not dhps mutations associated with sulphadoxine-pyrimethamine treatment failure and gametocyte carriage in northern Ghana

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Summary

Both use of sulphadoxine-pyrimethamine (SP) and SP-resistance of *Plasmodium falciparum* are increasing in sub-Saharan Africa. Mutations in the *P. falciparum* dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes can predict treatment failure of SP, however, the degree of this relationship varies regionally. In northern Ghana, pre-treatment *dhfr/dhps* genotypes were examined in 126 children and associations with PCR-corrected SP treatment outcome and gametocyte carriage were analysed.

SP treatment failure within four weeks of follow-up occurred in 28% (35/126) of the children. Among all pre-treatment isolates, the *dhfr* triple mutation (Ile-51+Arg-59+Asn-108) was detected in 47%. As compared to *dhfr* wildtype parasites, the presence of the *dhfr* triple mutation increased the risk of treatment failure ten-fold. Likewise, parasite clearance was delayed in the presence of *dhfr* variants. *Dhfr* mutants and *dhps* Gly-437 were selected in treatment failure isolates. Gametocytaemia one week following treatment was strongly associated with *dhfr* mutations. Remarkably, this was also true for the prevalence of gametocytes at recruitment. *Dhps* alleles did neither influence treatment outcome nor gametocyte carriage.

In northern Ghana, the prevalence of the *dhfr* triple mutation can be used as a tool to screen for and to monitor SP resistance. The lack of association between *dhps* alleles and SP treatment outcome suggests a minor role of these molecular markers in this region at present.

Introduction

Facing the aggravation of chloroquine (CQ) resistance [1], sulphadoxine-pyrimethamine (SP) has evolved into the first-line drug for uncomplicated malaria in several African countries. However, considering the ease of selecting resistant *Plasmodium falciparum* by SP pressure and the increasing spread and intensity of resistance, this drug is feared to have a short useful therapeutic life in Africa [2-5]. In Ghana, two recent trials demonstrated SP treatment failure within two weeks in 14% and 22% of the treated individuals [6,7]. Currently, amodiaquine-artesunate is being introduced as first-line antimalarial in this country [8]. Nevertheless, because SP is comparatively cheap and much if not most of antimalarial treatment is home-based [9] the drug's usage may still continue. Moreover, SP is used in intermittent preventive treatment in pregnancy [10] and, possibly, in infancy [11]. Monitoring SP resistance thus remains an important task. Molecular markers of SP resistance, i.e. mutations in the parasite's dihydrofolate reductase and dihydropteroate synthase genes (*dhfr*, *dhps*), are easy and relatively cheap to assess and reasonably predictive for treatment outcome. A *dhfr* codon 108 exchange (Ser-108 → Asn-108) represents the core mutation which in the presence of Ile-51 and Arg-59 confers pronounced resistance to pyrimethamine and predicts SP treatment failure in some areas. Thr-108 or Val-16 in combination with Asn-108 are linked with resistance to cycloguanil, and Leu-164 in addition to the *dhfr* triple mutation (Asn-108+Ile-51+Arg-59) with high-grade resistance to pyrimethamine. As for *dhps*, mutant Gly-437 is associated with sulphonamide resistance while additional changes (436-Ala, 540-Glu, Gly-581, 613-Ser) appear to increase its degree (reviewed in [12]).

The predictive value of these markers varies geographically, depending on baseline prevalences, age and immunity [13-15]. *Dhfr/dhps* patterns have not been related to SP treatment outcome in Ghana where the drug has served as a second-line antimalarial so far. Moreover, while gametocyte carriage is known to be elevated following SP failure (*Chapter 3*)[16], *dhfr* mutants were associated with increased gametocytaemia despite a high therapeutic efficacy of SP in Colombia [17]. In northern Ghana, we examined associations of *dhfr/dhps* mutations with SP treatment outcome and with pre- and post-treatment gametocyte carriage.

Methods

P. falciparum isolates were obtained from children with uncomplicated malaria who participated in a randomised, placebo-controlled, and double-blind treatment trial on SP, SP *plus* amodiaquine, and SP *plus* artesunate. That trial was conducted at the end of the rainy season of 2002 (August – December), in Tamale, Northern Region, Ghana, and the results are described elsewhere [6]. In the present report, data from 126 children with complete clinical and

parasitological follow-up of four weeks after treatment with SP alone are analysed. The study protocol was approved by the Ethics Committee, Ministry of Health, Tamale, Northern Region, by the Health Research Unit, Ministry of Health, Accra, and by the World Health Organization Secretariat Committee on Research Involving Human Subjects. Written informed consent of patients' parents was obtained.

Patients were febrile children aged 6 to 59 months with *P. falciparum* monoinfection (≥2,000 ≤200,000/μL; WHO 2002). None had severe malnutrition, a febrile disease other than malaria, "danger signs" [18], severe malaria [19], severe anaemia (haemoglobin (Hb) < 5 g/dl), or a history of hypersensitivity to SP. Patients with mixed *Plasmodium* species infections or developing a concomitant disease during follow-up were excluded from analysis. Treatment consisted of a single dose of SP (25/1.25 mg/kg, Fansidar, Roche, Switzerland). Children were observed for at least 30 minutes. If vomiting occurred, treatment was repeated. Paracetamol was given on days 0, 1, and 2 if the axillary temperature was >38°C. Patients were examined clinically and parasitologically on days 0, 1 (only clinically), 2, 3, 7, 14, 21, 28, and at unscheduled visits in case of fever.

Therapeutic outcome during four weeks of follow-up was classified into early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF) or adequate clinical and parasitological response (ACPR)[20]. Mefloquine (Lariam, Roche, Switzerland; 15 mg/kg on first day and 10 mg/kg on second day) was given in case of treatment failure.

Venous blood was collected. Asexual parasites and gametocytes (only 104 samples available for this purpose on day 7) were counted per ≥200 and 500 white blood cells, respectively, on Giemsa-stained thick blood films, and densities were calculated based on a putative mean WBC count of 8,000/μL. Pre-treatment levels of CQ and pyrimethamine in blood were assessed by ELISA assays with lower limits of detection of 10 ng/mL and 25 ng/mL, respectively [21,22]. Hb concentrations were measured by a HemoCue photometer (Ångelholm, Sweden). DNA was extracted by commercial kits (QIAmp, Qiagen, Germany). Differentiation of recrudescence from new infection was achieved by comparing PCR-generated *msp1* and *msp2* genotype patterns in matched pairs of isolates obtained on admission and on the day parasitemia reappeared [23].

P. falciparum dhfr and *dhps* alleles were identified by restriction fragment length polymorphisms of PCR-generated amplicons [24]. Genotyping included: *dhfr*: Ser-108 → Asn-108, Ser-108 → Thr-108, Asn-51 → Ile-51, Cys-59 → Arg-59, Ala-16 → Val-16, Ile-164 → Leu-164; *dhps*: Ser-436 → Ala-436, Ala-437 → Gly-437, Lys-540 → Glu-540, Ala-581 → Gly-581, and Ala-613 → Ser-613.

Frequencies were compared by χ^2-tests or Fisher's exact tests, and continuous variables by Student's t-tests, Mann-Whitney U-tests, or Kruskal-Wallis tests as applicable. To estimate the risk of treatment failure, mixed alleles

(both wildtype and mutant allele present) were considered mutant. A p-value < 0.05 was considered statistically significant.

Results

The baseline characteristics of the 126 patients are shown in Table 4.1 and the distribution of *dhfr* and *dhps* alleles in Figure 4.1. *Dhfr* Thr-108, Val-16, and Leu-164 were not detected. Assigning mixed alleles as mutant, Ile-51, Arg-59, and Asn-108 occurred in 56% (70/126), 65% (82/126), and 72% (91/126) of the isolates, respectively. Figure 4.2 illustrates the grouping of *dhfr* alleles for further analysis. Isolates with the *dhfr* triple mutation were seen in 47% (59/126), and quadruple (*dhfr* triple + *dhps* Gly-437) and quintuple (*dhfr* triple + *dhps* Gly-437 + Glu-540) variants in 44% (55/126), and 0.8% (1/126), respectively.

Table 4.1. Baseline characteristics of 126 Ghanaian children with uncomplicated malaria

Parameter	Value
No.	126
Female : male	68 : 58
Age in months, median(range)	28.5 (6-59)
Weight in kg, median (range)	10.4 (5.5-19.1)
Axillary temperature, median in °C (range)	38.8 (37.5-41.0)
Haemoglobin level, median in g/dL (range)	8.0 (5.0-12.9)
Geometric mean parasite density/µL (range)	36,358 (2,068-175,333)
Pre-treatment gametocytes	
Prevalence, % (n/N)	19 (24/126)
Geometric mean density/µL (range)	50 (16-1,120)
Pre-treatment chloroquine	
Prevalence, % (n/N)	66 (83/126)
Median concentration, ng/mL (range)	32 (10-718)
Pre-treatment pyrimethamine	
Prevalence, % (n/N)	1.6 (2/126)
Concentrations, ng/mL	78 & 326
PCR-corrected treatment outcome within 4 weeks	
Early treatment failure, % (n/N)	5.6 (7/126)
Late clinical failure, % (n/N)	3.2 (4/126)
Late parasitological failure, % (n/N)	19 (24/126)
Adequate clinical and parasitological response, % (n/N)	72.2 (91/126)

SP treatment failure was strongly associated with the *dhfr* mutations. The mere presence (as compared to absence) of the core mutation Asn-108 and of the triple *dhfr* mutation effected ORs (95%CIs) for treatment failure of 4.0 (1.2-16.9) and 2.9 (1.2-7.2), respectively. The risk estimate improved when comparing triple mutant strains to *dhfr* wildtype parasites (Table 4.2; positive predictive value, 39%; 95%CI, 32-42%; negative predictive value, 94%; 95%CI, 81-99%).

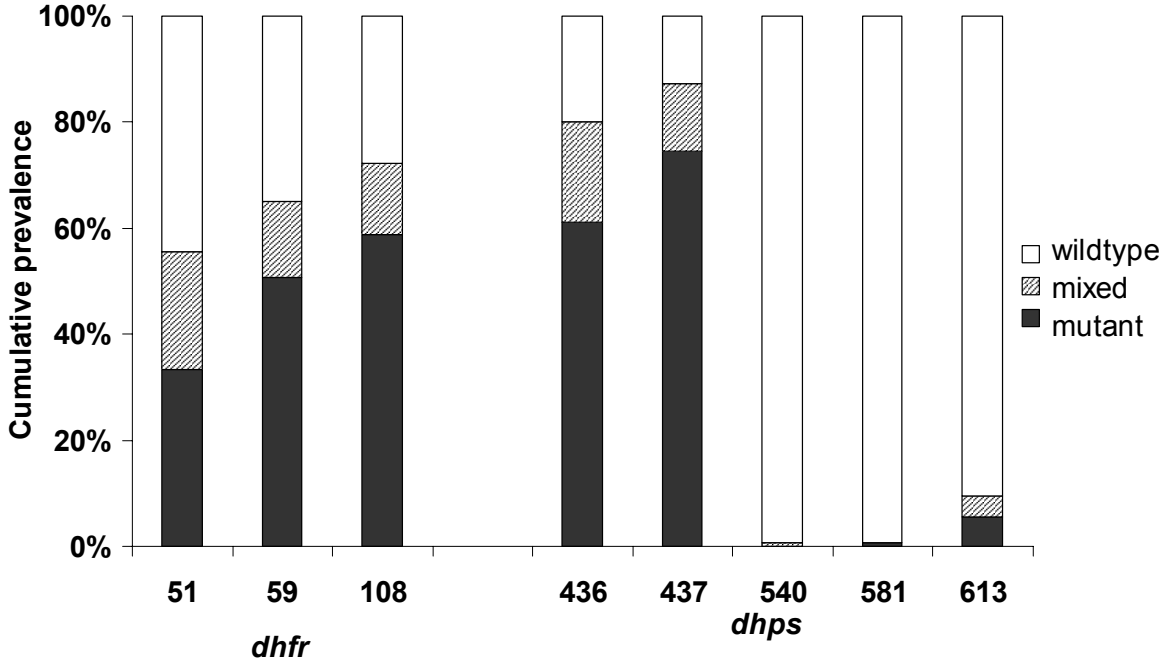

Figure 4.1. Prevalence of dhfr and dhps alleles in 126 Ghanaian children.

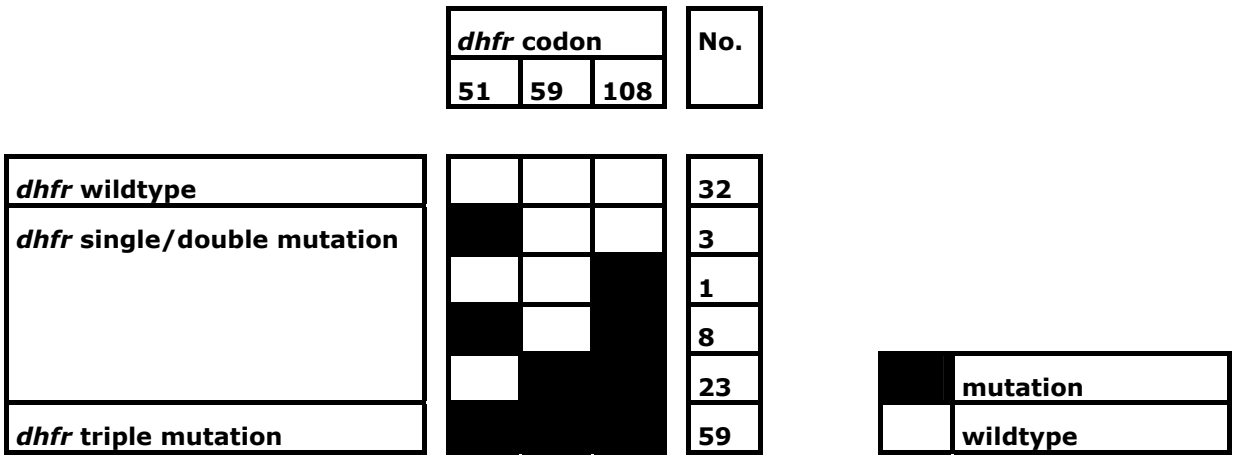

Figure 4.2. Scheme of grouping dhfr alleles.

Table 4.2. Associations between *dhfr/dhps* alleles and sulfadoxine-pyrimethamine treatment failure in 126 Ghanaian children

Mutation	No. Failure	No. ACPR	OR (95%CI)	*P*	Sensitivity (95%CI)	Specificity (95%CI)
dhfr						
WT	2	30	1			
S or D	10	25	6.0 (1.1-59.9)	0.02	83 (54-97)	55 (48-58)
T	23	36	9.6 (2.0-88.7)	0.0008	92 (75-99)	46 (39-48)
dhps[¥]						
WT	4	12	1			
S (Gly-437)	31	77	1.2 (0.3-5.5)	1.0	89 (77-96)	14 (9-16)
dhfr/dhps combinations						
dhfr WT	2	30	1			
dhfr S/D + *dhps* S	8	21	5.7 (1.0-58.9)	0.04	80 (47-96)	59 (52-62)
dhfr T + *dhps* S	22	31	10.7 (2.2-98.9)	0.0005	92 (74-99)	49 (42-52)

APCR = adequate parasitological and clinical response within four weeks of follow-up; OR = odds ratio; 95%CI = 95% confidence interval; WT = wildtype; S = single mutation; D = double mutation; T = triple mutation. [¥]dhps WT, wildtype alleles at codons 437, 540, 581, codons 436, 613 disregarded; dhps S, Gly-437, codons 540, 581 WT, codons 436, 613 disregarded

Adjusting for sex, pre-treatment CQ, pyrimethamine and parasite density (all not associated with treatment outcome) in a logistic regression model did not change these associations substantially (data not shown). After adjusting for age (in months, odds ratio (OR), 1.05; 95%CI, 1.02-1.08), the risk of treatment failure was increased nine-fold (OR, 8.6; 95%CI, 1.6-46.0; *P* = 0.01) and fifteen-fold (OR, 14.9; 95%CI, 3.0-74.0; *P* = 0.001) in children harbouring strains with *dhfr* single/double mutations and with the *dhfr* triple mutation, respectively, as compared to *dhfr* wildtype infections. When considering only failures occurring within the first two weeks of follow-up in this model (*n* = 19), the risk estimates were similar (single/double mutation, OR, 9.3; 95%CI, 1.0-86.8; *P* = 0.05; triple mutation; OR, 11.9; 95%CI, 1.4-101.7; *P* = 0.02). ETF was not predicted by any of the *dhfr* variants, the prevalence of isolates with the *dhfr* triple mutation in this group being 43% (3/7) compared to 40% (36/91) in ACPR (late failures, 71%, 20/28). The impact of *dhfr* mutations on parasite clearance is shown in Figure 4.3. Parasitaemia following SP treatment was more common in case of *dhfr* mutant parasites than with wildtype infections for all but one day of follow-up.

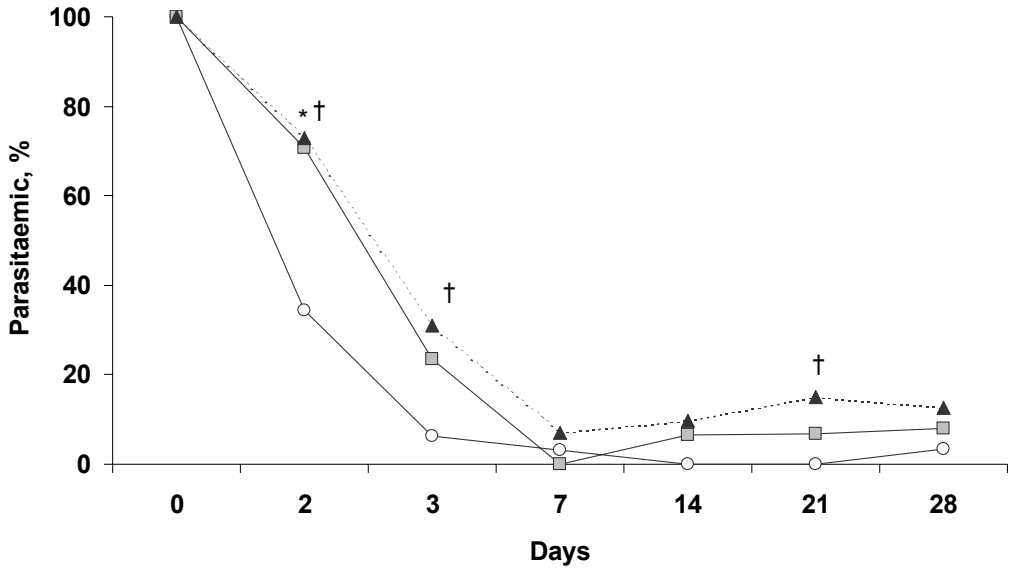

Figure 4.3. Proportion of parasitaemic children following treatment according to dhfr genotype of infecting parasite strains. *Triangles indicate dhfr triple mutation, squares single/double mutation and circles wildtype. Four re-infections were not considered. *wildtype vs. single/double mutant, P < 0.05; †wildtype vs. triple mutant, P < 0.05*

None of the *dhps* alleles was associated with treatment outcome (presence *vs.* absence, OR (95%CI), Ala-436, 1.3 (0.2-5.4); Gly-437, 1.2 (0.3-5.4); Ser-613, 0.2 (0-1.6)). Glu-540 and Gly-581 were too rare (one each) for sensible analysis. None of the *dhps* mutations significantly affected parasite clearance or substantially improved the predictive value of the *dhfr* alleles; results for Gly-437 are shown in Table 4.2. Albeit, Gly-437 was found at a significantly higher frequency in isolates obtained at treatment failure comparing to all recruitment isolates (P = 0.02) as was the case for all *dhfr* variants. Comparing the 35 pairs of recruitment and failure isolates, the increase in prevalence of Gly-437 was at borderline significance (P = 0.07; Figure 4.4).

Pre-treatment gametocyte carriage was associated with *dhfr* mutations, the highest prevalence of 29% occurring in children harbouring strains with triple mutations (Table 4.3). Gametocytaemia on day 7 after treatment was seen in 63% of the individuals and was also associated with *dhfr* variants, adjusting for the presence of gametocytes at enrolment (Table 4.3). Age, asexual parasitaemia at enrolment and previous drug use did not confound this relation. No associations between *dhfr* alleles and gametocyte density was observed. *Dhps* alleles were not associated with gametocyte prevalence or density at any time (data not shown).

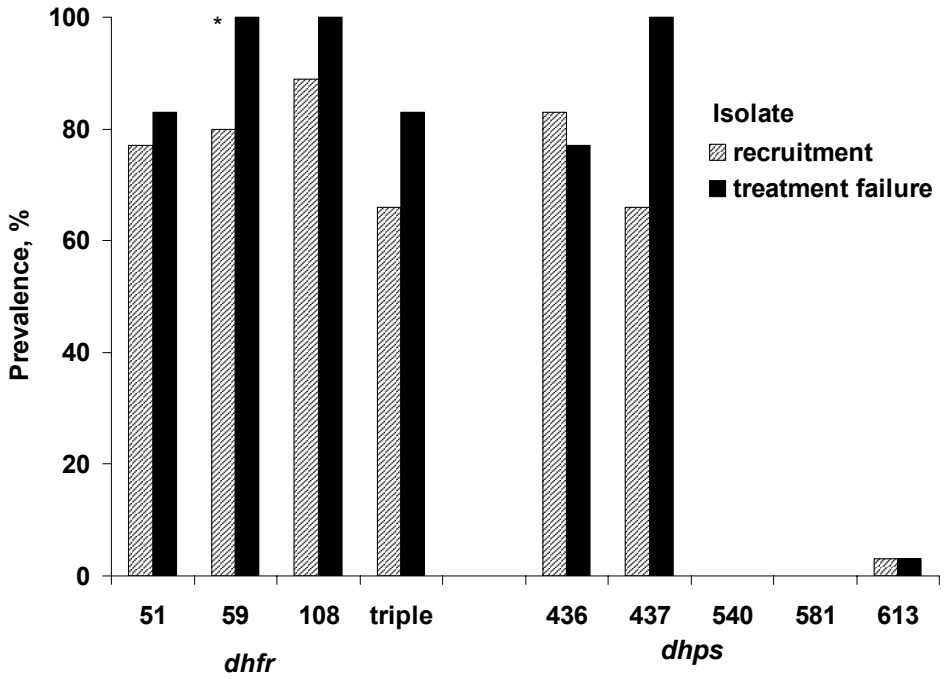

Figure 4.4 Prevalence of Plasmodium falciparum dhfr/dhps mutations in isolates obtained at recruitment and at treatment failure in 35 children. **P = 0.02 (McNemar paired test, exact significance probability)*

Table 4.3. Associations of *dhfr* alleles with gametocyte prevalence at enrolment and one week following treatment

dhfr mutation	Enrolment (*n* = 126)			Day 7 after treatment (*n* = 104)		
	Gametocyte prevalence (%)	OR (95%CI)	*P*	Gametocyte prevalence (%)	OR (95%CI)†	*P*
wildtype	9.4 (3/32)	1	-	43.3 (13/30)	1	-
single or double	11.4 (4/35)	1.3 (0.2-9.2)	1	80.8 (21/26)*	5.7 (1.7-19.4)	0.006
triple	28.8 (17/59)	3.9 (1.0-22.4)	0.03	66.7 (32/48)*	2.1 (0.8-5.7)	0.12

*OR = odds ratio; 95%CI = 95% confidence interval; *P < 0.05 (univariate analysis); †adjusted for enrolment gametocytaemia (OR, 4.1; 95%CI, 1.0-22.9).*

Discussion

In this study in northern Ghana, we show that the mere presence of the *dhfr* triple mutation confers a three-fold increased risk of treatment failure. In comparison to *dhfr* wildtype parasites, this risk is increased ten-fold. The relatively low specificity points to additional factors involved in treatment success, e.g. drug absorption, immunity, folate levels [25,26], parasite density [27], and impairs a reliable prediction of the individual treatment outcome. Inclusion of the above named and further factors in analysis would increase the precision of predicting resistance. However, in practice, moleculars marker need to be simple and easy to apply. Also for feasibility, we categorized mixed genotypes (both wildtype and mutant alleles present) as mutant. Essentially, for mapping SP resistance in simple cross-sectional surveys in this area, the ratio between the prevalence of the *dhfr* triple mutation and the treatment failure rate might serve as crude tool. Yet, it needs to be evaluated whether this index is similar in different locations and remains stable over time. Our findings also highlight the necessity to locally establish a relation between molecular markers and treatment outcome. In Malawi and Uganda [28,29], *dhfr* Arg-59/*dhps* Glu-540 strongly predicted treatment failure. In our study population this combination was seen only once rendering it inapplicable. One reason for this difference may be the quasi omnipresence of resistant *dhfr* alleles in the East African sites whereas in Tamale, roughly a third of the *dhfr* codons 51, 59, and 108, respectively, were wildtype.

Nevertheless, almost half of the isolates and thus substantially more than the one third observed in southern Ghana[30] exhibited the *dhfr* triple mutation. This was an unexpected finding because in Ghana, SP has been used as a second-line antimalarial so far and rarely in the study area which is corroborated by the very low prevalence of pyrimethamine in blood. Even considering that the ELISA assay applied can detect the drug for only two weeks after therapeutical SP intake [21] this renders exclusive selection by previous or home-treatment with SP unlikely. Trimethoprim-sulfamethoxazole is a cheap and widely used antibiotic. Cross-resistance between pyrimethamine and trimethoprim has been observed *in vitro* and on the molecular level [31-33]. Although one small-scale study reported opposite results[34], selection of *dhfr* variants by widespread use of trimethoprim-sulfamethoxazole might partially explain the abundance of the *dhfr* triple mutation in our study population. This notion is supported by a recent study from Guinea-Bissau[35]. Likewise, the use of sulfa drugs in bacterial infections has been suggested to contribute to a high prevalence of the *dhps* variants Ala-436 and Gly-437 in Brazil [36]. However, so far, there are no data to substantiate this hypothesis.

A minor role of *dhps* mutations in SP treatment failure has been reported from various sites [13,37-40]. In Cameroon, the *dhps* genotype did not affect the

efficacy of SP [39]. Similarly, in the present study, *dhps* alleles had no influence on treatment outcome, parasite clearance or gametocyte carriage. This contrasts with findings from East Africa where *dhps* variants increased the likelihood of treatment failure in the presence of *dhfr* triple mutants [28,29,41]. The reason for the complete independence of SP efficacy from *dhps* alleles, particularly from the key mutation Gly-437, in northern Ghana is unclear. A major role of exogenous folate in determining sulfadoxine resistance has been observed *in vitro* [42] but respective blood concentrations were not measured in our patients. Omar *et al.*[13] suggested that in highly endemic areas, immunity may mask the impact of the presumably less important *dhps* mutations to a larger extent than the one of *dhfr* alleles. In essence, our observations fit the concept that SP resistance is mediated only secondarily by *dhps* variants once *dhfr* triple mutant parasites predominate [38,41]. In the present study, only one isolate exhibited the highly resistant quintuple *dhfr/dhps* mutation [28]. The role of *dhps* variants may change once further mutations, particularly Glu-540, emerge under drug pressure. Selection of Gly-437 was observed in this trial. Of course, we cannot state whether this and resistant *dhfr* alleles emerged from below detection level or were induced by SP. Albeit, findings from Kenya suggest that selection for *dhps* mutants occurs more easily the more frequent *dhfr* mutations are[43].

Gametocyte carriage one week following treatment was increased in children harbouring *dhfr* mutant parasites. Irrespective of the notorious difficulties in microscopically detecting low density gametocytaemia[44] *dhfr* mutations influenced gametocyte prevalence rather than density, which was reported previously [17]. Even though this association held true after correcting for pre-treatment gametocytaemia, gametocytes observed on day 7 are likely to comprise portions formed before as well as during treatment. Prolonged clearance of resistant strains under drug pressure may increase the chance of parasites committed to gametocyte formation to progress. This has been suggested for both SP and CQ resistant infections [17,45]. Though infectivity of the gametocytes observed was not assessed. Increased gametocyte carriage following treatment of *dhfr* mutant strains will likely contribute to spreading resistance. Likewise, following CQ treatment, *P. falciparum* initially exhibiting the *chloroquine resistance transporter* key mutation were found to produce approximately 40-fold higher oocyst numbers in *Anopheles* than did sensitive strains[45].

Due to the methodology applied, it is impossible to separate *dhfr* genotypes in gametocytes and asexual parasites. Increased gametocytaemia at enrolment has also been observed for subsequently CQ resistant infections [46-48]. Previous drug use could have selected for mutant parasite strains with gametocytes persisting for a longer period than drug pressure being demonstrable. As mentioned before it seems unlikely that such selection can be

attributed exclusively to SP usage. In any case, the observation of *dhfr* mutations being associated with both pre- and post-treatment gametocyte carriage is worrying since it supports similar findings on CQ resistance[45] pointing to a transmission advantage of resistant parasites.

In conclusion, despite a lack of complete agreement, *P. falciparum dhfr* mutations predict treatment failure to SP in the study area of northern Ghana, and are associated with prolonged parasite clearance and increased gametocyte carriage. *Dhps* alleles show no such features. For now, the *dhfr* triple mutation may serve to monitor the distribution, emergence, and development of SP resistance in this region.

Acknowledgements

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Chapter 5

(Sub)microscopic *Plasmodium falciparum* gametocytaemia in Kenyan children after treatment with sulphadoxine-pyrimethamine monotherapy or in combination with artesunate

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Summary

The effects of drugs on *Plasmodium falciparum* transmission stages may reduce the spread of parasites in the population and contribute to malaria control. Detailed quantitative studies on (sub)microscopic gametocytaemia have become feasible with the availability of real-time *Pfs25* quantitative Nucleic Acid Sequence-based Amplification (QT-NASBA), which can be used to detect gametocyte densities above 20 gametocytes/mL from *in vitro* cultures.

Gametocyte dynamics were investigated in children with uncomplicated *P. falciparum* malaria after treatment with sulphadoxine-pyrimethamine (SP) or a combination of SP and artesunate (SP+AS), in a 28-days drug efficacy study.

This study demonstrated that gametocyte prevalence in 873 samples from symptomatic Kenyan children was 2.8 times higher by QT-NASBA compared to microscopy. Microscopy-positive cases showed a significant correlation with QT-NASBA for gametocyte density. At enrolment, gametocyte prevalence was 86% by QT-NASBA compared to 22% by microscopy. Gametocytes were detected in 97% of children in at least one blood sample and in 38% of children in all samples obtained during the 28-days follow-up. Both the risk of gametocyte carriage and gametocyte density were considerably higher after treatment with SP compared to SP+AS. Gametocyte prevalence and density decreased with time in the SP+AS group, but not in the SP-treated children.

Our data suggest that the potential of malaria transmission remains high even after treatment with artemisinin combination therapy, although prevalence and density of gametocytes is lower after SP+AS.

Introduction

Clinical symptoms of malaria are caused by cyclical proliferation of asexual *Plasmodium* parasites in the patient's red blood cells. Spread of the disease depends on the presence of mature sexual stage parasites (gametocytes), which do not replicate or cause clinical symptoms, but are essential for transmission from humans to the mosquito vector.

As measures to prevent malaria are not completely efficient, early treatment of symptomatic infections is a major component of malaria control strategies. Drugs are designed to cure clinical symptoms and therefore mostly target asexual stages; the impact of drug treatment on gametocytes and transmission has long been neglected. Some drugs increase (e.g. chloroquine and sulphadoxine-pyrimethamine) or decrease (e.g. artemisinin derivatives) gametocyte prevalence [1-4]. Due to increasing resistance to most affordable antimalarial drugs, many countries are forced to revise drug treatment strategies. Generally, artemisinin combination therapies (ACT) are recommended to replace current first line drugs such as chloroquine and sulphadoxine-pyrimethamine (SP).

Combination of artemisinin derivatives with other antimalarial drugs may protect the other drug from development of resistance. The benefit of better cure rates induced by ACT combined with its effect on transmission due to its alleged gametocytocidal effects [5] may justify increased costs for treatment.

The key factors that trigger sexual stage development are not yet understood. Gametocytes are derived only from a small subset of asexual parasites and only a fraction of patients develop patent gametocytaemia [6]. However, the apparently low prevalence of gametocytes contrasts with the successful spread of malaria and the difficulties to control malaria transmission [7]. Recently, a number of studies have addressed the effect of antimalarial drug treatment on gametocytes [3,8-13]. Most of these studies used microscopy for detection and quantification of gametocytes, but it has been shown that patients without microscopically detectable gametocytes can infect mosquitoes [14,15] and submicroscopic gametocytaemia can be common [16-19]. Detailed quantitative studies on submicroscopic gametocytes are now possible with the recently developed gametocyte-specific *Pfs25* quantitative Nucleic Acid Sequence-Based Amplification (QT-NASBA) [20]. The assay is performed in real-time, and quantifies mature *Plasmodium falciparum* gametocytes in blood samples with a lower detection limit of 20-100 gametocytes per millilitre of blood. The objective of this study was to investigate the effect of SP and of SP plus artesunate (SP+AS) treatment on *P. falciparum* gametocyte dynamics using *Pfs25* real-time QT-NASBA.

Methods

Real-time Pfs25 QT-NASBA and nucleic acid extraction

Nucleic acids were extracted from blood samples using the Guanidiumisothiocyanate (GuSCN)/silica procedure as described by Boom *et al.* (1990). Total parasite load was quantified by real-time 18S rRNA QT-NASBA as previously described [21]. *Pfs25* mRNA QT-NASBA was performed as described by Schneider *et al.* [20] with some adjustments to enable real-time detection. Briefly, real-time QT-NASBA for *Pfs25* mRNA (Genbank accession number AF193769.1) was performed on a NucliSens EasyQ analyser (bioMérieux) using the NucliSens Basic Kit for amplification according to manufacturer's manual at a KCl concentration of 80mM. Reactions were carried out in a total reaction volume of 10μL per reaction. Forward primer: 5'-gac tgt aaa taa acc atg tgg aga-3' (nucleotides 204-227); reverse primer: 5'-aat tct aat acg act cac tat agg gag aag gca ttt acc gtt acc aca agt ta-3' (T7 promoter sequence, linker and nucleotides 338-359); *Pfs25* molecular beacon: 5'-TexasRed-cga tcg ccc gtt tca tac gct tgt aac gat cg-DABSYL-3' (molecular beacon stem of 6 paired nucleotides and nucleotides 259-278). The time point during amplification at which the fluorescence, resulting from detection of target amplicons, exceeded the mean fluorescence of three negative controls + 20 standard deviations (SD) was calculated (time to positivity). The use of a standard gametocytes stage V dilution series allowed calculation of the number of gametocytes present in test samples [20].

Detection limit and interassay variability

Blood samples of in vitro parasite culture of *P. falciparum* NF54 [22] were collected as described by Schneider *et al.* [20], with the exception that internal controls for quantification were not added to the samples. Ten-fold dilution series of gametocytes ranging from 10^6 to 10 gametocytes per millilitre of blood were made, nucleic acids extracted using the GuSCN/silica procedure[23] and the number of gametocytes was quantified by real-time *Pfs25* QT-NASBA.

Field study

A drug efficacy study was performed from October to December 2003 in Mbita, Western Kenya (0° 25'S, 34° 13'E), according to the WHO protocol (WHO, 2002). Mbita is a rural region on the shores of Lake Victoria experiencing endemic malaria with seasonal variation in transmission intensity. A description of the study site can be found in *Chapter 2* [24,25]. Study approval was obtained from the ethical and scientific review boards of the Kenya Medical Research Institute (SSC 791).

In total, 245 children aged 6 months to 10 years with uncomplicated clinical *P. falciparum* malaria confirmed by microscopy (500-100,000

parasites/µL) were included in the study after their guardians gave informed consent. Children were randomised to receive either SP (Fansidar, Roche, Switzerland; 125mg sulphadoxine and 6.25mg pyrimethamine/ 5kg as a single dose) or SP+AS (Arsumax, Sanofi, France; 20mg/ 5kg daily for 3 days) treatment. Details of randomisation and treatment procedures are described elsewhere (*Chapter 6*) [26]. Follow-up was performed by the study clinician on days 1, 2, 3, 7, 14 and day 28 post-treatment and at any time the child showed symptoms of disease. At all sampling points, a blood smear for microscopic detection of parasites was made and stained with 10% Giemsa. Asexual parasites were counted against 200 leucocytes, gametocytes against 500 leucocytes and converted to number of parasites per volume assuming 8,000 white blood cells/µL blood. Slides were considered negative when no parasites were detected after viewing 100 microscopic fields at a 10x100 magnification. Additional to the standard protocol (microscopy), at all sampling times, a small blood sample on filter paper was collected for genotyping. A blood sample (50 µL) for QT-NASBA, stored in GuSCN lysis buffer until further analysis, was collected on the same days except for day 2. Samples for all methods were obtained from the same finger prick.

Statistical analysis

Statistical analyses were performed in SPSS version 12.0.1 and STATA 7.0. Mann-Whitney U test was used for comparisons between groups for continuous variables and Chi-square test for dichotomous variables. Results of microscopy and QT-NASBA were compared by Spearman correlation and paired t-test.

Gametocyte prevalence and density after treatment were assessed by use of QT-NASBA in a random subset of 118 children, aged 6 months to 5 years, who completed at least 7 days of follow-up (age groups of 1, 2, 3, 4 and 5 years consisted of 32, 23, 20, 21 and 22 children respectively). This group was representative for the entire study group regarding gender (50% male), mean weight (13.3 kgs) and mean age (2.8 years). Gametocyte prevalence at enrolment was not statistically different between the two treatment groups (Chi square tests, P=0.770 for microscopy and P=0.421 for QT-NASBA), neither were total parasite and gametocyte densities at enrolment (Mann-Whitney U tests, total parasite density P=0.345 and 0.516; gametocyte density P=0.732 and 0.538 for microscopy and QT-NASBA, respectively). Logistic regression was used to determine the relation between gametocyte prevalence and time after treatment and type of drug treatment (SP or SP+AS), adjusting for age in years and outcome of drug treatment (adequate clinical response or late treatment failure [27]). Odds Ratios (OR) were calculated with 95% confidence intervals (95% CI). For gametocyte density, linear regression was used with inclusion of the same parameters, but only gametocyte-positive samples were used in the

analysis. In case of multiple observations per individual, Generalised Estimating Equations[28] models (STATA 7.0) were used to allow for correlation of observations from the same individual.

Results

Detection limit and interassay variation of real-time Pfs25 QT-NASBA

Eighteen independent gametocyte series from in vitro parasite culture were collected and processed over a period of 2 months. Gametocyte densities from 10^2 to 10^6 gametocytes/mL could be detected consistently with 10 gametocytes/ml being close to the detection limit of 20-100 gametocytes/mL of blood. Interassay variability (SD/mean*100%), including variations in culture material and nucleic acid extractions, is below 10% for all measured gametocyte densities and is highest for the lower densities (table 5.1). Regression analysis on the ^{10}log number of parasites present in the control samples and the resulting time to positivity showed a consistently significant correlation ($P<0.001$) with a correlation coefficient of on average 0.98 (range 0.92-0.99).

For negative controls, blood samples (50 µL) were obtained from 20 malaria-naïve blood donors in Nijmegen, the Netherlands and from 10 healthy volunteers at Kenya Medical Research Institute, Nairobi, Kenya with no recent history of malaria. The 30 blood samples were all confirmed negative by *Pfs25* QT-NASBA.

Table 5.1 Interassay variability (SD/mean*100%) for real-time *Pfs25* QT-NASBA in 18 independent dilution series from in vitro cultured gametocytes.

Log gct/ml	TTP	SD	Interassay variability
6	46.60	1.95	4.2%
5	53.48	3.32	6.2%
4	64.56	4.26	6.6%
3	72.70	5.03	6.9%
2	84.06	6.29	7.5%
1[¥]	89.85	8.33	9.3%

Log gct/mL = ^{10}log of the number of gametocytes/ ml of blood originally determined by microscopy and diluted. TTP = Average time to positivity for Pfs25 QT-NASBA for each gametocyte concentration. SD = standard deviation. [¥]Calculations for 8 measurements only, 10/18 were below the detection limit and excluded from the analysis

Field study, comparison microscopy and QT-NASBA for gametocyte quantification

In total, 873 samples obtained during the drug efficacy study in Mbita were quantified for *P. falciparum* gametocytes both by microscopy and by *Pfs25* QT-NASBA. Of these samples, 72% were positive for gametocytes by QT-NASBA, while gametocytes were detected in only 26% of the blood smears by microscopy. The *Pfs25* QT-NASBA confirmed 92% of the microscopically positive samples; 65% of the microscopically gametocyte negative samples was positive in the QT-NASBA with a geometric mean of $6.0*10^2$ gametocytes/mL (IQR $1.7*10^2$–$2.1*10^3$), which is well below the detection limit of microscopy ($1.6*10^4$ gametocytes/mL). In total, inconsistent results between *Pfs25* QT-NASBA and microscopy were shown in 33/873= 3.7% of the samples. Quantification of gametocytes by *Pfs25* QT-NASBA and microscopy showed a statistically significant correlation (Spearman correlation, $\rho=0.452$, $P<0.001$). However, for samples with microscopically detected gametocytes, quantification by QT-NASBA was slightly lower than quantification by gametocyte microscopy (paired *t*-test, $P<0.01$).

Gametocyte dynamics after SP and SP+AS treatment

Of the 245 children included in the study comparing treatment efficacy of SP alone versus SP+AS (*Chapter 6*) [26] a random subset of 118 children was included for QT-NASBA analysis. Gametocyte prevalence at enrolment was 22% as detected by microscopy and 86% by *Pfs25* QT-NASBA. Gametocytes were detected in 97% of all children at some time point and in 38% at all time points during the 28-days follow-up period by *Pfs25* QT-NASBA.

The SP+AS treated group (n=55) showed a significantly lower risk of gametocyte carriage (OR 0.137; 95% CI 0.065 - 0.288) and a reduced gametocyte density (β -0.395; 95% CI -0.652 – -0.139) compared to the SP treated group (n=63) in the *Pfs25* QT-NASBA analysis. Microscopic analysis showed a less pronounced effect of treatment on prevalence (OR 0.322; 95% CI 0.185, 0.561) and no statistically significant effect on gametocyte density.

Since SP and SP+AS showed different effects on gametocytes, data were further analysed separately for each treatment group. Gametocyte prevalence by QT-NASBA decreased significantly with time after SP+AS treatment in days (OR 0.905; 95% CI 0.877 – 0.934) but not after SP treatment (Fig. 5.1). Similar results were obtained with microscopy. However, microscopy data show a peak in gametocyte prevalence at day 7 after SP treatment that was not detected by *Pfs25* QT-NASBA (Fig. 5.1). Gametocyte density decreased significantly (β -0.032; 95% CI -0.048 – -0.017) after treatment with SP+AS but not after treatment with SP, where gametocyte densities peaked at day 7. This is shown

in figure 5.2 where the median of gametocyte densities relative to the day 0 density are shown.

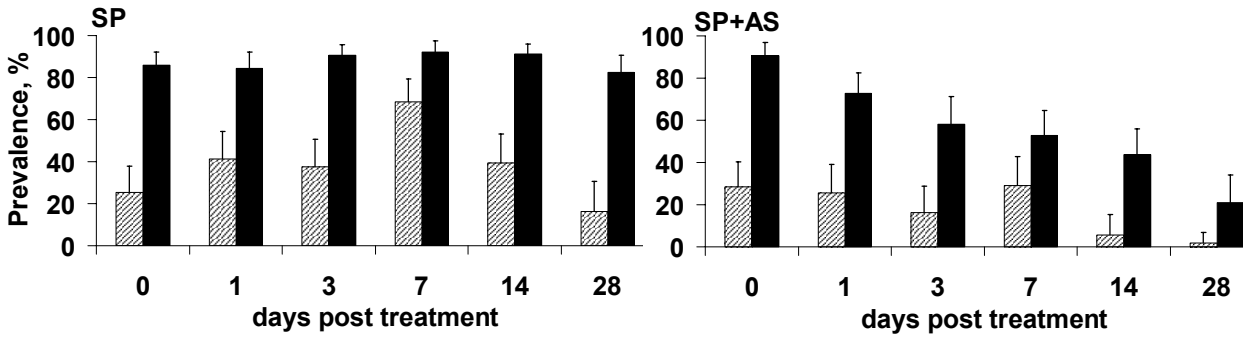

Figure 5.1. Gametocyte prevalence from day 0 to 28 after treatment, calculated by microscopy (shaded bars) and Pfs25 QT-NASBA (black bars) for SP monotherapy and SP+AS combination therapy. *Error bars indicate the upper limit of the 95% confidence intervals.*

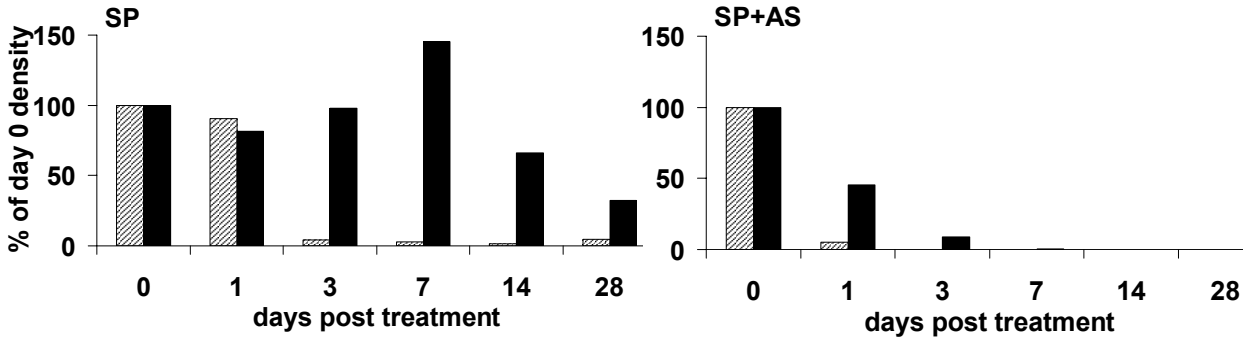

Figure 5.2. Median QT-NASBA total parasite (shaded bars) and gametocyte (black bars) densities relative to day 0. *Figures are given for SP monotherapy and SP+AS combination therapy.*

Discussion

The real-time *Pfs25* QT-NASBA is more sensitive for quantification of *P. falciparum* gametocytes compared to standard microscopy (detection limits of 20-100 and 16,000 gametocytes/mL of blood, respectively). Inconsistent results between gametocyte detection by microscopy or *Pfs25* QT-NASBA, including both false positive and false negative results, occurred in 3.7% of all samples. Similar low percentages of inconsistent results were found during a cross-sectional study in Burkina Faso (Ouédraogo *et al.* unpublished). The observation that QT-NASBA results in lower gametocyte densities compared to microscopy may be explained by an overestimation of gametocyte densities by microscopy.

Counting of gametocytes against 500 leucocytes was started only after the first gametocyte has been detected, which introduces a positive bias in quantification.

Over 50% of the microscopically gametocyte-positive *in vivo* samples from the study in Kenya contained only 1 gametocyte/500 white blood cells, a density just above the detection limit of microscopy. The increased sensitivity of gametocyte detection by *Pfs25* QT-NASBA revealed high prevalences of submicroscopic levels of gametocytes at enrolment and during the 28-days follow-up period. This confirms previous studies showing submicroscopic gametocytaemia in endemic samples [15,18,19,29,30]. Almost all children harbour gametocytes at some time point during the study and 38% show persistent gametocytaemia during follow-up. This suggests that the majority of children with uncomplicated symptomatic *P. falciparum* infection contribute to the infectious reservoir both before and after treatment with antimalarial drugs.

Our data further show that the gametocyte reservoir in symptomatic children may be much larger than previously indicated by microscopic analyses. Many gametocyte carriers carry very low densities of gametocytes (geometric mean 600/mL) in the circulation. Data suggest that gametocyte densities below the microscopy detection limit can infect *Anopheles stephensi* mosquitoes in membrane feeding experiments (*Chapter 7*)[31]. Therefore, low gametocyte densities *in vivo* may infect mosquitoes, especially when taking into account that *P. falciparum* gametocytes in the peripheral blood may not be homogeneously distributed [32,33].

Both gametocyte carriage and higher gametocyte densities are considerably more prevalent in the SP group compared to SP+AS. A post-treatment reduction of gametocyte prevalence and density over time was present in the SP+AS but not in the SP treatment group. The peak gametocyte prevalence at day 7 after SP treatment observed by microscopy confirms data from previous studies [1,8,34] but was not confirmed by QT-NASBA. In contrast, QT-NASBA showed a peak in gametocyte density in the SP group at day 7. This difference may be explained as higher gametocyte densities being more likely to be detected by microscopic analysis. The peak of gametocyte density at day 7 after SP treatment cannot be the result of *de novo* induction under drug pressure, as gametocyte development takes longer than 7 days. This peak may reflect the slower clearance of immature gametocytes after SP treatment compared to SP+AS treatment. These results show that data on gametocyte prevalence are highly dependent on the sensitivity of the detection method and microscopy is inferior for this purpose. In conclusion, the *Pfs25* QT-NASBA is the preferred technique to detect and quantify *P. falciparum* gametocytes. The use of *Pfs25* QT-NASBA in transmission studies provides more detailed data and makes studies, especially those with small sample sizes, more efficient.

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Chapter 6

Moderate effect of artemisinin-based combination therapy on transmission of *Plasmodium falciparum*

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Summary

Artemisinin-based combination therapy (ACT) reduces microscopically confirmed gametocytaemia and mosquito infection. However, molecular techniques have recently revealed high prevalences of submicroscopic gametocytemia. Our objective here was to determine the effect of sulphadoxine-pyrimethamine (SP) monotherapy and treatment with SP plus amodiaquine (AQ), SP plus artesunate (AS), and artemether-lumefantrine (AL; Coartem) on submicroscopic gametocytaemia and infectiousness.

Kenyan children (*n* = 528) 6 months–10 years of age were randomized to 4 treatment arms. Gametocytaemia was determined by both microscopy and *Pfs25* RNA–based quantitative nucleic acid sequence–based amplification (*Pfs25* QT-NASBA). Transmission was determined by membrane-feeding assays.

Gametocyte prevalence, as determined by *Pfs25* QT-NASBA, was 89.4% (219/245) at enrollment and decreased after treatment with SP plus AS, SP plus AQ, and AL. Membrane-feeding assays for a group of randomly selected children revealed that the proportion of infectious children was as much as 4-fold higher than expected when based on microscopy. ACT did not significantly reduce the proportion of infectious children but did reduce the proportion of infected mosquitoes.

Submicroscopic gametocytemia is common after treatment and contributes considerably to mosquito infection. Our findings should be interpreted in the context of transmission intensity, but the effect of ACT on malaria transmission appears to be moderate and restricted to the duration of gametocyte carriage and the proportion of mosquitoes that are infected by carriers.

Introduction

Antimalarial drug treatment targets the asexual blood stages of *Plasmodium falciparum,* which are responsible for clinical disease and death. Sexual-stage parasites—that is, gametocytes—do not cause clinical disease, and they can also be found in the circulation of infected persons and can infect mosquitoes taking a blood meal. After fertilisation and further sporogonic development, infectious parasites appear in the mosquito salivary gland. With each subsequent blood meal, parasites are transmitted to a new person, resulting in the spread of malaria among the human population. The majority of the currently used antimalarial drugs do not kill gametocytes, and the effects of drugs on these stages are often ignored during the development of antimalarial drugs and policies.

The resistance of *P. falciparum* to such antimalarial drugs as chloroquine and sulfadoxine-pyrimethamine (SP) is rapidly increasing in many areas of sub-Saharan Africa. This has forced many African countries to abandon monotherapy with these drugs as first-line antimalarial treatment [1]. Because drug resistance is less likely to develop when combination therapy is used [2,3], there is increasing acceptance that it, rather than monotherapy, is the way forward, with artemisinin being one of the preferred components [1,2,4]. Compared with monotherapy, artemisinin-based combination therapy (ACT) provides higher clinical efficacy [5,6] in the absence of drug resistance. An additional advantage of ACT is its effect on sexual stages—it lowers the prevalence and density of posttreatment gametocytemia [5,7,8] and, subsequently, the prevalence of mosquito infection [7,9,10]. ACT may reduce malaria transmission at the population level [8,11], and the specific reduction in the transmission of resistant strains [8,11,12] further suggests that ACT may counteract the spread of drug resistance.

In contrast to asexual parasites, gametocytes often circulate at such low densities that they may not be detected by standard microscopy [13-15]. Indeed, *Pfs25* RNA–based quantitative nucleic acid sequence–based amplification (*Pfs25* QT-NASBA) [16] recently revealed that a large proportion of children with symptomatic malaria had submicroscopic gametocytemia (*Chapter 5)* [17].

Our objective here was to determine, in Kenyan children treated for uncomplicated malaria, the clinical efficacy of drug regimens that are currently in use or are being considered for inclusion in national guidelines in different African countries; gametocyte prevalence by *Pfs25* QT-NASBA [16]; and posttreatment infectiousness to *Anopheles gambiae* mosquitoes. The regimens analyzed were SP monotherapy and 3-day courses of SP plus amodiaquine (AQ), SP plus artesunate (AS), and artemether-lumefantrine (AL) [1,6].

Methods

The present study was conducted from October to December in 2003 and 2004 in Mbita, a rural village on the shores of Lake Victoria in Suba District, western Kenya. The main malaria vectors in the area are *A. gambiae*, *A. funestus*, and *A. arabiensis*. Malaria transmission is high and perennial, with parasite prevalences in the human population ranging from 24.4% to 99.0% [18]. Generally, the rainfall pattern is bimodal, with a long rainy season between March and May and a short rainy season between October and December. The study protocol (SSC 791) was approved by the Scientific Steering Committee and the Ethical Review Committee of the Kenya Medical Research Institute. Written informed consent was obtained from a parent or guardian of the participating children.

Study population and treatment

Subjects were recruited at the clinic of the International Centre of Insect Physiology and Ecology (ICIPE) Mbita Point Field Station and came from an area of ~10 km around the ICIPE compound. Children 6 months–10 years of age with either a temperature of >37.5°C or a history of fever and with *P. falciparum* monoinfection at an asexual parasite density of ≥500 parasites/μL were eligible for recruitment. Exclusion criteria were the inability to take drugs orally, known hypersensitivity to any of the drugs given, reported treatment with antimalarial chemotherapy during the previous 2 weeks, evidence of chronic disease or of an acute infection other than with a malarial parasite, residence outside of the study area, and signs of severe malaria. Children were randomly allocated to receive one of the four following treatment regimens: (i) SP monotherapy, which consisted of 25 mg/kg sulfadoxine and 1.25 mg/kg pyrimethamine as a single dose (Fansidar; Roche) plus placebo once daily for 3 days; (ii) SP plus 4 mg/kg AS (Arsumax; Sanofi) once daily for 3 days; (iii) SP plus 10 mg/kg AQ (Camoquine; Pfizer) once daily for 3 days; and (iv) AL (Coartem; Novartis Pharma), administered as half a tablet (20 mg of artemether and 120 mg of lumefantrine) per 5 kg of body weight in a 6-dose regimen (at enrollment and 8, 20, 32, 44, and 56 h (±90 min) after the initiation of treatment).

Children were randomized to the SP monotherapy and the SP plus AS arms in 2003 and to the SP monotherapy, SP plus AS, SP plus AQ, and AL arms in 2004. The sizes of the arms were not equal, for several reasons. At the time of the study, AL was not recommended for children who weighed <10 kg. In addition, the 6-dose AL regimen required treatment to be administered at the child's home. Therefore, only those children who weighed ≥10 kg and resided <5 km from the clinic (to allow supervised treatment at home) were eligible for randomization to the AL arm. This resulted in a smaller number of children in this treatment arm. The numbers of children in the SP monotherapy arm and the SP plus AS arm were highest because the relevance of the random-feeding assays

became evident during the latter part of data collection in 2004. For these assays, an additional number of children had to be randomized to the SP monotherapy and the SP plus AS arms. Therefore, to supplement the number of children who received these regimens in 2003 (n = 245), 81 children were randomized to the SP monotherapy and the SP plus AS arms at a 1 : 2 ratio in 2004. This ratio was chosen to facilitate comparison between the SP plus AS arm and the AL arm, for which parasitological differences were expected to be smallest.

For SP monotherapy and treatment with SP plus AQ and SP plus AS, treatment was administered by staff at the recruitment clinic; for treatment with AL, the first dose was administered by staff at the recruitment clinic, and subsequent doses were administered by field assistants at the child's home. All medication was given with a local fatty food (mandazi), to facilitate absorption. Each child was observed for 20 minutes after administration of treatment, and a replacement dose was given if vomiting occurred. Repeated vomiting led to exclusion from the study; paracetamol (10 mg/kg) was given until symptoms had subsided.

Children were encouraged to return to the recruitment clinic on days 1, 2, 3, 7, 14, and 28 after initiation of treatment and at any time the child became ill. Field assistants visited the homes of children who failed to appear at the clinic, to collect additional samples. Other than those administering medication, all staff engaged in the trial were blinded to the treatment arm of each child.

Microscopy and *Pfs25* QT-NASBA

Blood smears were stained with 10% Giemsa for 10 min and then screened for asexual parasites and gametocytes at enrollment and on days 3, 7, 14, and 28. Slides were considered to be negative if no parasites were observed in 100 microscopic fields, resulting in a sensitivity of ~5 gametocytes/μL of blood. Asexual parasites and gametocytes were counted against 200 and 500 white blood cells (WBCs), respectively, and the counts were converted to parasites per microliter on the assumption of a density of 8,000 WBCs/μL. For quality control, 10% of the slides were reread. Parasite detection by *Pfs25* QT-NASBA was done as described elsewhere [16,19], using a NucliSens EasyQ analyser (bioMérieux) as described elsewhere for *Pfs25* mRNA [16]. Nucleic acid was extracted from 50 μL blood samples as described by Boom et al. [20]. The *Pfs25* QT-NASBA technique is gametocyte specific and has a detection limit of 20–100 gametocytes/mL. NucliSens Basic kits were used for amplification, in accordance with the manufacturer's instructions. A standard dilution series of mature, *in vitro* cultured NF54 gametocytes [21] was included in each run. Detection by *Pfs25* QT-NASBA was done for a random selection of children from each treatment arm and for all of the samples included in the membrane-feeding assays.

Mosquito membrane feeding and dissection

Membrane-feeding assays were conducted on day 14, when the gametocytes that develop after treatment are expected to be mature [22]. Children >2 years of age whose parent or guardian gave specific consent for the procedure were eligible for the membrane-feeding assays. Two groups of subjects were selected: (i) children with microscopically confirmed gametocytemia on day 7, which has been shown to be the time when the peak prevalence after treatment occurs [7,23]; and (ii) a random selection of children from each treatment arm (including children with and without gametocytemia by both microscopy and *Pfs25* QT-NASBA).

For all membrane-feeding assays, 3 mL venous blood samples were obtained and fed to ~150 locally reared [24] 4 to 5-day-old female *A. gambiae* sensu stricto mosquitoes via an artificial membrane attached to a water-jacketed glass feeder maintained at 37°C. After 10–15 min, fully fed mosquitoes were selected and kept on glucose for 7 days at 27°C–29°C, at which time midguts were dissected in 2% mercurochrome. Midguts were examined microscopically for oocysts, with a second microscopist confirming their presence if observed. Assay results were considered to be valid if a minimum of 20 mosquitoes were dissected. For the randomly selected children, exactly 30 mosquitoes were dissected per child.

Molecular genotyping

For DNA analyses, blood was collected as dry blood on Whatman glass microfiber filter papers. DNA was extracted using a chelex-based method. Alleles of the polymorphic locus *msp2* were compared between pretreatment and posttreatment parasite isolates by polymerase chain reaction, as described elsewhere [10]. The procedure of Cattamanchi *et al.* [25] was followed in that indeterminate samples for which a majority of novel bands appeared for the posttreatment infection were scored as new infections.

Statistical analyses

Treatment outcome was classified as early treatment failure, late treatment failure, reinfection, or adequate clinical response [26]. In the event of treatment failure, rescue treatment was administered in accordance with Kenyan national guidelines. Statistical analyses were done using SPSS for Windows (version 12.0; SPSS) and Stata (version 7.0; Stata Corporation). Percentages were compared using the χ^2 test, and trends in percentages were analyzed using the Cochran-Armitage test for trend. Parasite densities were analyzed after log transformation. Normally distributed continuous variables were compared using analysis of variance or Student's t test. Variables that were not normally distributed were compared using the Kruskal-Wallis or Wilcoxon rank-sum test.

For analyses of gametocyte prevalence during follow-up, multiple logistic regression models and generalized estimating equations were used, to allow for autocorrelation. Regression coefficients (β) were calculated.

The results of the membrane-feeding assays were analyzed on two outcomes. The percentage of the population that was infectious was estimated by multiplying the percentage of children with gametocytemia on day 7 and the percentage of these children who infected at least one mosquito; this method was used because it is in accordance with that of previous studies of malaria transmission after antimalarial drug treatment [7,9,10] and, hence, allowed comparison of results. In addition, the percentage of infectious children and the percentage of infected mosquitoes were directly determined for the randomly selected children. Relative risks and 95% confidence intervals were calculated using the SP monotherapy arm as the reference group.

Results

Clinical efficacy

A total of 528 children were randomized to the four treatment arms (figure 6.1). Two children died within one day of enrollment, as a result of factors other than malaria. Of the remaining children, 6.3% (33/526) were lost for evaluation during the 28-day follow-up period. Asexual parasite density, gametocyte prevalence, and frequency of fever at enrollment were not significantly different among the four treatment arms (table 6.1).

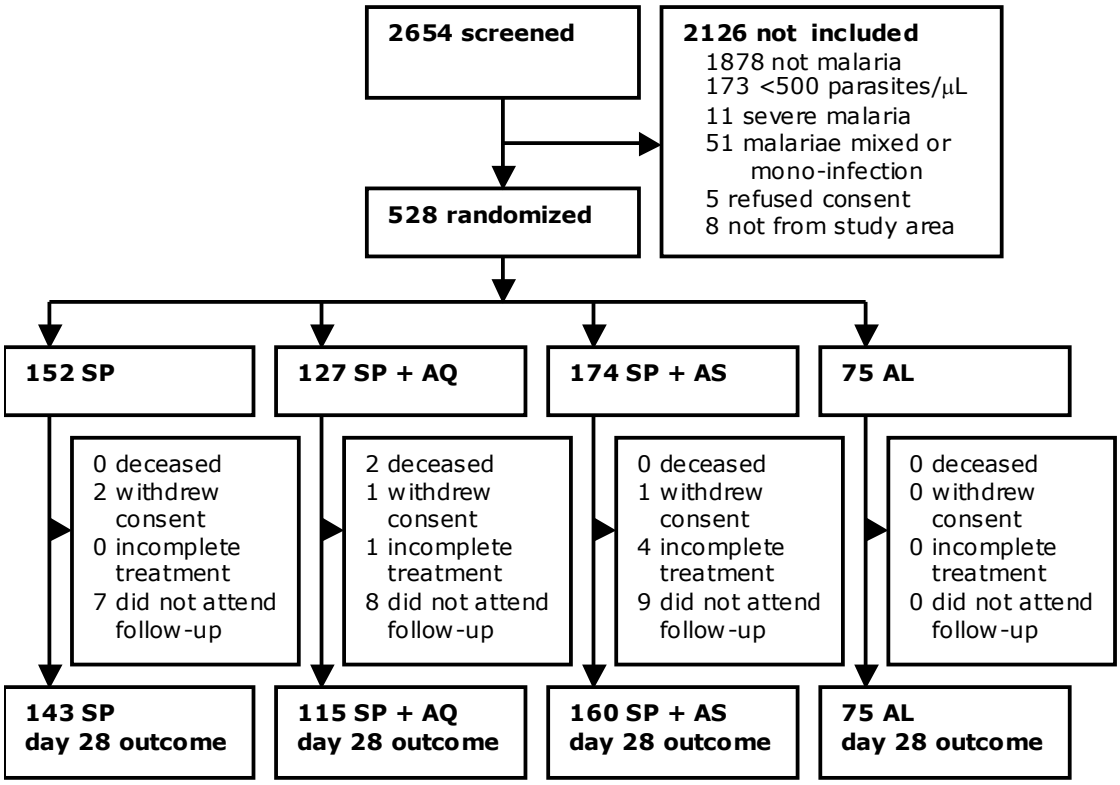

Figure 6.1 Profile of the study.

The median age (P = 0.03), weight (P = 0.03), and age-associated hemoglobin level (P = 0.01) were slightly higher for the children in the AL arm than for the children in the other arms, because only subjects who weighed ≥10 kg were included in the AL arm. This imbalance was accounted for in the statistical analyses and did not confound any of the observed relationships. During follow-up, SP monotherapy produced an adequate clinical response in 44.1% (63/143) of the children, and early treatment failure was observed in 9.8% (14/143) of the children in this arm (table 6.2). The combination regimens resulted in fewer treatment failures, and adequate clinical response was observed in 87.0% (100/115), 81.9% (131/160), and 96.0% (72/75) of the children in the SP plus AQ, SP plus AS, and AL arms, respectively.

Table 6.1. Characteristics of the study population at enrolment

	SP	SP+AQ	SP+AS	AL
N	152	127	174	75
Age, median (IQR)	3.5 (2.0–5.3)	2.6 (1.4-4.9)	3.1 (1.8-5.1)	4.1 (2.0-6.4)
Sex, % male (n/N)	50.7 (77/152)	52.8 (67/127)	49.4 (86/174)	53.3 (40/75)
Weight, median (IQR)	14.0 (11.0-18.4)	13.0 (10.0–16.1)	13.5 (10.0–18.0)	16.0 (12.0-20.2)
Temperature, % >37.5ºC (n/N)	66.2 (100/151)	53.5 (68/127)	64.7 (112/173)	54.7 (41/75)
Hemoglobin, median (IQR)	9.4 (7.9-10.7)	9.3 (8.0-10.7)	9.4 (7.8-10.6)	10.4 (8.6 – 11.8)
Asexual parasite density, GM (IQR)	10,122 (3,680-29,593)	11,452 (5,040-31,679)	12,355 (5,417-28,521)	11,887 (3,680-32,161)
Gametocyte prevalence, % (n/N)	20.9 (31/148)	22.3 (27/121)	24.4 (42/172)	24.6 (17/69)

IQR = interquartile range; GM = geometric mean

Table 6.2. Treatment outcome for the different treatment regimens on day 28.

	SP	SP+AQ	SP+AS	AL
Number evaluated	143	115	160	75
Treatment outcome, %				
ACR	44.1	87.0	81.9	96.0
ETF	9.8	0.9	0.6	0.0
LTF	44.8	7.8	13.8	2.0
Re-infection	1.4	4.3	3.8	2.0

ACR = adequate clinical response, ETF = early treatment failure, LTF = late treatment failure

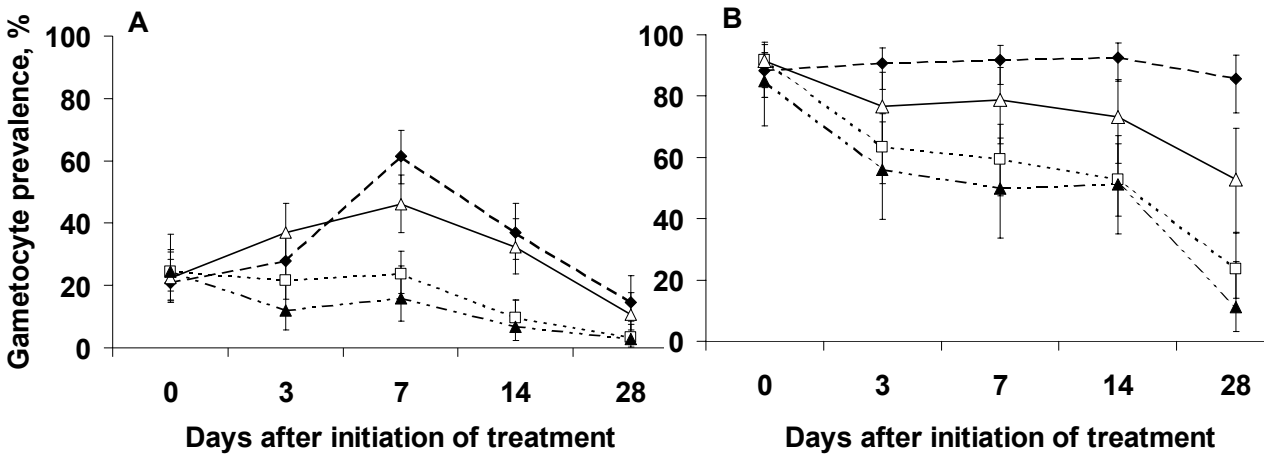

Figure 6.2 Gametocyte prevalence by microscopy (A) and Pfs25 QT-NASBA (B) after sulphadoxine-pyrimethamine (SP) monotherapy (black diamonds), SP plus amodiaquine (AQ) (white triangles), SP plus artesunate (AS) (white squares), and artemether-lumefantrine (AL) (black triangles).

Bars indicate the 95% confidence intervals around the proportions.

***6.2A**: Differences in microscopic gametocyte prevalence were statistically significant for the comparison between SP and SP+AS (β=-0.78, se=0.17, p<0.001) and AL (β=-1.14, se=0.26, p<0.001); between SP+AQ and SP+AS (β=-0.70, se=0.19, p<0.001) and AL (β=-1.06, se=0.27, p<0.01). Differences in gametocyte prevalence were not statistic ally significant for the comparison between SP and SP+AQ (β=-0.08, se=0.18, p=0.67) and between SP+AS and AL (β=-0.35, se=0.27, p=0.18).*

***6.2B**:Differences in Pfs25 QT-NASBA gametocyte prevalence were statistically significant for the comparison between SP and SP+AQ (β=-1.31, se=0.38, p=0.001); SP+AS (β=-2.14, se=0.33, p<0.001) and AL (β=-2.44, se=0.37, p<0.001); between SP+AQ and SP+AS (β=-0.83, se=0.30, p=0.007) and AL (β=-1.12, se=0.34, p=0.001). There was no statistically significant difference in gametocyte prevalence between SP+AS and AL (β=-0.30, se=0.27, p=0.27).*

Gametocyte prevalence by Pfs25 QT-NASBA

During follow-up, gametocyte prevalence by microscopy was significantly lower among the children in the SP plus AQ, SP plus AS, and AL arms than among the children in the SP monotherapy arm (figure 6.2*A*). Gametocyte prevalence by *Pfs25* QT-NASBA was much higher than by microscopy (figure 6.2B). The overall gametocyte prevalence was 22.9% (117/510) at enrollment by microscopy, compared with 89.4% (219/245) by *Pfs25* QT-NASBA (χ^2 = 295.9; P < 0.001).

Although gametocyte prevalence by *Pfs25* QT-NASBA was almost universal at enrollment (range for the treatment arms, 85%–92%), it decreased gradually during the follow-up period for the SP plus AQ (β= -0.060, se= 0.014; P < 0.001), SP plus AS (β= -0.090, se= 0.012; P < 0.001), and AL (β= -0.099, se=

0.015; $P < 0.001$) arms but not for the SP monotherapy arm (β= -0.0032, se= 0.15; $P = 0.83$).
During the entire study period, gametocyte prevalence by *Pfs25* QT-NASBA was lower in the SP plus AQ, SP plus AS, and AL arms than in the SP monotherapy arm. The children treated with AL had the lowest gametocyte prevalence by *Pfs25* QT-NASBA, although the difference between the prevalence in this arm and that in the SP plus AS arm was not statistically significant (figure 6.2B).

Infectiousness of children in different treatment arms

A total of 118 successful membrane-feeding assays were conducted for children who had microscopically confirmed gametocytemia on day 7 and were >2 years of age. Three assays were not included in the analyses because <20 mosquitoes were dissected. A high percentage of the gametocyte carriers were infectious to mosquitoes (table 6.3). The estimated percentage of the population who were infectious to mosquitoes was significantly lower after treatment with either SP plus AS or AL, compared with that after SP monotherapy. No statistically significant difference was observed between SP monotherapy and treatment with SP plus AQ. However, the high gametocyte prevalence by *Pfs25* QT-NASBA prompted us to randomly test blood samples from treated children by the membrane-feeding assay. Across treatment arms, the percentages of the randomly selected children who were infectious to mosquitoes were consistently higher than the estimated percentages of the children with microscopically confirmed gametocytemia (table 6.3). Although this result was observed for SP monotherapy (64.0% vs. 48.4%), the relative increase was most evident for the combination therapies, for which the infectious proportion increased 2-fold for SP plus AQ treatment (80.0% vs. 37.9%), 3-fold for SP plus AS treatment (44.0% vs. 14.7%), and 4-fold for AL treatment (60.0% vs. 14.0%). Among the randomly selected children themselves, there were no statistically significant reductions in the infectious proportion for treatment with SP plus AQ, SP plus AS, or AL, compared with that for SP monotherapy (table 6.3).

The force of malaria infection in the natural situation is determined by the infectious mosquito reservoir. This reservoir is related to the infectious proportion of the human population but also to the percentage of mosquitoes that become infected when feeding on humans. Among the randomly selected children, the percentage of mosquitoes that became infected was quantified for each treatment arm and was found to be 6.9% for SP monotherapy, 5.5% for SP plus AQ treatment, 2.3% for SP plus AS treatment, and 3.6% for AL treatment (table 6.4). Compared with that for SP monotherapy, the probability of a mosquito becoming infected was significantly lower for treatment with SP plus AS and with AL but not for treatment with SP plus AQ.

Table 6.3. The estimated proportion of infectious children per treatment arm in membrane feedings in microscopically confirmed gametocyte carriers (A) and a random selection of patients (B)

	Gametocytaemic children, % (n/N)	Infectious gametocyte carriers, % (n/N)	Proportion infectious children, %*	RR (95% CI)†
A. Microscopically confirmed gametocyte carriers				
SP	61.5 (83/135)	78.7 (48/61)	48.4	1
SP+AQ	46.2 (55/119)	82.1 (23/28)	37.9	0.79 (0.59–1.05)
SP+AS	23.8 (39/164)	61.9 (13/21)	14.7	0.30 (0.20–0.46)
AL	16.0 (12/75)	87.5 (7/8)	14.0	0.30 (0.17–0.53)
B. Random selection of patients				
SP	n.a.	n.a.	64.0 (16/25)	1
SP+AQ	n.a.	n.a.	80.0 (20/25)	1.25 (0.88–1.78)
SP+AS	n.a.	n.a.	44.0 (11/25)	0.69 (0.40–1.17)
AL	n.a.	n.a.	60.0 (15/25)	0.94 (0.61–1.45)

*RR = relative risk; 95% CI = 95% confidence interval; n.a.= not applicable. The median number of dissected mosquitoes per experiment was 31.0 (IQR 30-48) for table 6.3A and not different between the treatment groups (Kruskal-Wallis χ^2 =0.88; p=0.83). The number of dissected mosquitoes for table 6.3B was exactly 30 for each experiment.*The proportion of infectious children was estimated for table 6.3A by multiplying the gametocytaemic proportion of children with the proportion of infectious gametocyte carriers. The number of children who were included in the membrane feedings was smaller than the number of gametocytaemic children because only children above 2 years of age were eligible for membrane feedings. For table 6.3B, this proportion was determined directly. †The relative risk for the proportion of infectious children with SP as reference group.*

Table 6.4 The average number of infected mosquitoes in different treatment arms

	No. of children	Proportion infected mosquitoes, % (n/N)	RR (95% CI)†
SP	25	6.9 (52/750)	1
SP+AQ	25	5.5 (41/750)	0.79 (0.53 – 1.17)
SP+AS	25	2.3 (17/750)	0.33 (0.19 – 0.56)
AL	25	3.6 (27/750)	0.52 (0.33- 0.82)

†The relative risk for the probability of mosquito infection with SP as reference group.

Discussion

The present study shows that the effect of ACT on the infectiousness of young children to mosquitoes is moderate in an area of Kenya where malaria is highly endemic. Although ACT reduces the density of gametocytemia and the proportion of infected mosquitoes, submicroscopic gametocytemia appears to be sufficient to drive posttreatment malaria transmission.

Our microscopy findings on posttreatment gametocyte prevalence are similar to those of other studies of SP monotherapy [7,23] and of treatment with SP plus AS [7,23,27,28], SP plus AQ [6,23,27], and AL [6,10]. The infectiousness of gametocyte carriers was somewhat higher in our study than in the studies by Targett *et al.* [7] and Sutherland *et al.* [10], possibly because the membrane-feeding assays were conducted on different days. We conducted ours on day 14 after initiation of treatment, whereas Targett *et al.* and Sutherland *et al.* conducted theirs on day 7, when gametocytes may not be fully mature [22] or may be less infectious as a result of residual drug concentrations [29]. We nevertheless consider this possibility to be unimportant for interpretation of our data, because our findings on infectiousness by microscopy are comparable with those of these two previous studies and lead to the same conclusions. We observed a clear reduction in malaria transmission after administration of ACT (i.e., treatment with SP plus AS [7] or with AL [10]). No estimate for infectiousness has been given previously for SP plus AQ treatment, which appears to be similar to SP monotherapy with respect to its effect on posttreatment malaria transmission.

The results of the membrane-feeding assays for the randomly selected children, however, lead to very different conclusions. The most likely explanation for this is that detection of gametocytes by microscopy represents only the tip of the iceberg. The results for the randomly selected children suggest that relying on microscopy to select gametocyte carriers may lead to an overestimation of the effects of treatment regimens on malaria transmission. This was evident for all tested regimens and is attributable to low-density gametocytemia. The proportion of gametocyte carriers among children presenting with clinical malaria was as much as 4-fold higher when based on *Pfs25* QT-NASBA than when based on microscopy, and certainly this estimate is closer to the truth. This proportion decreased markedly after administration of ACT, suggesting a direct negative effect of artemisinin derivatives on gametocyte survival [10,30]. In contrast to SP monotherapy, ACT seemed to limit the period of infectiousness. Despite the evident reduction in gametocytemia after the administration of ACT, during the first two weeks after treatment, the majority of children harbored gametocytes at a density that was at or below the microscopic limit of detection, and a large proportion of these carriers were capable of infecting mosquitoes. The observation of infectiousness in children with gametocytemia below the

microscopic threshold is not unexpected [31-33], but the relative contribution made by these carriers to posttreatment malaria transmission in the present study is remarkable. Although a recent study concluded that AL treatment is highly effective in preventing posttreatment malaria transmission [10], we found here that 60% of children treated with AL were capable of infecting mosquitoes. The present study shows that ACT does not substantially reduce the proportion of infectious children but results in a significantly lower proportion of infected mosquitoes. In this manner, ACT is likely to have beneficial effects on transmission.

Submicroscopic gametocytemia is a relevant factor in mathematical models of general malaria transmission [34] and the development and spread of drug resistance [35]. Resistance of malarial parasites to SP is common in our study area, with more than half of the children experiencing treatment failure during follow-up. This finding is similar to those of recent studies in Kenya and Uganda [27,36,37]. The efficacy of SP combination therapy is high, as has been previously reported for SP plus AQ treatment [27,38] and SP plus AS treatment [27]. Here, we found that AL treatment provides the best clinical response, with only 2% treatment failure; this is identical to recent findings from Uganda and Tanzania [6,39].

Transmission intensity varies significantly in Africa and is determined by the local reservoir of infectious mosquitoes and the gametocyte prevalence in a population. We dissected ~30 fully fed mosquitoes per child, which is approximately equivalent to 1 week of exposure to mosquito bites in our study area [40]. Considering a person's infectious period, this makes our estimates reasonable for the local situation, but our results clearly need to be interpreted in the context of biting rates in areas with other levels of endemicity. In our study area, the gametocyte prevalence at enrollment was ~90%. In a previous study of malaria transmission after AL treatment in the Gambia [10], where transmission intensity is much lower and highly seasonal, a number of children without microscopically detectable gametocytemia before or after treatment were included in membrane-feeding assays. Only 3.2% (1/31) of these assays resulted in mosquito infection (G. Targett, R. Ord, M. Jawara, and C. Sutherland, unpublished data), which is much lower than our present findings, despite comparable levels of infectiousness among children with microscopically confirmed gametocytemia. This suggests that infectious submicroscopic gametocyte densities may be less common in the Gambia. Findings from an area of low endemicity in Sudan, on the contrary, suggested a high prevalence (12%–45%) of submicroscopic gametocyte densities, although infectiousness was not assessed [41]. Future studies conducted in the general populations of areas with different transmission intensities and seasonalities should determine the

relationships among the level of endemicity, the prevalence of submicroscopic gametocytemia, and infectiousness to mosquitoes.

Although *Pfs25* QT-NASBA is much more sensitive than microscopy and may prove to be very valuable in estimating gametocyte prevalences, some reservations should be considered. It is not the mere presence of gametocytes but their capacity to infect mosquitoes that is relevant for transmission. For this, the accepted assay is measurement by membrane feeding. In addition, the sensitivity of *Pfs25* QT-NASBA is 20–100 gametocytes/mL (i.e., 0.02–0.1 gametocytes/μL). The average size of a blood meal of a mosquito is 2μL, and, to result in mosquito infection, it should contain a minimum of 1 male and 1 female gametocyte. Therefore, the lowest densities detected by *Pfs25* QT-NASBA may be too low to be relevant for malaria transmission.

In conclusion, our findings need to be interpreted in the context of transmission intensity but indicate that none of the tested drug regimens can clear gametocytes from all children during the month after treatment. ACTs in general, and AL in particular, are efficacious as treatments for uncomplicated malaria and are able to limit the period of posttreatment gametocyte carriage. However, because of the contribution made by submicroscopic gametocytemias, infectiousness appears to be the rule rather than the exception after treatment. These findings highlight the possible limitations of interventions that aim to reduce transmission by use of antimalarial drugs and indicate that gametocyte detection by microscopy is insufficient for future studies of malaria transmission.

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Chapter 7

Submicroscopic Plasmodium falciparum gametocyte densities frequently result in mosquito infection

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Summary

Submicroscopic *Plasmodium falciparum* gametocytaemia (<5,000 gametocytes/mL) is common and may result in mosquito infection. We assessed the relation between gametocyte density and mosquito infection under experimental and field conditions using real-time quantitative nucleic acid sequence-based amplification (QT-NASBA) for gametocyte quantification.

Serial dilutions of NF54 *P. falciparum* gametocytes showed a positive association between gametocyte density and the proportion of infected mosquitoes (β= 6.1, 95% CI 2.7 – 9.6, p=0.001). Successful infection became unlikely below an estimated density of 250-300 gametocytes/mL. In the field, blood samples of 100 naturally infected children showed a positive association between gametocyte density and oocyst counts in mosquitoes (β = 0.38, 95% CI 0.14 – 0.61, p = 0.002). The relative contribution to malaria transmission was similar for carriers with submicroscopic and microscopic gametocytaemia.

Our results show that transmission occurs efficiently at submicroscopic gametocyte densities and that carriers harbouring submicroscopic gametocytaemia constitute a considerable proportion of the human infectious reservoir.

Introduction

Transmission of *Plasmodium falciparum* from man to mosquito depends on the presence of infectious sexual stage parasites (gametocytes) that are ingested by mosquitoes taking a blood meal. The likelihood of mosquito infection can be expected to increase proportionally with increasing gametocyte density. However, this relation remains equivocal. A positive relation between gametocyte density and the proportion of infected mosquitoes was detected in experimental [1-3] and natural [2,4-6] *P. falciparum* infections but this relation was weak or non-existing in other studies [7-11]. Several studies show that mosquitoes can become infected with gametocyte densities below the detection level of standard microscopy [3,6,8,9,12-14].

Importantly, the recent application of molecular techniques for the detection [15,16] and quantification (*Chapter* 5) [17,18] of gametocytes indicated that submicroscopic gametocyte densities are common (*Chapter 5*) [18-20]. In a previous study we have shown that submicroscopic gametocyte densities appear sufficient to drive post-treatment malaria transmission (*Chapter 6*) [21]. It is important to quantify this potential reservoir and to determine the relation between submicroscopic gametocyte densities and the infectiousness to mosquitoes in order to plan and evaluate transmission reducing interventions [22]. This can be done in natural infections and under laboratory conditions where the use of cultured, synchronized gametocytes excludes potential confounders such as gametocyte maturity and host immune factors.

In this study we used quantitative nucleic acid sequence-based amplification (QT-NASBA) to determine the relation between *P. falciparum* gametocyte density and the infectiousness to mosquitoes under field and laboratory conditions.

Methods

The infectiousness of cultured gametocytes.

Plasmodium falciparum NF54 gametocytes were obtained from *in vitro* culture [23]. Experiments were performed with mature gametocytes. Serial dilutions were made in whole blood to obtain gametocyte dilutions at approximately 50% haematocrit ranging from 10^2 to 10^6 gametocytes/mL. Gametocyte density in serially diluted suspensions was calculated from the number of gametocytes present in the original sample. Of all dilutions, a 50 µL sample was stored at -70ºC for subsequent quantification of gametocytes by real-time *Pfs25* QT-NASBA. All dilutions were fed through a membrane feeding system to 50-100 female *Anopheles stephensi* mosquitoes [24] that were 3-5 days old and starved for 1-2 hours prior to feeding. After feeding, mosquitoes were kept at 26ºC,

80% relative humidity, on glucose until dissected in 1% mercurochrome for microscopical assessment of oocyst infection on days 7-9.

Infectiousness of naturally infected children

Membrane feedings were conducted on samples from children naturally infected with *P. falciparum* malaria in Mbita, Suba district, western Kenya. Details of the study can be found elsewhere (*Chapter 6*). The study protocol was approved by the scientific steering committee and ethical review committee of the Kenya Medical Research Institute (SSC No. 791). Children were treated for uncomplicated *P. falciparum* malaria with sulphadoxine-pyrimethamine (SP), SP plus artesunate, SP plus amodiaquine or artemether-lumefantrine. All children older than 2 years who attended the clinic for their day 14 post-treatment visit in November-December 2004 were invited for membrane feeding experiments, regardless of microscopic gametocytaemia. We enrolled 25 children consecutively per treatment arm, giving a total of 100 membrane feeding experiments.

Venous blood samples of 3 mL were obtained from children whose parent or guardian gave specific consent for the procedure. Samples were fed to approximately 150 locally reared 4 to 5-day-old female *Anopheles gambiae s.s.* mosquitoes via an artificial membrane attached to a water-jacketed glass feeder maintained at 37ºC. After 10 to 15 minutes, fully fed mosquitoes were selected and kept at 27-29ºC for 7 days on glucose (10%) until midguts were dissected in 2% mercurochrome. The midguts were examined microscopically for oocysts with a second microscopist confirming their presence if observed. For each experiment, exactly 30 randomly selected mosquitoes were dissected. From each blood sample included in the membrane feedings, 50μL was used for quantification of gametocytes by real-time *Pfs25* QT-NASBA.

Gametocyte detection by *Pfs25* QT-NASBA

Quantification of gametocytes by real-time *Pfs25* mRNA QT-NASBA was performed as described elsewhere (*Chapter 5*)[18]. Briefly, nucleic acids were extracted from the blood samples using the guanidium isothiocyanate/silica procedure [25]. QT-NASBA was performed on a NucliSens EasyQ analyser (bioMérieux) using the NucliSens Basic Kit for amplification according to manufacturer's manual at a KCl concentration of 80mM in a total reaction volume of 10 μL per reaction. The number of gametocytes was calculated in relation to a standard gametocyte stage V dilution series, using the time point of amplification at which the fluorescence detecting target amplicons exceeded the mean fluorescence of three negative controls + 20 standard deviations. A similar protocol was used for the 18S rRNA QT-NASBA [17] to confirm the presence of *P. falciparum* parasites.

Data analyses

Statistical analyses were carried out in SPSS version 12.0 and STATA 8.0. *Pfs25* QT-NASBA gametocyte density was analyzed on a log-scale with negative samples included as zero's. The relation between gametocyte density and mosquito infection rates was fitted to linear, logistic and exponential functions. The best results were presented based on r^2. For field samples, the relation between gametocyte density and oocyst counts in individual mosquitoes was determined using negative binomial models. Uninfected mosquitoes were included in these analyses and a random effect was added to allow for the correlation between observations from the same individual. Regression coefficients β with 95% confidence intervals (95% CI) were presented. Proportions were compared using odds ratio's (OR), the trend in proportions with the Cochran-Armitage test for trend. Normally distributed continuous variables were compared using the Student t-test and the correlation between continuous normally distributed variables was quantified with the Pearson correlation coefficient.

Results

Infectiousness of cultured gametocytes

The percentage of infected mosquitoes was positively associated with gametocyte density from *in vitro* culture (β = 6.1, 95% CI 2.7-9.6) and was described by a linear function (r^2 = 0.39, p = 0.001)(figure 7.1). Non-linear functions did not improve the fit. Infected mosquitoes harboured 1-4 oocysts and only 14% of the infected mosquitoes had more than one oocyst, making analyses on oocyst counts uninformative. An estimated 7.6% of the feeding mosquitoes was infected at the microscopic detection limit of 5 gametocytes/μL. The trendline crossed the Y-axis at 250-300 gametocytes/mL, indicating that mosquito infection under the used conditions is unlikely below this threshold. There was a good consensus between the gametocyte density measured in the Pfs25 QT-NASBA and the estimated density from diluted starting concentrations (Pearson correlation coefficient = 0.88, p < 0.001).

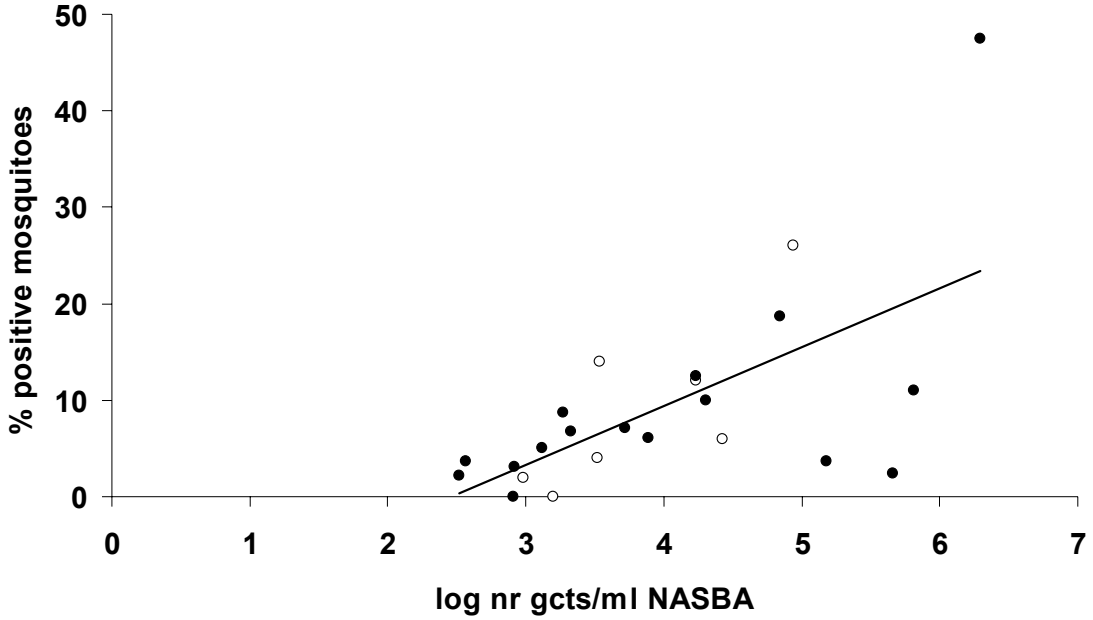

Figure 7.1 Mosquito infection rates against log gametocyte density for feeding experiments on serial dilutions of *in vitro* cultured *P. falciparum* gametocytes. *Results are shown from three membrane feeding experiments with serial dilutions of non-synchronised (open circles) and synchronized (filled circles) gametocytes. Gametocyte density was determined by Pfs25 QT-NASBA; mosquito infection rates by examination of 50-100 dissected mosquitoes per sample. The curve represents the linear relation between log gametocyte density and mosquito infection rates (y = 6.13 x – 15.06; r^2 = 0.39, p = 0.001).*

Infectiousness of naturally infected children

A total of 100 membrane feedings were successfully carried out in children with a median age of 2.8 (interquartile range 2-4) years. At the time of membrane feedings 25.0% (25/100) of the children harboured microscopically detectable gametocytes and 75.0% (75/100) were gametocyte positive by *Pfs25* QT-NASBA. A total of 62.0% (62/100) of the children was infectious to mosquitoes, of whom 69.4% (43/62) had no microscopically detectable gametocytes. There was a positive association between gametocyte density and the percentage of infected mosquitoes, described by an exponential function (r^2 = 0.48, p = 0.026)(figure 7.2).

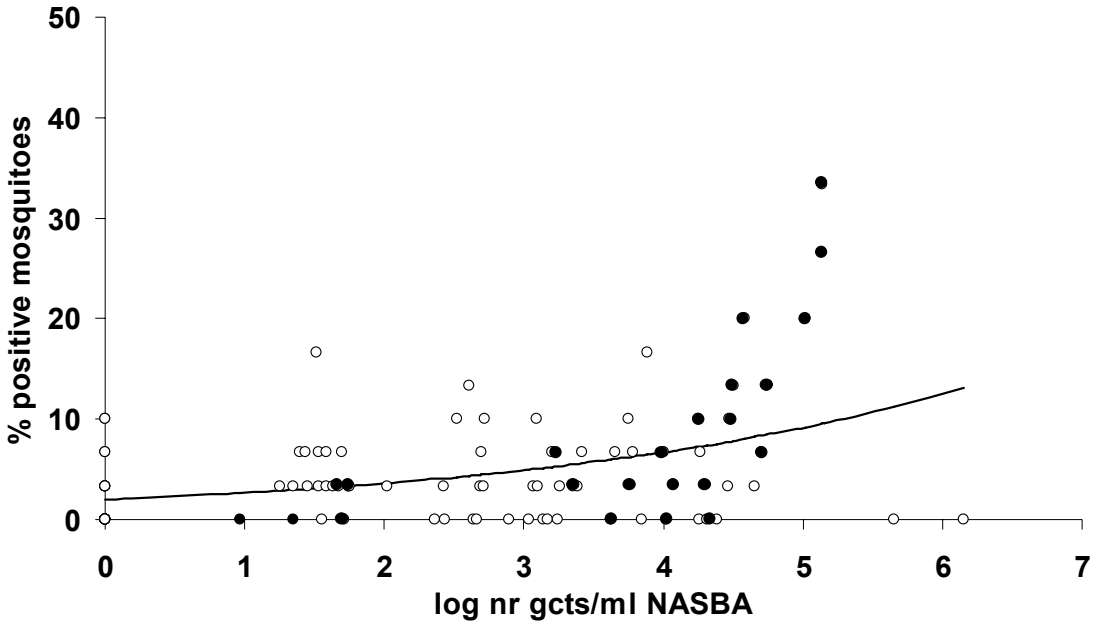

Figure 7.2. Mosquito infection rates against log gametocyte density for feeding experiments on venous blood samples of naturally infected Kenyan children. *Results are shown for individual venous blood samples; microscopically confirmed gametocyte carriers are indicated with filled circles. Gametocyte density was determined by Pfs25 QT-NASBA; mosquito infection rates by examination of 30 dissected mosquitoes per sample. The curve represents the exponential relation between log gametocyte density and mosquito infection rates ($y = 1.93\ e^{0.31\ x}$; $r^2 = 0.48$, $p = 0.026$).*

Infectiousness of children with submicroscopic gametocytaemia

Successful mosquito infection was observed in children with microscopic and submicroscopic gametocyte densities (table 7.1). Compared to children with microscopically detectable gametocytes, the proportion of infectious children was lower in children with submicroscopic gametocytaemia and lowest in children who were negative in both microscopy and *Pfs25* QT-NASBA (Cochran-Armitage test for trend, $p = 0.001$). The same trend was observed for the proportion of infected mosquitoes (Cochran-Armitage test for trend, $p < 0.001$). Importantly, 70.0% (35/50) of the children with submicroscopic gametocytaemia were capable of infecting at least one mosquito.

Infected mosquitoes harboured 1-13 oocysts and 26% of the infected mosquitoes had more than one oocyst. We observed a positive association between gametocyte density and oocyst counts in mosquitoes ($\beta = 0.38$, 95% CI 0.14-0.61, $p = 0.002$) and an independent borderline significant positive association between age and oocyst counts ($\beta = 0.11$, 95% CI -0.01-0.23, $p = 0.075$). Eight children were infectious to mosquitoes with no gametocytes detected in their samples by *Pfs25* QT-NASBA. *P. falciparum* 18S ribosomal RNA was detected in all these samples (8/8), indicating that RNA extraction had been successful and that *P. falciparum* parasites were present. Of those children who were gametocyte negative in both microscopy and *Pfs25* QT-NASBA, 32.0% (8/25) were infectious to mosquitoes. In general, the association between gametocyte prevalence and the proportion of infectious children in the random feeds was weak for microscopy (OR = 2.36, 95% CI 0.85-6.57) and much stronger for the *Pfs25* QT-NASBA (OR = 5.46, 95% CI 2.05-14.56).

The contribution to malaria transmission of children with microscopically confirmed gametocytes and children with submicroscopic gametocytaemia was estimated by determining the proportion of the total number of infected mosquitoes that they infected. Although individual submicroscopic gametocyte carriers infected fewer mosquitoes, submicroscopic gametocytaemia was more common than microscopic gametocytaemia, resulting in an estimated contribution to transmission of 45.3% for both groups (table 7.1).

Table 7.1. Gametocyte carriage detected by microscopy and *Pfs25* QT-NASBA and infection of mosquitoes after membrane feeding.

	Gametocyte carriage			p-value
Microscopy	-	-	+	
Pfs25 QT-NASBA	-	+	+	
Number of samples	25	50	25	
Density, GM (IQR) *	-	592.5 (42-2487)	4058.1 (301-34798)	0.007†
Infectious children, % (n/N)	32.0 (8/25)	70.0 (35/50)	76.0 (19/25)	0.001‡
Infected mosquitoes, % (n/N)	1.7 (13/750)	4.1 (62/1500)	8.3 (62/750)	<0.001‡
Contribution to transmission,%§	9.5	45.3	45.3	

*- = gametocyte negative; + = gametocyte positive; GM = geometric mean. IQR = interquartile range; *Geometric mean density of gametocytes per mL of blood in the Pfs25 QT-NASBA for positives only; †P-value for the Student t-test on log-transformed gametocyte density; ‡P-value for the Cochran-Armitage test for trend; § The contribution of each group to transmission was estimated by dividing the number of mosquitoes infected by this group by the total number of mosquitoes infected by all 100 children. Equal mosquito exposure of all groups is assumed.*

Discussion

In this study, we show that submicroscopic gametocyte densities can efficiently infect mosquitoes under both laboratory and field conditions. Although it has previously been suggested that submicroscopic gametocyte densities can infect mosquitoes, [3,6,8,9,12-14] this is the first study that actually quantifies submicroscopic gametocytaemia in relation to mosquito infection. We found that the contribution of submicroscopic gametocytaemia to malaria transmission can be similar to that of microscopically confirmed gametocyte carriers.

Data from cultured gametocytes show a clear reduction in the proportion of infected mosquitoes with decreasing gametocyte density. Such a relation has been observed before [24,26] but could not be extended to submicroscopic densities until recently. An estimated 7.6% of mosquitoes became infected after feeding on a sample with a gametocyte concentration at the threshold of microscopical detection. Successful mosquito infection becomes unlikely below an estimated density of 250-300 gametocytes per mL.

Under field conditions, submicroscopic gametocyte densities can also infect mosquitoes, although the association between gametocyte density and mosquito infection rates was less straightforward than in the laboratory experiments. A direct comparison between the experiments in the laboratory and the field can not be made for a number of reasons. There may be

differences in gametocyte fitness or maturity between cultured NF54 gametocytes and natural infections as well as differences in compatibility of parasite isolates and vector species [27,28]. Natural infections also regularly contain multiple parasite genotypes, opposed to a single infecting genotype in the in vitro culture. This could affect gametocyte development through in-host competition between parasite genotypes [29,30]. Sexual stage-specific immune responses may also have influenced transmission efficiency in the field experiments (*Chapter 12*) [31].

In the field experiments, we frequently observed mosquito infection at very low gametocyte densities. Successful mosquito infection by eight children with negative Pfs25 QT-NASBA results suggests that infection can even occur below the detection level of the *Pfs25* QT-NASBA, which is 20-100 gametocytes/mL. [17] Unlike for the laboratory experiments, we could not estimate a minimum gametocyte density for mosquito infection in the field. Gametocytes below levels of 100/mL may not be detected by *Pfs25* QT-NASBA and this may explain transmission without detection of *Pfs25* mRNA. Findings from laboratory and field experiments indicate that the threshold gametocyte density necessary to infect mosquitoes is below the theoretical threshold of 2 gametocytes per mosquito blood meal (2μL). Since the uptake of gametocytes by mosquitoes follows a Poisson or negative binomial distribution [32], some successful infections can occur when feeding on samples with lower gametocyte densities. Several biological mechanisms could facilitate successful mosquito infections at low gametocyte densities. Motility or aggregation of gametocytes may occur, favouring the encounter of males and females [32]. This phenomenon could have influenced transmission efficiency in our experiments. Gametocytes may also preferentially accumulate in the subdermal capillaries [33,34]. Although this could increase the transmission success of individuals with low peripheral gametocyte density under natural conditions, it will not have influenced our findings since we used the same venous blood samples for feeding experiments and gametocyte quantification.

Both laboratory and field experiments clearly show the efficient transmission of low-density gametocytaemia, despite *Anopheles* immune responses that can substantially reduce transmission success [35]. We observed a 2-fold lower proportion of infected mosquitoes for children harbouring submicroscopic gametocyte densities (4.1%) compared to microscopic gametocyte densities (8.3%). This lower proportion of infected mosquitoes was counterbalanced by the higher prevalence of submicroscopic gametocytaemia in our population, resulting in a similar contribution to malaria transmission of carriers with microscopic and submicroscopic gametocytaemia at the time we conducted our experiments. The duration of gametocyte carriage is obviously a crucial factor to determine the overall contribution of submicroscopic

gametocytaemia to malaria transmission and this requires further research. Further studies should also determine the prevalence and infectiousness of submicroscopic gametocytaemia in other populations and the possible relations with seasonality and transmission intensity.

Despite our selected population of children after antimalarial treatment, we feel that our findings are of public health importance. Interventions that aim to interrupt transmission will have to reduce gametocytaemia to densities well below the microscopic detection limit and should also target carriers with submicroscopic gametocytes.

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Chapter 8

Submicroscopic *Plasmodium falciparum* gametocyte carriage is common in an area of low and seasonal transmission in Tanzania

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Summary

Recently developed molecular gametocyte detection techniques have shown that submicroscopic *P. falciparum* gametocytes are common in symptomatic patients and can infect mosquitoes. The relevance for the infectious reservoir of malaria in the general population remains unknown. In this study we investigated submicroscopic asexual parasitaemia and gametocytaemia in inhabitants of an area of hypo-endemic and seasonal malaria in Tanzania.

Two cross sectional malariometric surveys were conducted in the dry and wet season of 2005 in villages in lower Moshi, Tanzania. Finger prick blood samples were taken to determine the prevalence of *P. falciparum* parasites by microscopy, rapid diagnostic test and real-time nucleic acid sequence-based amplification (QT-NASBA).

In total, 2752 individuals participated in the surveys, of whom 1.9% (51/2721) had microscopically confirmed asexual parasites and 0.4% (10/2721) gametocytes. In contrast, QT-NASBA revealed that 32.5% (147/453) of the individuals harboured asexual parasites and 15.0% (68/453) gametocytes. No age dependency or seasonality was observed in submicroscopic parasite carriage.

In conclusion, molecular detection techniques reveal that carriage of submicroscopic asexual parasite and gametocyte densities is relatively common in this low transmission area. Submicroscopic gametocytaemia is likely to be responsible for maintaining malaria transmission in the study area.

Introduction

Gametocytes are the sexual stage malaria parasites responsible for transmission from man to mosquito. Successful mosquito infection requires the ingestion of mature gametocytes by mosquitoes taking a blood meal. The production of gametocytes is therefore essential for maintaining malaria transmission. This is particularly important in areas of low or seasonal malaria transmission where levels of man-vector contact are unpredictable and a consistent production of gametocytes appears necessary for parasite survival [1,2].

Gametocyte carriage is most commonly assessed microscopically and is related to malaria transmission intensity. In high endemic areas most microscopic gametocyte carriers are children, while this negative age dependency disappears as transmission intensity decreases [3,4].

Microscopically confirmed gametocyte carriage is generally uncommon in low transmission areas, especially in the dry season [1,5,6] although gametocyte carriers are vital in re-initiating malaria transmission in the subsequent transmission season. During the dry season, asexual parasite carriage can persist during below the microscopic threshold [7] and this may also be true for gametocyte carriage.

Molecular gametocyte detection techniques have recently addressed submicroscopic *Plasmodium falciparum* gametocytaemia in microscopically confirmed asexual parasite carriers *(Chapter 6, 7)*[1,2,8,9]. Studies using either reverse transcriptase PCR (RT-PCR) or quantitative nucleic acid sequence-based amplification (QT-NASBA) have revealed that submicroscopic gametocytaemia is common in symptomatic parasite carriers and can persist for several months *(Chapter 6, 7)* [1,2,8,9]. The prevalence and importance of submicroscopic gametocytaemia has however yet to be investigated in the general population. In this study we address this issue by determining the prevalence of (submicroscopic) asexual parasites and gametocytes in inhabitants of an area of low and seasonal malaria endemicity in Tanzania.

Methods

Study site

This study was conducted in the villages Msitu wa Tembo and Magadini in Lower Moshi. The study area lies on the plains beneath Kilimanjaro in northern Tanzania (latitude 3° 33′ – 3° 44′ S; longitude 37°17′ – 37° 24′ E). Malaria transmission in the area is largely restricted to the long rainy season (May-July) and an unpredictable short rainy season (October-November). The remainder of the year is dry and hot and transmission is practically absent .The average annual rainfall is 615 mm (10 year average) and the entomologic inoculation rate was recently estimated at 2.3 infectious bites per person per year with *Anopheles arabiensis* as principle vector [10]. Villagers predominantly belong to

Masai, Pare and Arusha tribes, and are mostly involved in self-subsistence farming and fishing. Two cross sectional surveys were conducted during the dry season (April) and wet season (August) in the year 2005, targeting a minimum of 1000 participants per survey. Families were selected using village censuses and computer randomised tables. A central point in each village was identified and villagers attended the survey on a first come first serve basis.

Data collection

Information on demographic, anthropometric and general health indicators were collected by means of a questionnaire. Each participant was examined by a qualified clinical attendant for signs of anaemia, fever and splenomegaly. A single blood sample was obtained by finger prick from each participant and used in the field for haemoglobin (Hb) measurement using a haemocue photometer (Angelholm, Sweden), detection of malaria parasites using a rapid diagnostic test (RDT) detecting *P. falciparum* specific histidine rich protein-2 (Paracheck®, Orchid Biomedical Systems, India; Eurocheck®, Euromedi Equip Limited, UK) and the preparation of microscopy and QT-NASBA samples. The RDT and Hb measurement were performed according to the manufacturer's instructions and scored in the field. The comparability in sensitivity of the two rapid tests was confirmed in dilutions of cultured NF54 *P. falciparum* parasites [11] (Kappa-value: 0.82, p=0.001). Those found to have malaria by RDT were given free treatment with sulphadoxine-pyrimethamine, according to the national guidelines. Individuals requiring further attention were treated accordingly or referred to the nearby hospital.

Thin and thick blood smears were made and Giemsa stained in the field. Slides were subsequently double read in two different stations by experienced microscopists. Readings were compared for validation and slides giving discordant results were read by a third reader. The majority result was taken as final.

Exactly 50μL of blood was collected in EDTA tubes for QT-NASBA. *P. falciparum* parasite detection by QT-NASBA was done as described elsewhere [12,13]. Nucleic acids were extracted from blood samples as described by Boom *et al.* [13]. The first part of the RNA extraction was done in the field following the original Guanidinium isothiocyanate (GuSCN) RNA extraction method [14] until the nucleic acids were bound to silica dioxide particles. At this point, samples were stored at -20°C and transferred to the laboratory for completion of the extraction and QT-NASBA analysis. QT-NASBA was performed on a NucliSens EasyQ analyser (bioMérieux) for 18S rRNA and *Pfs25* mRNA. The 18S QT-NASBA detects all circulating parasite stages; the *Pfs25* QT-NASBA is gametocyte specific. Although the number of asexual parasites can not be directly determined by QT-NASBA, it can be estimated by subtracting the concentration

of gametocytes in the *Pfs25* QT-NASBA from the total concentration of parasites in the 18S QT-NASBA. Nuclisens Basic kits were used for amplification according to the manufacturers manual. The number of gametocytes was calculated in relation to a standard gametocyte stage V dilution series [11], using the time point of amplification at which the fluorescence detecting target amplicons exceeded the mean fluorescence of three negative controls + 20 standard deviations. Parasite detection by QT-NASBA was done for a selection of 140-160 individuals from three age groups (< 5 years, ≥5 to 15 years and ≥ 15 years). Samples were randomly selected within age-strata using computer generated randomisation tables.

Data analysis

All data was double entered in a created computer access database and imported in SPSS 12.01 after validation. Age was categorized into < 5 years, ≥5 to 15 years and > 15 years; haemoglobin status in severe (< 5g Hb/dl), moderate (< 8g/dl), and mild anaemia (<11g/dl). Proportions were compared between groups with the χ^2-test or Fisher's Exact test; means were compared with one-way ANOVA, student t-test or Wilcoxon Rank-sum test, where appropriate. The correlation between normally distributed continuous variables was quantified using the Pearson's correlation coefficient, the relation between gametocyte prevalence and asexual parasite density by logistic regression models. Parasite density was analyzed on a log-scale and geometric mean densities per mL were calculated for parasite carriers with interquartile ranges (IQR).

Ethical aspects

All villagers who were invited for the survey were given detailed information about the study aim and procedures before they were asked to sign individual informed consent. Parents/guardians were asked to sign on behalf of children. The study protocol was approved by the ethical committees of both the Tanzanian National Institute of Medical Research (NIMR/HQ/Vol.IX/343) and the London School of Hygiene & Tropical Medicine.

Results

In total, 2752 individuals participated in the cross-sectional surveys, 1593 in the dry season and 1159 in the wet season (table 8.1). The prevalence of moderate or severe anemia (Hb<8g/dl) was 6.7% (185/2752) in the general population but was higher in children under five years of age, 15.6% (96/614) (χ^2=97.73, p<0.001). Splenomegaly was uncommon with only 0.5% (15/2752) of the individuals having a Hackett's score ≥2.

Table 8.1. Characteristics of the study population

N		2752
	Dry season, n	1593
	Wet season, n	1159
Age (Years), % (n)		
	< 5	22.3 (614)
	5 – 15	27.3 (752)
	>15	50.4 (1386)
Sex, % male (n)		39.1 (1075)
Tribe		
	Masai	25.9 (713)
	Arusha	12.4 (340)
	Pare	12.2 (336)
	Others	49.5 (1362)
Anaemia, % (n)		
	Normal	69.8 (1921)
	Mild	23.0 (634)
	Moderate	6.1 (168)
	Severe	0.6 (17)
Reported fever in previous two weeks, % (n)		20.4 (562)
Splenomegalia (Hackett's score ≥2), % (n)		0.54 (15)

Anaemia haemoglobin was defined as nomal (≥11 g Hb/dl), mild (<11g/dl), moderate (<8 g/dl) and severe (<5 g/dl) anaemia.

The rapid diagnostic test (RDT) detected parasites in 3.0% (48/1593) of the individuals in the dry season and 1.7% (20/1156) in the wet season (χ^2=4.67, p=0.03). Asexual parasite prevalence as detected by microscopy was 1.9% (51/2721) and similar in the dry and wet season (table 8.2). The geometric mean asexual parasite density in parasite carriers was 2,225.6 (IQR 80.0-29,396.8) $*10^3$ parasites/mL. Although there was a statistically significant positive association between the presence of parasites by RDT and microscopy (χ^2=257.35, p<0.001), 72.1% (49/68) of the RDT positive samples were negative by microscopy and 62.7% (32/51) of the microscopy positive samples were negative by RDT. In contrast to the low prevalence of microscopically confirmed parasite carriers, the 18S QT-NASBA detected parasites in 32.5% (147/453) of the individuals. Parasite prevalence by 18S QT-NASBA was non-significantly higher in the dry season (χ^2=2.96, p=0.09, table 8.2).

Table 8.2. Parasite prevalence per season by different detection techniques

	Parasite prevalence, % (95% CI)				
Season	Rapid test*	Microscopy Asexual	Microscopy Gametocytes	QT-NASBA Asexual	QT-NASBA Gametocytes
Dry	3.0 (48/1593)	2.0 (32/1571)	0.4 (6/1571)	36.4 (78/214)	15.9 (34/214)
Wet	1.7 (20/1156)	1.7 (19/1150)	0.3 (4/1150)	29.9 (69/239)	14.2 (34/239)
Total	2.5 (68/2749)	1.9 (51/2721)	0.4 (10/2721)	32.5 (147/453)	15.0 (68/453)

*95% CI = 95% confidence interval; *Difference between dry and wet season: χ^2 = 4.57, p = 0.03*

For those individuals who were 18S positive and *Pfs25* QT-NASBA negative, 18S QT-NASBA parasite concentration was assumed to equal asexual parasite density. In those individuals who were both 18S and *Pfs25* QT-NASBA positive, the total parasite concentration by 18S QT-NASBA was always higher than the gametocyte concentration by *Pfs25* QT-NASBA, indicating that both asexual parasites and gametocytes were present. In these individuals asexual parasite density was estimated by subtracting the concentration in the *Pfs25* QT-NASBA from the total parasite concentration in the 18S QT-NASBA. In those 18S QT-NASBA positive, the estimated geometric mean density of asexual parasites was 491.9 parasites/ml (IQR 32.6 - 5607.9). A positive RDT was positively associated with 18S QT-NASBA parasite prevalence (χ^2=15.46, p<0.001) although 1.6% (5/306) of the 18S QT-NASBA negatives were positive in the RDT. Gametocytes were detected by microscopy in 0.4% (10/2721) of the slides, the *Pfs25* QT-NASBA detected gametocytes in 15.0% (68/453) of the individuals, independent of season (table 8.2). The geometric mean gametocyte density by *Pfs25* QT-NASBA was 108.2 gametocytes/mL (IQR 20.0 – 1049.0). There was a strong correlation between the estimated density of asexual parasites by 18S QT-NASBA and *Pfs25* QT-NASBA gametocyte prevalence (β=0.35, se 0.07, p<0.001) and density (r^2 0.40, p=0.001).

Parasite densities of those individuals whose samples were tested by QT-NASBA and microscopy (n=453) are presented in figure 8.1. The vast majority of parasite carriers detected by 18S QT-NASBA harboured parasites at a low density and were not detected by microscopy (figure 8.1A). Only 7.5% (11/147) of 18S QT-NASBA positives were also detected by microscopy. One microscopic parasite carrier was negative in the 18S QT-NASBA. A similar observation was seen for gametocytes where 4.4% (3/68) *Pfs25* QT-NASBA positives were confirmed by microscopy and all microscopic gametocyte carriers were positive in the *Pfs25* QT-NASBA figure 8.1B).

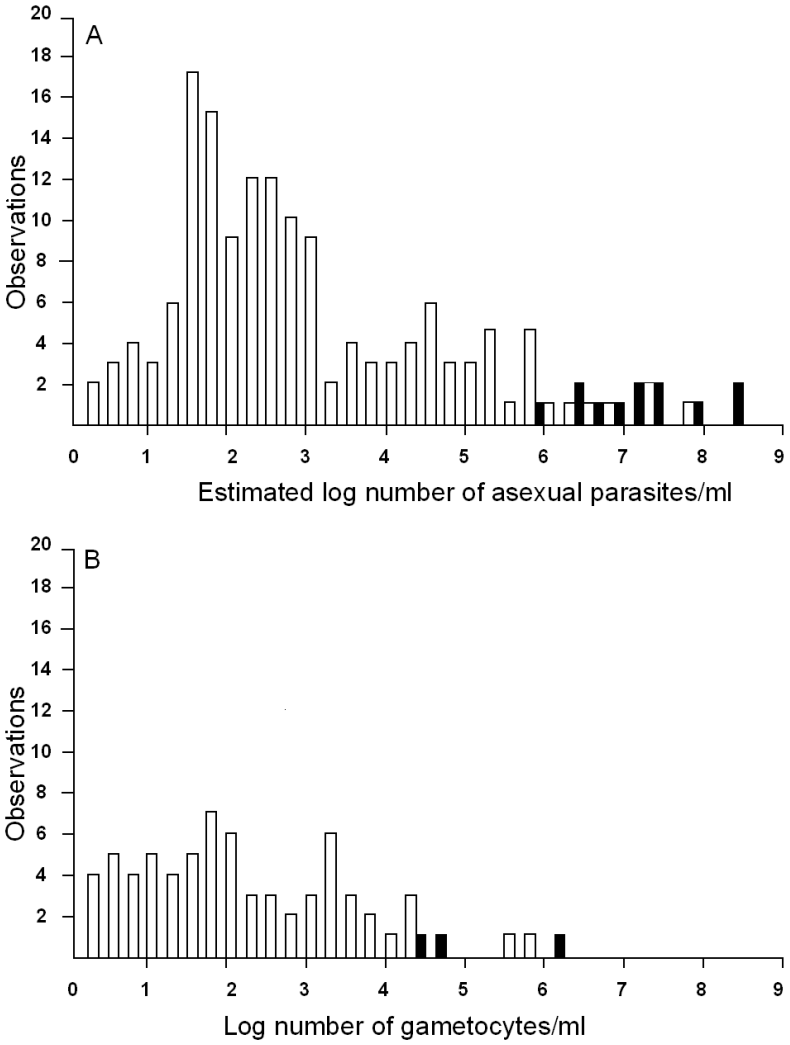

Figure 8.1 Asexual parasite and gametocyte detection by microscopy and QT-NASBA.

A. Open bars indicate the estimated asexual parasite density by QT-NASBA using the formula: total parasite density by 18S QT-NASBA – gametocyte density by Pfs25 QT-NASBA (n=147). For those samples that were positive by microscopy, microscopic asexual parasite densities are presented in solid bars (n=12).

B. Open bars indicate gametocyte density determined by Pfs25 QT-NASBA (n=68). For those samples that were positive by microscopy, microscopic gametocyte densities are presented in solid bars (n=3).

There was no statistically significant difference in QT-NASBA asexual parasite or gametocyte carriage in the different age groups, χ^2=3.07, p=0.22 and χ^2=1.11, p=0.57, respectively (figure 8.2).

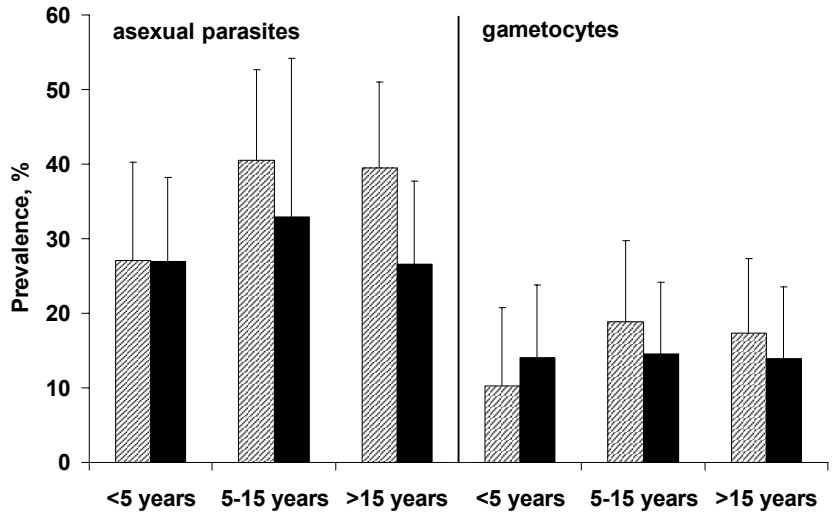

Figure 8.2 The age-dependency of asexual parasite carriage and gametocyte carriage as determined by QT-NASBA. *Shaded bars indicate parasite prevalence in the dry season, solid bars in the wet season. Error bars indicate the upper limit of the 95% confidence interval. Asexual parasite prevalence was detected by 18S QT-NASBA; gametocyte prevalence by Pfs25 QT-NASBA.*

Discussion

In this study we investigated submicroscopic *P. falciparum* parasitaemia in a cross section of the general population of a low transmission area in Tanzania. While microscopically confirmed carriage of asexual parasites and gametocytes was very low, the QT-NASBA revealed that 32.5% of the individuals carried asexual parasites and 15% gametocytes.

This high prevalence of submicroscopic parasitaemia was not related to age or season. The absence of a clear seasonal difference in parasite prevalence is most likely due to the limited rainfall in the year we conducted our study. The total rainfall in 2005 was only 34% of the 10-year average and was particularly poor in the months of April and May, usually the months of peak numbers of mosquitoes [10]. The lack of age-dependency of parasite prevalence is in line with previous work in low transmission settings in our study area [15]. Although asymptomatic parasite carriage is well known in areas with perennial transmission, it is remarkable in an area of low and seasonal malaria transmission. The prevalence of submicroscopic parasitaemia was previously determined in areas of low malaria transmission intensity although results are not directly comparable with ours because of differences in malaria transmission patterns [6] and the selection of individuals [16]. Only 6-15% of individuals from an epidemic-prone area of low transmission in Kenya harboured parasites by PCR in the dry or wet season [6] and 40-50% of treated symptomatic patients retained

persisting parasitaemia by PCR during the dry season in an area of unstable malaria in Sudan [16].

Our findings suggest that even in an area of very low transmission intensity, a substantial part of the population is capable of controlling infections in such a way that parasite densities remain extremely low. These asymptomatic parasite carriers can be expected to become gametocytaemic at some point during their infection if a regular number of asexual parasites is committed to the sexual pathway, as is suggested by mathematical models [17]. Indeed, we observed that a relatively large proportion of parasite carriers harboured gametocytes at submicroscopic densities at the time of sampling. Our gametocyte prevalence of 15% measured by QT-NASBA compares with 12-52% in longitudinally followed individuals after antimalarial treatment in an area of unstable malaria in Sudan [1]. Sequestration of gametocytes in sub dermal capillaries [18] suggests that some gametocyte carriers may have remained undetected by examining finger prick blood samples. The true proportion of gametocyte carriers in our population may therefore be even larger than detected by *Pfs25* QT-NASBA.

The relevance of these low-density gametocyte infections to the overall infectious reservoir of malaria needs some consideration. It is generally accepted that the presence of gametocytes does not always render the human host infectiousness to mosquitoes. Infectivity is influenced by several factors, including immune responses, gametocyte fitness and density (*Chapter 12*) [19-21]. Thus, rather than gametocyte density per se, malaria transmission potential in mathematical models is often defined as the probability that a mosquito blood meal contains the threshold density of at least one gametocyte of each sex [20]. This threshold is 1 gametocyte per μL assuming an average mosquito blood meal size of 2μL. In this study, only 30% of the gametocyte carriers harboured gametocytes at or above this theoretical threshold density. However, data are accumulating to suggest that this threshold density is too high; submicroscopic gametocyte densities are infectious to mosquitoes [22,23] and this has recently been shown using the Pfs25 QT-NASBA to detect gametocytes (*Chapter 6*) [8]. Using this technique, mosquito infection can be successful at densities below 1 gametocyte/μL, for both natural infections and cultured gametocytes (*Chapter 7*) [24]. Although the proportion of mosquitoes infected is lower for carriers with submicroscopic gametocytaemia [17] (*Chapter 7*) [24] they are present in substantial numbers and their contribution to malaria transmission can be considerable (*Chapter 6, 7*) [8,22,24]. Population based infectiousness can be crudely based on the relative proportion of gametocyte carriers multiplied by their infectivity. When assuming a two-fold lower infectivity of submicroscopic gametocytaemia compared to microscopically detectable gametocyte densities, as was found in a study in western Kenya (*Chapter 7*) [24], the estimated transmission potential in

our population would be five-fold higher when based on the *Pfs25* QT-NASBA compared to microscopy.

The use of molecular techniques provides more precise measures of parasite prevalence. These measures suggest more asymptomatic infections and longer periods of carriage of asexual parasites, which will presumably affect the generation of effective immunity. The subsequent development of sexual stage parasites in these infections would mean that malaria transmission is maintained by a much larger number of low-density gametocyte carriers than previously thought.

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Chapter 9

Rapid onset of transmission-reducing antibodies in Javanese migrants exposed to malaria in Papua, Indonesia

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Summary

Transmission of *Plasmodium falciparum* malaria is initiated by sexual stages in the mosquito. Anti-Pfs48/45 and anti-Pfs230 sexual stage antibodies that are ingested together with parasites can reduce parasite development and subsequently malaria transmission.

Acquisition of sexual stage immunity was studied in a cohort of 102 non-immune Javanese individuals migrating to hyperendemic Papua Indonesia. Seroprevalence of antibodies against Pfs48/45, Pfs230 and functional transmission reducing activity (TRA) were measured upon arrival and at 6, 12 and 24 months. Asexual parasitaemia and gametocytaemia were assessed fortnightly.

TRA and seroreactivity increased with the number of *P. falciparum* infections. The longitudinally sustained association between TRA and antibodies against Pfs48/45 (OR 3.74, 95% CI 1.51-9.29) and Pfs230 (OR 3.72, 95% CI 1.36-10.17) suggests that functional transmission reducing immunity is acquired following limited exposure to infection.

Introduction

Successful transmission of *Plasmodium falciparum* parasites from humans to mosquitoes depends on the presence of infectious gametocytes in the circulation, which are derived from a small fraction of asexual parasites. After ingestion of the blood meal by mosquitoes, fertilization takes place in the midgut, which will ultimately result in the generation of infectious sporozoites in the salivary glands. Specific antibodies against sexual stages can interfere with fertilization and subsequent sporogonic development when co-ingested with the blood meal, as shown in natural and experimental infections [1-4]. This transmission reducing activity (TRA) is associated with antibodies directed against the sexual stage specific antigens Pfs48/45 and Pfs230 [5-8]. The rate of development and persistence of TRA is largely unknown. While it was suggested to be low-grade and rapidly transient [9] persistent TRA has also been observed [10]. Acquisition of immunity directed against asexual blood stage parasites has been intensively studied people migrating from a non-endemic to a malaria endemic area [11-15]. In these transmigrants, clinical and parasitological immunity developed quickly after exposure [13] with inconsistent evidence for an independent role of age [11-16].

The objective of this study is to examine the development of sexual stage specific immunity in a longitudinal cohort of Javanese children and adults from non-endemic Java, following migration to malaria endemic Papua. Antibody reactivity against sexual stage specific antigens Pfs230 and Pfs48/45 were determined and compared to antibodies against asexual stage antigen glutamate rich protein (GLURP) and whole parasite extracts. GLURP-specific antibodies have previously been associated with protection against high levels of parasitaemia [17] and clinical disease [18-20]. The development of sexual stage specific immunity was evaluated by detecting circulating antibodies against Pfs230 and Pfs48/45 and by assessing functional TRA in the standard membrane-feeding assay.

Methods

Study population.

The study site was a newly created transmigration village (designated SP2), located near the north-eastern coast of Papua, Indonesia. After informed consent, healthy volunteer adults between 20-40 years of age and children between 6-12 years of age were included in the study, which was approved by all convening scientific and ethical review boards under U.S. Department of Defence Protocol #30820. Subjects were excluded if admitting a history of residence in a malaria endemic area in the previous five years. Details on recruitment, follow-up and the epidemiology of malaria in this cohort have been

described elsewhere [16]. Enrolment started in 1996 and follow-up ended in 1999. The subjects originated from Java and were enrolled in the study within 48 hrs of arrival in Papua, Indonesia. Malaria transmission in Java has been very low for many decades, typically around 0.01 malaria cases/1000 person-years after the exclusion of the few foci of hypo-endemic transmission [21]. In contrast, malaria transmission in Papua is perennial and often hyper- to holo-endemic, with incidence rates from 500 to 5000 infections/1000 person-years[22].

Follow up.

Malaria episodes were actively detected over a period of 24 months by periodic clinical assessments and blood smears. Blood smears were collected at two-week intervals or at any time a subject presented to the clinic with symptoms (chills, fever, etc.) suggesting malaria infection. Blood was microscopically examined for *P. falciparum* and *P. vivax* asexual parasites and gametocytes by Giemsa-stained blood films. Parasites were counted against the number of white blood cells. A slide was considered negative after examination of 200 microscopic fields, corresponding to 1600 to 2400 white blood cells and a estimated diagnostic threshold of 5 parasites per μl. Parasite densities were calculated using a normal density of 8000 white blood cells per μL blood. The first episode of symptomatic or asymptomatic *P. falciparum* malaria was recorded as first infection. Each subsequent *P. falciparum* parasitaemia was classified as a new infection if it occurred more than 28 days after directly observed chemotherapy of the previous infection. Sera were collected on the day of arrival and after 6, 12 and 24 months at SP2 [16]. In order to study the acquisition of immunity in time, we selected samples from subjects for whom at least 3 samples were available. We selected 51 subjects aged 6-12 years and 51 subjects aged 20-40 years. The incidence of malaria infection in these individuals was not different from that of the general population.

Enzyme Linked Immunosorbent Assays.

Antigen preparation. Mature gametocytes of *P. falciparum* (NF54 strain) were produced in an automated static culture system as described by Ponnudurai *et al*.[23], isolated [4] and stored at -70^{0}C until used. NF54 gametocytes were extracted in 25 mM Tris-HCl (pH 8.0) supplemented with 150 mM NaCl, 1.0% sodium desoxycholate (DOC) and 1 mM phenylmethylsulphonyl fluoride. Centrifugation pelleted insoluble debris (13,000g for 5 min at room temperature) and the supernatant provided antigen for ELISA's (whole parasite, Pfs48/45 and Pfs230).

Pfs230 and Pfs48/45 capture ELISA. Anti-Pfs230 IgG antibodies were assayed by coating 10μg/mL of anti-Pfs230 mouse mAb 63F6D7-F(ab)2 fragments in phosphate buffered saline (PBS, pH 7.4) on Sterilin® ELISA plates (PS, ref 53011, International Medical Products B.V., Zutphen, the Netherlands).

For Pfs48/45, the plates were coated with anti-Pfs48/45 rat mAb 85RF45.3. Plates were blocked with 5% (wt/vol) low-fat dry milk (Marvel, Premier International Foods Ltd, Spalding, Lines, United Kingdom) in PBS. For Pfs230 or Pfs48/45 antigen capture, plates were incubated with gametocyte extract (250,000 parasite equivalents/well). Sera (1:100 dilution) were added to the wells and incubated for 2 hrs. Bound IgG antibodies were detected by horse-radish-peroxidase (HRPO)-labeled goat anti-human IgG (Pierce 31412). Wells were washed three times with PBS and subsequently incubated with tetramethyl benzidine (TMB) substrate solution for 20 min. The colour reaction was stopped with 4N H_2SO_4, and optical density (OD) read at 450 nm (Anthos 2001 microplate reader: Labtec BV). All incubations were carried out at room temperature. All serum samples were tested in duplicate with a concurrent positive control and a minimum of 4 negative (Dutch blood bank donor) controls per plate. Sera were considered positive if OD exceeded three standard deviations above the mean of negative controls.

Whole parasite and GLURP ELISA. Antibodies against whole parasite extract and GLURP served as markers for general anti-parasite and specific asexual stage immunity, respectively. Sterilin® ELISA plates (PS, ref 53011, International Medical Products B.V., Zutphen, the Netherlands) were coated overnight with 250,000 parasites per well by diluting a stock containing $30x10^6$ parasites/mL in extraction buffer with PBS (as above). After blocking with 5% milk/PBS, sera were incubated 2 hr at room temperature (1/100 dilutions in 0.1% milk-TPBS). Anti-$GLURP_{85-213}$ antibodies were measured in plasma samples by ELISA[24]. Microtiter plates (NUNC™ Maxisorp, Nalge Nunc International Corp, Life Techn, The Netherlands) were coated overnight at 4°C with 50 μL of 0.2μg $GLURP_{85-213}$ /mL final concentration in 0.05 M carbonate buffer pH 9.6. After washing, wells were incubated with 2.5 % (wt/vol) milk in PBS. Plasma samples were diluted in PBS/0.05% Tween 20/1.25% milk. For the detection of anti- $GLURP_{85-213}$ IgG antibodies, rabbit anti-human IgG peroxidase (Dako, P-214) was used 1/10.000 in PBS/0.05% Tween 20/1.25% milk. The remainders of both tests were carried out as for Pfs230 and Pfs48/45described above.

Standard Membrane Feeding Assay (SMFA).

Experimental infections of mosquitoes were carried out as previously described [25,26]. Three to five-day old, colony-reared *Anopheles stephensi* were allowed to membrane feed on freshly cultured mature NF-54 *P. falciparum* gametocytes in batches of 50 mosquitoes each in the presence of serum. A maximum of 17 transmigrant serum samples were compared to three batches of a single (Dutch blood bank donor) control serum. Immediately after the feed, non-fed and partially fed mosquitoes were removed. Blood-fed mosquitoes were kept at 26°C and 80% humidity for seven days. Surviving mosquitoes (>90%) were dissected

and oocysts counted from extracted midguts. Twenty mosquitoes per batch were examined for oocysts. An SMFA experiment was considered valid when in all three control batches the percentage of infected mosquitoes was 90% or more. The observed transmission reducing activity (TRA) of serum was determined as the percentage reduction in oocyst numbers in test samples as compared to controls [27]. Functional TRA was defined as a minimum of 50% reduction in oocysts numbers in the standard SMFA. Not all sera were analysed in the SMFA because of resource limitations. A random sample of individuals was therefore selected and all their available serum samples were tested in the same SMFA experiment.

Statistical analyses.

Analyses focused on the relation between the development of (functional) sexual stage specific immunity and cumulative exposure to *P. falciparum*, quantified as the cumulative number of infections with asexual stage *P. falciparum* parasites. Statistical analyses were carried out using SPSS for Windows, version 10 (SPSS Inc., Chicago, IL) and Stata 7.0 (Stata Corporation, 4905 Lakeway Drive, College Station, Texas 77845 USA). Prevalence of antibodies and functional TRA (dichotomous variables) as a function of exposure to asexual *P. falciparum* parasites was tested using multiple logistic regression models by Generalized Estimating Equations (GEE). Regression coefficients β were calculated with 95% confidence intervals (95% CI). Prevalence was logit-linked analyzed. The number of infections was included as a continuous variable and a random effect was included in the models to allow for correlations within individuals. Age group (20-40 years vs. 6-12 years) and number of *P. vivax* infections were included in the models as covariates. Interaction terms were added to the models to assess a possible modifying effect of age on the relation between exposure to infections and the development of immunity.

Results

The malariometric indices of the study population are presented in table 9.1. The vast majority of individuals experienced at least one episode of malaria, with a median number of 3 infections during the 24-month period of follow-up. While prevalence of infection was similar for adults and children, the latter were exposed to higher densities of asexual parasites (P = 0.02, table 9.1). Gametocytes were detected in 26% of the children and 29% of the adults with no statistically significant difference in gametocyte prevalence or density.

Table 9.1 Characteristics and *Plasmodium falciparum* malariometric indices of 102 study subjects from the SP2 cohort during 24 months of follow-up in Papua, Indonesia

	Children	Adults	P-value
N	51	51	
Age, median (IQR)	9 (8-11)	32 (28-37)	
Number (percentage) of individuals infected	49 (96.0%)	49 (96.0%)	1.0
Number of infections per person, median (IQR)	3 (1-3)	3 (2-4)	0.53
Asexual parasite density, GM (IQR)	2792 (879-11,962)	1697 (520-5920)	0.02
Number (percentage) of individuals gametocytaemic	13 (25.5%)	15 (29.4%)	0.66
Gametocyte density, GM (IQR)	112 (40-280)	176 (48-579)	0.21

IQR = interquartile range; GM = geometric mean parasite density in parasites/μL for P. falciparum asexual parasite or gametocyte carriers only.

The cumulative number of infections per 6 months is presented in figure 9.1. By six months, 20% of the 102 migrants had experienced two or more infections; by 24 months, this proportion had increased to 73%. The cumulative number of subjects in whom gametocytes were observed in peripheral blood films was 0, 2, 8 and 18 for time points 0, 6, 12 and 24 months post migration, respectively.

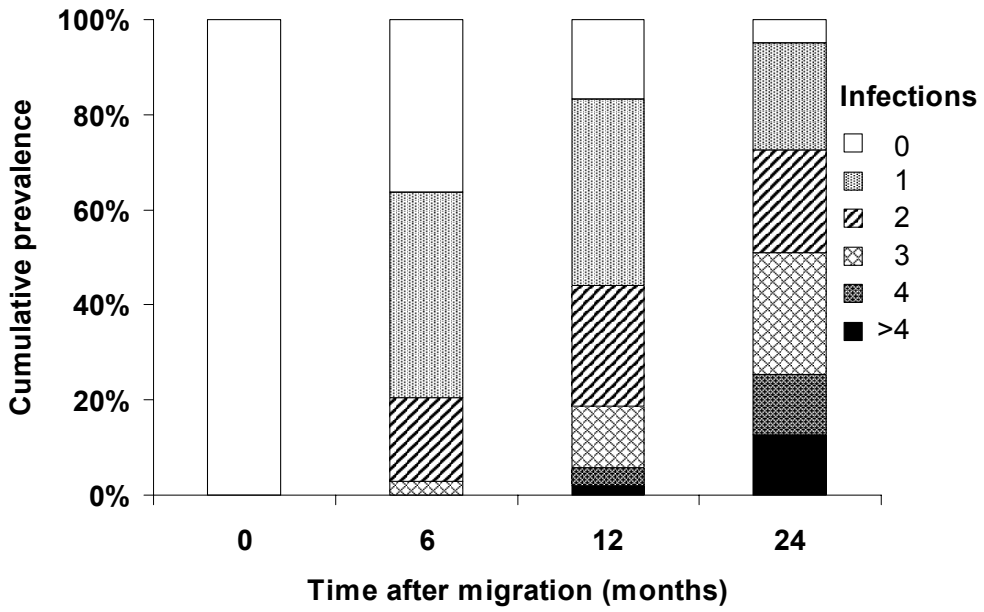

Figure 9.1. Cumulative prevalence of asexual stage infections in a cohort of 102 non-immune migrants from Java to Papua, Indonesia experiencing one to over four Plasmodium falciparum infections during 2 years of follow-up in a malaria-endemic area.

Seroreactivity against whole *P. falciparum* extracts and asexual stage specific antigens increased with cumulative exposure (figure 9.2A). Antibody prevalence in the whole parasite ELISA rose to 98% after two infections and maintained high after further infections. The prevalences of antibodies against sexual stage specific antibodies Pfs48/45 and Pfs230 were also increased after two infections (figure 9.2B). The proportion of serum samples with TRA gradually rose from 10% (3/29) prior to migration, increasing to 27% (3/11) after more than four infections.

There was a clear association between the presence of gametocytes at the time of sampling and TRA (OR 5.22, 95% CI 1.87-14.56). Nevertheless, only 31.3% (10/32) of sera with TRA were derived from individuals with patent gametocytaemia at the time (n=9) or prior to sampling (n=1). After adjustment for the number of infections, TRA was significantly associated with the prevalence of anti-Pfs48/45 antibodies (OR 3.74, 95% CI 1.51-9.29) and anti-Pfs230 antibodies (OR 3.72, 95% CI 1.36-10.17). No statistically significant association existed between TRA and the prevalence of anti-whole parasite (OR 4.21, 95% CI 0.53-33.18) or anti-GLURP (OR 1.90, 95% CI 0.83-4.34) antibodies.

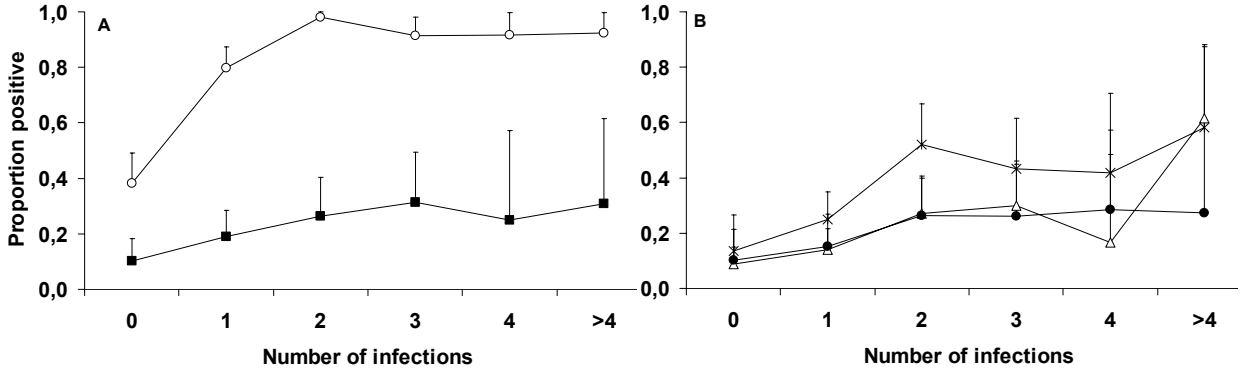

Figure 9.2. Proportion of serum samples with *P. falciparum* specific antibodies as a function of the number of prior *P. falciparum* infections.

A. *seroreactivity for whole parasite (open circles) and asexual-stage specific GLURP (closed squares)*

B. *seroreactivity for sexual-stage specific Pfs230 (asterisks) and Pfs48/45 (open triangles), and functional TRA>50% (closed circles). For TRA, exactly 20 mosquitoes were dissected per serum sample included in the SMFA. The control samples resulted in a median number of 21 oocysts (IQR 7-46) per mosquito. Error bars indicate the upper limit of the 95% confidence interval.*

In order to adjust for correlation between observations from the same individual and for potential confounders, a GEE model was used for statistical

analyses of the data presented in figure 9.2 (table 9.2). There was a statistically significant increase in positive response after *P. falciparum* infections for all ELISA's, which was strongest for the whole parasite assay. There also was a statistically significant positive association between the prevalence of TRA and the number of infections (table 9.2).

Table 9.2 The regression coefficient of the number of prior *Plasmodium falciparum* infections as a predictor of seroreactivity or transmission reducing activity

	N*	Positive response (95% CI) ¥	*P-value*
Whole parasite	102 (296)	1.30 (0.64-1.96) †	<0.001
GLURP	102 (296)	0.49 (0.21-0.77) †, ¶	0.001
Pfs48/45	95 (268)	0.40 (0.24-0.57)	<0.001
Pfs230	89 (252)	0.46 (0.25-0.67) †	<0.001
TRA	76 (161)	0.39 (0.07-0.71) ¶	0.016

** Number of individuals (number of measurements); ¥regression coefficient β (95% confidence interval); †estimate adjusted for age group; ¶estimate adjusted for number of P. vivax infections*

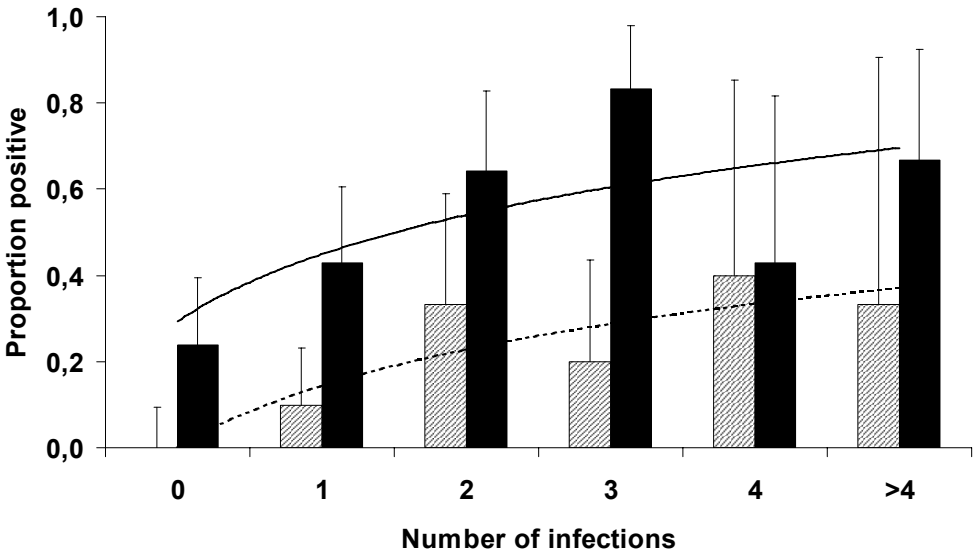

Figure 9.3. The prevalence of Pfs230 seroreactivity as a function of the number of prior P. falciparum infections. *The prevalence of Pfs230 antibodies is depicted for children (shaded bars) and adults (black bars). Error bars indicate the upper limit of the 95% confidence interval. The number of observations for children: 0 (n = 32); 1 (n = 41); 2 (n = 18); 3 (n = 20); 4 (n = 5); >4 infections (n = 3). For adults: 0 (n = 42); 1 (n = 35); 2 (n = 28); 3 (n = 12); 4 (n = 7); >4 infections (n = 9). Logistic trend lines are added to the figures for children (dashed line) and adults (solid line).*

Seroprevalence of antibodies against Pfs230 (figure 9.3), whole parasite and GLURP were consistently higher in adults (data not shown). In adults, 23.8% (10/42) of individuals had anti-Pfs230 antibodies prior to exposure, compared to 0% (0/32) in children. This higher seroprevalence for adults remained apparent throughout follow-up. While the intercept of the trend lines was higher in adults (P = 0.001), the slope of both lines was not different (P = 0.62), indicating a similar relation between number of infections and Pfs230 seroreactivity for adults and children. In the GEE models (table 9.2), the relation between number of infections and whole parasite, GLURP and Pfs230 seroreactivity was not different between children and adults.

Discussion

This study shows that sexual stage specific seroreactivity develops after a limited number of *P. falciparum* infections in a cohort of non-immune transmigrants. The prevalence of anti-Pfs48/45 and anti-Pfs230 antibodies and functional TRA increase with the number of infections experienced.

Development of sexual stage specific immunity resembles that of immunity against asexual stages with similar patterns for acquisition of antibodies against GLURP, Pfs230 and Pfs48/45. We found that reactivity against all antigens develops quickly after exposure, with seroprevalence increasing with number of infections. In line with previous studies, immune response therefore seems to require recent but not necessarily long-term exposure [11-13]. Sexual stage specific antibody responses seem to be part of the initial immune response, which corroborates previous studies in individuals after a primary infection with *P. falciparum* [28,29]. The finding is nevertheless remarkable since exposure to sexual stage parasites is generally considered to be much lower than to asexual parasites. In this study, less than one third of the individuals presented with microscopically detectable gametocytes on any occasion during the two-year follow-up. This apparent low exposure may be due to the poor sensitivity of microscopy for detecting low-density gametocytaemia [30], which can contribute substantially to the total exposure to gametocytes [31]. Sexual stage specific antibodies may be induced by these low density gametocytes or by gametocytes that are sequestered in capillary vessels [32]. The combined possibilities may explain the frequently observed reactivity against sexual stage specific antigens in the absence of microscopically detectable gametocytes. In addition to these antibody responses, we also frequently observed functional TRA without concurrent or prior detection of gametocytes. Although we may have missed some gametocytaemic periods as a result of our fortnightly screening we

conclude that submicroscopic levels of gametocytaemia do play an important role in eliciting sexual stage specific immune responses.

We found no evidence for age as key determinant for the development of immune responses in our population of 6-40 year old transmigrants. Although other studies reported an age-dependent acquisition of clinical and parasitic immunity [12,13], we do not find a similar effect for our selected markers of asexual and sexual stage specific immunity. The reactivity in ELISA's was higher in adults but could reflect background reactivity due to the presence of cross-reactive antibodies reacting with common epitopes [15]. We did not detect a difference in the relation between exposure to infection and the acquisition of (sexual stage specific) immunity for different age groups.

Our most important finding is the presence of functional TRA in a substantial proportion of the individuals increasing with exposure. This has not been shown before for *P. falciparum*, but is analogous to transmission blocking immunity in *P. vivax*, where TRA is more prevalent and efficient after repeated infections [33-35] . In this study, over one quarter of the individuals experiencing more than four *P. falciparum* infections showed TRA, i.e. >50% reduction in oocysts numbers. This pattern of increasing prevalence of TRA accompanies the occurrence of anti-Pfs230 and anti-Pfs48/45 antibodies, as previously shown [8,36-39]. The rapid development of TRA and associated antibodies is particularly promising, since Pfs230 and Pfs48/45 are considered as candidates for inclusion in future transmission-blocking vaccines [28].

Acknowledgments

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Chapter 10

Transmission reducing immunity is inversely related to age in *P. falciparum* gametocyte carriers

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Summary

Immunity to the sexual stages of *Plasmodium falciparum* is induced during natural infections and can significantly reduce the transmission of parasites to mosquitoes (transmission reducing activity; TRA) but little is known about how these responses develop with increasing age/exposure to malaria. Routinely TRA is measured in the standard membrane feeding assay (SMFA).

We collected sera from a total of 199 gametocyte carriers (median age 4 years, quartiles 2 and 9 years) near Ifakara, Tanzania. 128 samples were tested in the SMFA and generated TRA data classified as a reduction of >50% and >90% of transmission. TRA of >50% was highest in young children (aged 1-2) with a significant decline with age (χ^2 trend = 5.79, p=0.016). TRA was associated with prevalence of antibodies to both Pfs 230 and Pfs48/45 (OR 4.03, p=0.011 and OR 2.43 p=0.059 respectively). A TRA of >90% reduction in transmission was not age related but was associated with antibodies to Pfs48/45 (OR 2.36, p=0.055).

Our data confirm that antibodies are an important component of naturally induced TRA. However, whilst a similar but small proportion of individuals at all ages have TRA >90%, the gradual deterioration of TRA>50% with age suggests decreased antibody concentration or affinity. This may be due to decreased exposure to gametocytes probably as a result of increased asexual and/or gametocyte specific immunity.

Introduction

Immunity against the sexual stages of the *Plasmodium falciparum* parasite can develop after exposure to gametocytes [1]. This immunity is primarily antibody mediated and affects transmission by preventing fertilisation [2,3]. Sexual stage specific antigens that induce transmission-blocking immunity (TBI) can thus form potentially useful components of a malaria vaccine [2,4,5]. However complete blockade of transmission is rare [6] and it is more appropriate to define this as transmission reducing activity (TRA) [7].

It is well known that naturally occurring immunity to asexual stages of the malaria parasite develops with age and exposure [8,9] and that anti-parasite immunity follows the development of anti-disease immunity. By comparison relatively little is known about the development of immunity to the sexual stage of the malarial parasite, its age-dependency and the dynamics of the antibodies responsible for TRA. Studies have shown that antibody responses to the key pre-fertilisation antigens are induced as part of the initial immune response during infection [1,5,6]. However, whether maturation of these responses occurs with subsequent exposure is unclear [1]. In the Gambia there was no correlation with age and prevalence and intensity of immune responses to native Pfs230 [10] whilst in Papua New Guinea responses to Pfs48/45 and Pfs230 in competition ELISAs are suggestive of increase in prevalence and titre of antibodies with age [11]. The importance of these antigens is emphasised by the correlation between antibodies induced against them and TRA (Pfs48/45 [6,12-15] & Pfs230 [4,12,16,17]). Such a correlation has not been shown for the gametocyte protein Pfs16 though antibodies to Pfs16 are present in many individuals living in malaria endemic areas [18].

TRA is typically assessed using the standard membrane feeding assay (SMFA) [19]. Although the SMFA is labour intensive and therefore not ideally suited for testing large numbers of sera, it remains the gold standard and was used in this study on TRA in Tanzanian gametocyte carriers. In a previous study we examined the dynamics of gametocyte production in a population of symptomatic *P. falciparum* malaria patients from a hyperendemic area of Tanzania. Findings suggested age/exposure related immunity manifested as lower gametocyte prevalence with increasing age [20]. The current study investigates the relationship between age and functional TRA of sera from the gametocyte carriers in this population, against cultured parasites of the NF54 strain of *P. falciparum*. We also evaluate the correlation between TRA and several serological assays.

Methods

Gametocyte carriers and control sera

Field sera were obtained from gametocyte carriers recruited at a dispensary in Ifakara, Tanzania, as described elsewhere [20]. Briefly, all individuals presenting at the dispensary with *P. falciparum* parasitaemia were asked to return for several follow-up visits. On each return visit a blood slide was taken and examined extensively for gametocytes. A slide was declared negative if no parasites were seen after examination of 100 fields. Gametocyte density was assessed by counting against 500 leucocytes, trophozoite density by counting against 200 leucocytes. Gametocyte and trophozoite counts were converted to numbers of parasites per μL by assuming a standard leucocyte count of 8000/μL [21]. Afebrile individuals found positive for gametocytes were asked to give a venous blood sample. After obtaining written informed consent, venous blood samples were collected and allowed to clot. Sera were then aliquoted and stored at -20°C. A brief questionnaire was completed for each volunteer after collection of blood, detailing history of symptoms, drug consumption and other demographic details. All individuals were assessed by trained clinical staff and treated in accordance with national guidelines. Ethical permission for the study was given by the Tanzanian National Institute for Medical Research and informed consent was obtained from all participants or their parents/guardians.

Positive control sera were obtained from a Dutch expatriate who worked in an endemic malaria area in Tanzania for more than 30 years.

Negative control sera were obtained from Dutch bloodbank donor volunteers, without any history of malaria, collected in the absence of a patent infection.

Parasites

Gametocytes of the NF54 strain of *P. falciparum* were cultivated using the 'shaker' system [22]. Mature gametocytes were isolated at 37°C. To prevent activation parasite culture was diluted ten fold in M199, containing 0.1% glucose (M199-G). After centrifugation for 15 minutes at 2,000g the pelleted parasites were resuspended in M199-G, loaded on a cushion of 18% Nycodenz [Axis-Shield PoC AS, Oslo, Norway] and centrifuged for 30 minutes at 5,000g as described by Vermeulen *et al*. [23].

NF54 gametocytes were extracted in 25 mM Tris-HCl (pH 8.0) supplemented with 150 mM NaCl, 1.0% Sodium Desoxycholate (DOC), 1 mM phenyl-methylsulphonyl fluoride. Insoluble debris was pelleted by centrifugation (13,-000g, 5 min at room temperature) and the supernatant used as the antigen source in the different ELISAs (Pfs48/45 and Pfs230).

Standard membrane feeding assay (SMFA)

Experimental infections of mosquitoes were carried out as previously described [24,25]. Three to five day old *A. stephensi* were allowed to feed on cultured *P. falciparum* gametocytes of NF54, in the presence of control or test serum. Test serum was diluted in non-immune control serum (0.75 v/v) and suspension dilution was 0.33 v/v. After feeding, blood-fed mosquitoes were kept at 26°C. Surviving mosquitoes were dissected seven days later, and oocysts counts were made. Midguts of 20 mosquitoes were examined for oocysts. The test was considered valid when all three batches fed on samples containing control sera showed at least 90% infection. The observed transmission reducing activity (TRA) of serum was determined as the percentage reduction in oocyst numbers in test samples as compared to paired controls [26]. Functional transmission reducing activity was defined as a minimum of 50% reduction in oocysts numbers in the standard SMFA. Individuals with TRA>50% were considered transmission reducers and those with TRA>90% as transmission blockers.

Enzyme Linked Immunosorbent Assays

IgG1 antibodies against Pfs230. The presence of anti Pfs230 specific human antibodies of the IgG1 isotype was determined by coating 10μg/mL of anti-Pfs230 mouse mAb 63F6D7-F(ab)2 fragment in phosphate buffered saline (PBS pH7.2) on ELISA-plates (Sanbio U-bottom, high binding). Free sites were blocked with 5% milk in phosphate buffered saline (PBS), and Pfs230 was captured from a gametocyte extract (200.000 parasite equivalents/well). Three-fold serial dilution's of the test sera (1:25, 1:100, 1:200) was added to the wells and incubated for 60 min. Bound IgG1 subclass antibodies were detected by HRPO-labelled goat anti-human IgG1[Pierce]. Wells were washed with PBS and subsequently incubated with tetramethyl benzidine (TMB) substrate solution for 20 min. The colour reaction was stopped with 4N H_2SO_4, and the optical density was read in a Titertek Multiscan spectrophotometer at 450 nm. All serum samples were tested in duplicate. Negative and positive controls were included on each plate. Sera were considered positive if the optical density (OD) was greater than two standard deviations above the mean of the negative control sera.

IgG1 antibodies against Pfs48/45. Anti-Pfs48/45 specific human antibodies were measured by coating 10μg/mL of anti-Pfs48/45 rat mAb 85RF45.2b in PBS on ELISA-plates (Sanbio U-bottom, high binding). The remainder of the test was carried out as described above. Each serum was tested individually with and without gametocyte extract. Sera were considered positive by the same criteria as for Pfs230 ELISA.

Circumsporozoite antibodies ($NANP_5$) ELISA. Samples were tested for the presence of circumsporozoite antibodies as a measure of exposure to infectious

mosquito bites using a one-stage ELISA [27]. Briefly, one µg/mL ($NANP_5$) conjugated to bovine serum albumin, [F Esposito, Università di Camerino, Italy] in PBS was coated on round-bottom PVC Microtitre ® plates [Dynex Technologies INC., USA] for 60 minutes. After washing and blocking open wells with 5% milk (marvel dried skim milk) in PBS (pH 7.2), 50 µL of a 1:100 serum dilution in PBS with 0,05% Tween and 0,2% milk (MTPBS) was added and incubated for 90 min. After washing, 50 µL of goat anti human immunoglobulin labelled with peroxidase (ImmunoPure, diluted 1:40 000 in MTPBS) was added for 60 min. The remainder of the procedure and the evaluation of sera were carried out as described above for IgG1 antibodies against Pfs230. All incubations were carried out at room temperature.

Data Analysis

Data were analysed using SPSS statistical software version 9.0 and STATA version 7.0. The presence of an age-dependent trend in parasitological characteristics was tested using linear regression. For this purpose gametocyte and trophozoite densities were log-transformed. Age dependency of TRA and reactivity in ELISA assays was initially tested using the χ^2 test for trend. The association between seroreactivity in serological assays and reduction of transmission in the SMFA was tested in logistic regression and quantified using odds ratios.

Results

Sera were collected from a total of 199 gametocyte carriers (median age 4 years, quartiles 2 and 9 years), 47% (94/199) of the subjects were male. The parasitological characteristics of the gametocyte carriers are summarised in Table 10.1. Trophozoite prevalence, trophozoite density and gametocyte density decreased with age.

Standard membrane feeding assay (SMFA)

Sera of 128 subjects were tested in the membrane feeding assay. A total of 53.9% (69/128) of the sera showed ≥50% reduction in number of oocysts. Figure 10.1 shows the age dependent transmission-blocking activity as assessed in the SMFA. The proportion of sera resulting in ≥50% reduction was 64.6% (31/48) in 1-2 year old children and decreased with age (p=0.016). The proportion of sera able to reduce transmission by 90% or more remained consistent at all ages.

Table 10.1 Parasitological characteristics of study population: trophozoite prevalence and geometric means of the gametocyte density and trophozoite density.

Age group	Number	Gametocyte density/μL (IQR) *	Trophozoite prevalence % ¥	Trophozoite density/μl (IQR) ¶
1-2 years	76	81.2 (32 -200)	59.2	899.4 (118- 6420)
3-6 years	60	98.2 (40 -253)	50.0	521.8 (95 - 1156)
7-15 years	31	83.0 (32 -208)	48.4	674.8 (160 - 2340)
≥ 16 years	32	41.4 (16 -105)	43.8	192.2 (34 - 966)
Total	199	77.7 (32 -200)	52.3	599.4 (120 -1889)

*IQR = interquartile range; * Geometric mean gametocyte density; linear regression, age group: β = -0.18 (se=0.080); p = 0.029; ¥ Trophozoite prevalence, Chi-square test for trend: χ^2= 2.43 p=0.12; ¶ Geometric mean asexual parasite density; linear regression, age group: β = -0.42 (se=0.18); p = 0.025*

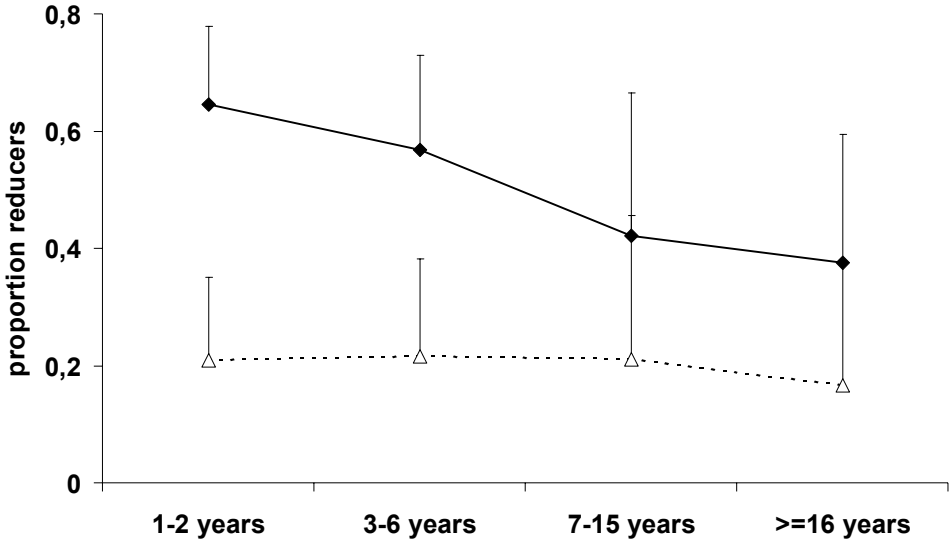

Figure 10.1. Age dependence of transmission reducing activity in the standard membrane feeding assay. *Solid line = transmission reduction (>50%), chi square test for trend χ^2 = 5.79, p=0.016. Broken line = transmission reduction (>90%), chi square test for trend χ^2 = 0.13, p=0.71. Error bars indicate the upper limit of the 95% confidence interval. The number of individuals was 48, 37, 19 and 24 for age groups 1-2 years, 3-6 years, 7-15 years and >15 years respectively.*

Antibody responses and transmission reduction

Serological reactivities as assessed by ELISA are summarised in Table 10.2. Testing for anti- circumsporozoite antibodies was performed to assess the exposure to infectious mosquito bites [28]. Overall 59.2% (116/196) of the gametocyte carriers were positive for anti-$NANP_5$ antibodies, indicating a high

exposure. The prevalence of anti-$NANP_5$ antibodies increased with age, probably as a result of cumulative exposure to malaria infections. There was no age dependency observed for the presence of anti-Pfs48/45 antibodies, which was poorly recognised. Antibodies to Pfs 230 were weakly correlated with age (p=0.093).

Table 10.2: Prevalence of serological reactivity to sporozoite ($NANP_5$) and sexual stage antigens (Pfs230 and Pfs48/45)

Age group	$NANP_5^*$ % (N)	Pfs230[¥] % (N)	Pfs48/45 % (N)
1-2 years	45.2 (73)	16.7 (72)	23.3 (73)
3-6 years	60.0 (60)	13.8 (58)	13.3 (60)
7-15 years	67.7 (31)	20.0 (30)	12.9 (31)
≥ 16 years	81.3 (32)	31.3 (32)	28.1 (32)
Total	59.2 (196)	18.8 (192)	19.4 (196)

[*]$NANP_5$ and age: χ^2 for trend 13.1 p=0.0003; [¥]Pfs230 and age: χ^2 for trend 2.8 p=0.093

A TRA of 50% was negatively associated with age (OR=0.62; 95% CI 0.43-0.88) as is shown in table 10.3. Sera with detectable levels of antibodies to Pfs230 and, to a lesser extent, Pfs48/45 were significantly associated with this level of TRA. A TRA of >90% was associated with antibodies to Pfs48/45 (table 10.3) at borderline level of significance. The prevalence of anti $NANP_5$ antibodies was not associated with TRA at either level (data not shown). Neither asexual prevalence, density nor gametocyte density were significant predictors of TRA in the logistic regression models nor did they confound any of the associations described above.

Table 10.3: Adjusted odds ratio (OR) of the probability of functional transmission reducing activity (TRA) in the SMFA.

	TRA –50		TRA –90	
	OR (95% CI)	P-value	OR (95% CI)	P-value
Age group	0.62 (0.43 – 0.88)	0.017	0.90 (0.58 – 1.37)	0.63
Pfs230 seroprevalence	4.03 (1.37 – 11.86)	0.033	2.46 (0.83 – 7.31)	0.105
Pfs48/45 seroprevalence	2.43 (0.97-6.12)	0.068	2.62 (0.98-7.00)	0.055

TRA-50 = transmission reducing activity with >50% reduction; TRA-90 = transmission reducing activity with >90% reduction.

Discussion

In this study 54% of the sera from gametocyte carriers were capable of reducing transmission of gametocytes to mosquitoes in standard membrane feeding assay (SMFA) with cultured parasites. This prevalence is comparable with results from Cameroon [19,29]. However the ability to reduce transmission of gametocytes to mosquitoes by more than 50% of controls was highest in the youngest age group and then decreased significantly with age. The most efficient level of TRA (a reduction in transmission of 90%) was stable at ~20% in all age groups. These data are intriguing and seem to contrast with immune responses to the asexual stages of *P. falciparum* where effective immunity matures with age.

A recent study from Cameroon found no relation between age and TRA [7] and an earlier study from the same area suggested a positive relation [29], contrary to our findings in Tanzania. However, both studies used the direct feeding assay (DMFA) to assess TRA which is often less sensitive due to its reliance on circulating gametocyte density in the infected individual. In this assay mosquitoes feed on peripheral blood washed and resuspended in either autologous or a control serum. Natural gametocyte densities and resultant oocyst loads are typically low making statistical assessment of TRA difficult. Moreover neither study included the youngest children where we observed the highest level of TRA>50%, this may have obscured the trend. In the most recent of these studies the proportion of individuals defined as transmission reducers dropped from 30% in the 5-10 year age group to 10.3% in the 11-20 year age group [7].

It is interesting to postulate why, with increasing age and cumulative exposure to parasites, TRA seems to decrease rather than increase. Firstly, it may be a variety of mechanisms which reduce the numbers of sexual forms to a level which is insufficient for maintenance of effective TRA. This could be due to asexual stage immunity where numbers are controlled such that subsequent gametocyte densities are also reduced. Secondly, cross-stage immunity against PfEMP1 affecting both asexual and the earlier developmental sexual stages of the parasite which have similar sequestration profiles has also been described [30]. A third possibility is a human immune response specifically directed against later stage sexual parasites which differ from asexual forms in their sequestration site and presumably use different ligands yet to be described [31]. There is epidemiological support for this as an explanation [32] that could reduce gametocyte prevalence or longevity (*Chapter 2*) [33]. Thus the higher gametocyte densities in children induce higher levels of transmission reducing activity. As age increases, gametocyte densities and the stimulus for TRA decrease. The dynamics of antibody development will also be affected by the frequency of infection. In Sri Lanka, an area of low transmission intensity, no correlations

were seen with the antibodies to Pfs230 and Pfs48/45 and the ability to block transmission, suggesting the rate of re-infection influences maturity of the immune response [34].

The finding may also be a consequence of poor immunogenicity of the sexual stage antigens investigated [35]. It has been hypothesised that the poor memory response generated by the antigens Pfs230 & Pfs48/45 is a result of a T-cell independent response [36] and/or clonal imprinting [37]. The gradual decline of TRA>50 in these individuals may be due to changes in the antibody repertoire. Although TRA was correlated with antibodies against Pfs48/45 and Pfs230, none of those antibodies showed a similar age-dependent decrease in prevalence. Our analysis was restricted to antibodies of the subclass IgG1 it may be that isotype switching (noted with other malaria antigens notably MSP-1 –19 [38]) results in a less effective TRA. This may be particularly relevant for Pfs230 as complement mediated lysis of gametes has been correlated with antibodies to this antigen [17] and murine monoclonal antibodies to Pfs230 which block transmission are all of complement fixing isotypes [39,40].

Clear relationships between serology, TRA and age may have been obscured by the fact that gametocyte carriers were sampled at first presentation with gametocytes and the development of sexual stage immunity may take longer during the course of a natural infection. Data from Papua New Guinea documented a complicated profile of anti Pfs48/45 antibody kinetics (defined by competition ELISA) in children one month after a clinical attack [11]. It may be that effective immunity is only apparent once gametocytes have disappeared from the peripheral blood having been cleared and presented to the immune system. This is an important area for future study and could provide important information on the influence of age related TRA on the infectious reservoir for malaria.

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Chapter 11

A longitudinal study of immune responses to *Plasmodium falciparum* sexual stage antigens in Tanzanian adults

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Summary

Next to children, adults form a substantial part of the infectious reservoir that is responsible for the spread of malaria in human populations. The objective of this longitudinal study was to determine sexual stage immune responses to *Plasmodium falciparum* and the infectiousness to mosquitoes in adults from an area with intense malaria transmission.

A cohort of 43 Tanzanian adults was followed for 18 months. Mosquitoes were collected fortnightly; parasitological data monthly and serum every 3 months. Serological assays were performed for antibodies to sexual stage antigens Pfs230 and Pfs48/45 and circumsporozoite protein ($NANP_5$)(n=199). Functional transmission reducing activity (TRA) was assessed by standard membrane feeding assay (n=85).

The overall EIR was 165.5 infectious bites per person per year (95% CI 117.2 – 227.2). Cumulative parasite prevalence was 67.4 % (29/43) for asexual stages and 34.9% (15/43) for gametocytes. Enrolment antibody prevalence was 95.3% (41/43) for $NANP_5$, 18.9% (7/37) for Pfs230 and 7% (3/43) for Pfs48/45 epitope 3. TRA >50% reduction was seen in 48.2% (41/85) and TRA >90% reduction in 4.7% (4/85) of the samples.

Thus, sexual stage immune responses were low, inconsistent over time and not related to age. Our findings suggest that the low level sexual stage and transmission blocking immunity is likely to be the result of an effective asexual stage immunity that limits gametocyte numbers and thereby the antigenic stimulus necessary to maintain effective sexual stage immunity. As a consequence, the relative infectivity of older individuals is less likely to be affected by sexual stage immunity.

Introduction

Successful transmission of *Plasmodium falciparum* from man to mosquitoes depends not only on the presence of mature gametocytes in the peripheral bloodstream but also on human host immunity. Host immunity can affect transmission indirectly by attacking asexual stage parasites [1] and directly by cross-stage immunity affecting asexual and sexual stage parasites [2] or sexual stage specific immunity. The latter may reduce gametocyte prevalence [3] or longevity (*Chapter 2*) [4] but can also reduce the infectiousness of gametocytes by preventing fertilization or arresting the development of zygotes in the mosquito midgut [5-7]. This phenomenon is called transmission reducing activity (TRA) and is observed in sera from individuals from malaria endemic countries [8,9]. TRA has been correlated with the presence of antibodies against *P. falciparum* gamete surface antigens Pfs230 [8,10,11] and Pfs48/45 [8,12].

Sexual stage specific immunity appears to develop rapidly after exposure (*Chapter 9*) [13,14], but may be short-lived [15] and is hypothesised to depend on recent rather than cumulative exposure (*Chapter 12*) [16]. Exposure to gametocytes is lower in adults compared to children [17,18] and, as a consequence, sexual stage specific antibody responses would be expected to decrease rather than increase with age. The recently reported negative age dependency of TRA in gametocytaemic children (*Chapter 10*) [19] seems to support this hypothesis although data confirming this trend in adults is lacking. Information on sexual stage specific immunity in adults is relevant for future transmission reducing interventions since adults contribute considerably to the human infectious reservoir [17].

Our objective was therefore to determine sexual stage immune responses to *P. falciparum* in a cohort of Tanzanian adults resident in an area of high but seasonally variable transmission intensity. We determine the prevalence of sexual stage antibodies and functional TRA in relation to age and compare two villages of different transmission intensity.

Methods

Study site

This study was conducted in Ichima and Miwangani, hamlets of the village of Idete 30 kilometres distance from Ifakara, south-eastern Tanzania. The transmission of *P. falciparum* is different for both villages although intense and perennial with *Anopheles gambiae* and *A. funestus* as the major vectors. *P. falciparum* is present in over 90% of malaria cases [20]. Six households were selected from Ichima and from Miwangani. All occupants who were 16 years of age or older were asked to participate in the study. Only those subjects who voluntarily gave signed informed consent were enrolled. Scientific and ethical clearance for the project was obtained from the Tanzanian Commission for Science and Technology (COSTECH) and the Ifakara Health Research and Development Centre (IHRDC).

Entomology

CDC light traps were used as described previously [21] to catch mosquitoes fortnightly in each room that was occupied by study participants. The presence of sporozoites was determined by ELISA and entomologic inoculation rate (EIR) was calculated using a conversion factor (1.605), which takes into account the difference between light trap catches and human landing catches [22]. EIR was expressed as number of infectious bites per person per year for both villages separately and the method described by Drakeley *et al*. was used for calculating confidence intervals [23]. Season was defined as wet season (March - May); cool season (June – September) and hot season (October – February) based on meteorological data [23].

Parasitology

Screening for asexual parasites and gametocytes of *P. falciparum* took place monthly for a period of 16 months. Finger prick blood samples were collected and thick blood smears were dried, stained with 10% Giemsa and examined microscopically. A slide was considered negative if no asexual parasites were seen after examination of 100 fields. Gametocyte and trophozoite densities were assessed by counting against 500 and 200 leukocytes, respectively, and converted to parasites per μl by assuming a standard leukocyte count of 8000/μL. Every three months a venous blood sample was taken, centrifuged and serum separated and frozen at -80° for laboratory analyses.

Antigen preparation

Mature gametocytes of *P. falciparum* (NF54 strain) were produced as described elsewhere [19] in an automated static culture system as described by Ponnudurai *et al*. [24] and stored at -70^{0}C until used. NF54 gametocytes were extracted in 25

mM Tris-HCl (pH 8.0) supplemented with 150 mM NaCl, 1.0% sodium desoxycholate (DOC) and 1 mM phenylmethylsulphonyl fluoride. Centrifugation pelleted insoluble debris (13,000g for 5 min at room temperature) and the supernatant provided antigen for ELISA's.

Standard Membrane Feeding Assay

Experimental infections of mosquitoes were carried out as previously described [25,26]. Three to five day old *Anopheles stephensi* mosquitoes were allowed to feed on cultured NF54 *P. falciparum* gametocytes, in the presence of control or test serum. Heat-inactivated test serum was diluted in non-immune control serum that was not heat-inactivated (0.75 v/v). The suspension dilution was 0.33 v/v. After feeding, blood-fed mosquitoes were kept at 26°C. Surviving mosquitoes were dissected seven days later, and oocysts counts were made. Midguts of 20 mosquitoes were examined for oocysts. The test was considered valid when all three batches fed on samples containing control sera showed at least 90% infection. The observed transmission reducing activity (TRA) of serum was determined as the percentage reduction in oocyst numbers in test samples as compared to paired controls [27]. Not all sera were analysed in the standard membrane feeding assay (SMFA) because of resource limitations. We therefore selected four months to obtain information on TRA in the three different seasons as well as from the beginning and the end of the study-period. Thus, all available sera from September, December 1997 and March, December 1998 were assayed.

Enzyme Linked Immunosorbent Assays (ELISA)

ELISA assays for circumsporozoite protein ($NANP_5$) and Pfs230 IgG1 were preformed as described elsewhere [19]. Serum was diluted 1/100 for Pfs230 antibody detection and the titer was determined for $NANP_5$ antibodies, starting with a 1/100 serum dilution.

The presence of anti-Pfs48/45 specific human antibodies was determined for different epitopes since there may be variation in their functionality [28]. Antibodies were measured by coating 10μg/mL of anti-Pfs48/45 mouse mAb 32F3 or 32F1 recognizing epitope 1 and 2b of Pfs48/45) in PBS for one hour on ELISA-plates (Sanbio U-bottom, high binding). Free sites were blocked with 5% milk in phosphate buffered saline (PBS) for 30 minutes, and gametocyte extract (200,000 parasite equivalents/well) was incubated for two hours. After three washes with PBS, wells were incubated with a mixture of 30 μl 1/20 diluted test serum and 30 μL HRPO-labeled anti-Pfs48/45 mAb for one hour (final dilution 1/40). Different anti-Pfs48/45 mAb's were used for epitope 1: 85RF45.1 (0.1μg/mL); epitope 2b: 85RF45.2b (1μg/mL); epitope 3: 85RF45.3 (1μg/mL) and epitope 5: 85RF45.5 (1μg/mL). Wells were washed with PBS and

subsequently incubated with tetra methyl benzidine (TMB) substrate solution for 20 min. The color reaction was stopped with 4N H_2SO_4, and the optical density was read in a Titertek Multiscan spectrophotometer at 450 nm. All serum samples were tested in duplicate. Negative and positive controls were included on each plate. In this competition ELISA, sera were considered positive when the optical density (OD) was equal or lower than the mean plus two standard deviations of the negative control blood bank sera.

Data analyses

Parasite densities were analyzed after natural log-transformation. Geometric mean parasite density with inter quartile range (IQR) was calculated for parasite carriers only. Functional transmission reducing activity was categorized as >50% reduction in the number of oocysts in the mosquito midgut (TRA >50%) or >90 reduction (TRA >90%) (*Chapter 9*) [13,27]. Nonparametric Wilcoxon Rank-sum tests were used to test differences between groups for statistical significance in case of continuous variables. Pearson correlation coefficients were calculated to assess the association between continuous variables. Multiple logistic regression models with Generalized Estimating Equations (GEE) were used to test the influence of age as continuous variable on dichotomous variables such as parasite prevalence and immune reactivity. A random effect was included in the models to allow for correlations within individuals. A similar procedure was carried out using GEE for continuous dependent variables. Statistical analyses were preformed using procedures available in SPSS 12.0 and STATA 8.0.

Results

Entomology

The overall EIR was 165.5 ib/p/yr (95% CI 117.2 – 227.2). The EIR was consistently higher in Ichima (276.9 ib/p/yr, 95% CI 189.5 – 390.9) compared to Miwangani (61.9 ib/p/yr, 95% CI 22.7 – 134.7). In the cool season (June – September) the EIR was lowest with 227.5 infectious bites per person per year in Ichima and 0 in Miwangani. In the hot season (October – February) these values were 274.6 and 80.2, respectively, and in the wet season (March – May) 376.7 and 83.7. The number of mosquitoes that was caught per month shows a similar picture, fluctuating over time but being consistently higher in Ichima (figure 11.1)

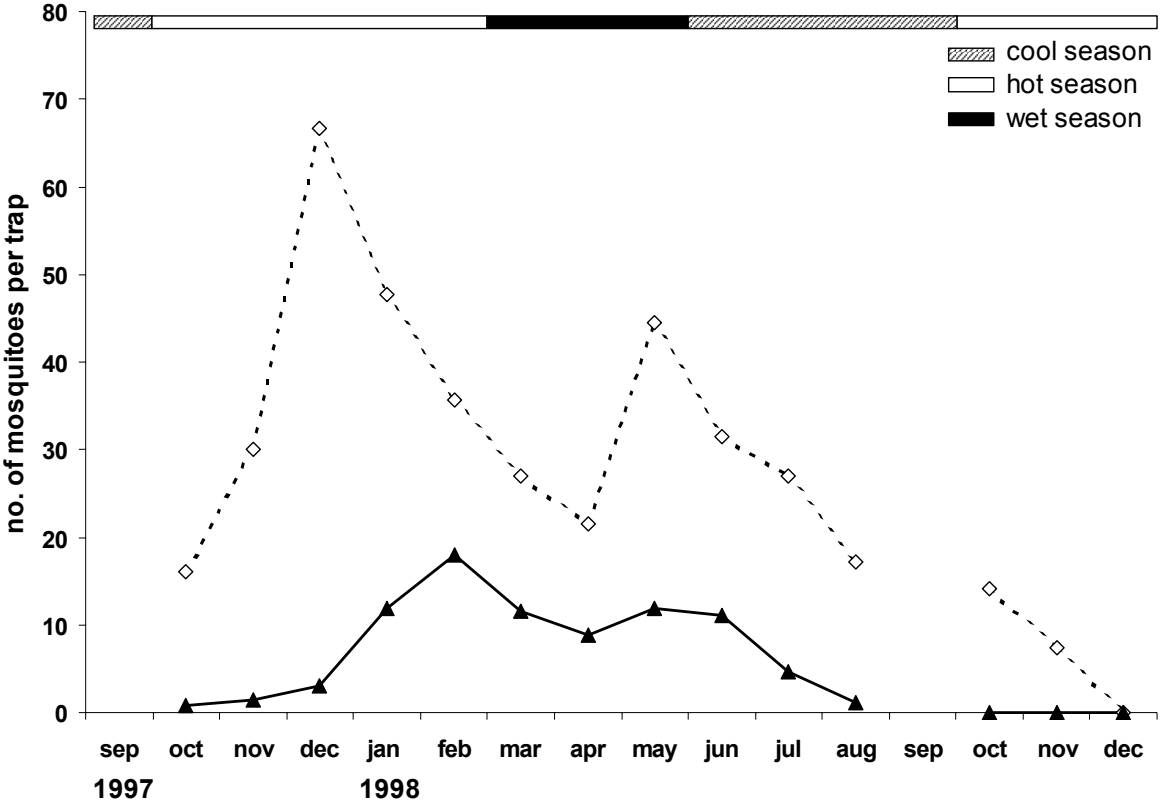

Figure 11.1. The average number of mosquitoes per trap per month in Ichima and Miwangani. *Diamonds and dotted line = Ichima, triangles andsolid line = Miwangani. Data were missing for September 1998 because the roads were inaccessible due to heavy rains.*

Parasitology

Forty-three adults with a median age of 28 years (IQR 21-50) were followed for 16 months. Asexual parasite prevalence at inclusion was 14.0% (6/43) with a geometric mean parasite density of 228.3 parasites/μL (IQR 108.4 – 463.3). One individual had detectable gametocytes at enrolment. During the 16 month study, the cumulative asexual parasite prevalence was 67.4% (29/43); for gametocytes this was in 34.9% (15/43). The peak point prevalence was 33.3% (11/33) for asexuals and 9.1% (3/33) for gametocytes. Only 27.6% (8/29) of the asexual parasite carriers harboured asexual parasites on at least two consecutive visits. One person harboured gametocytes on more than one occasion. Gametocytaemia coincided with asexual parasitaemia in 25% (4/16) of the cases. The association between parasitaemia and immune responses with age is presented in table 11.1. While data are presented in age categories, statistical analyses were performed using age as a continuous variable. The adjusted estimates show a statistically significant negative relation between age and both asexual parasite prevalence (GEE: β = -0.052; se = 0.016; p = 0.001) and asexual parasite density (GEE: β = -0.024; se = 0.0084; p = 0.004).

Estimates were adjusted for potential confounding factors such as the village of residence and season and a random effect was added to allow for within individual correlations.
There was no statistically significant relation between age and either gametocyte prevalence or gametocyte density (table 11.1).

Serological reactivity

Reactivity to circumsporozoite protein derived $NANP_5$, was highly prevalent in this population where 95.3% (41/43) of the subjects had detectable antibodies at enrolment (range of titers 50 – 1600). Despite the study population consisting of adults only (range 16 – 63 years), there was an age dependent increase in anti-$NANP_5$ antibody prevalence (table 11.1, GEE: β = 0.26; se = 0.10; p = 0.001). Individuals who remained free of asexual parasites throughout the study (n = 14) had a median $NANP_5$ titer at enrolment of 400 (IQR 200 – 800) compared to 200 (IQR 100 – 400) for those newly infected during follow-up (n = 23) (Wilcoxon rank-sum test, p = 0.01), suggesting an association of circumsporozoite antibodies with responses that are protective against infection with *P. falciparum*.

In contrast to antibodies to $NANP_5$, the prevalence of antibodies against sexual stage specific antigens was relatively low. Antibodies against Pfs48/45 at enrolment were detected in 2.3% (1/43) of the subjects for epitope 1, in 2.3% (1/43) for epitope 2b, in 7% (3/43) for epitope 3 and in none of the subjects for epitope 5. None of the individuals showing reactivity against Pfs48/45 epitope 1, 2 or 5 had antibodies on more than one occasion. Eleven individuals showed reactivity against Pfs48/45 epitope 3 (table 11.2). Of those, 63.6 % (7/11) was seropositive only once. Two individuals were consistently seropositive and although these individuals harboured asexual parasites during the study period, gametocytes were not detected. Seroconversion from Pfs48/45 epitope 3 positive to negative was observed in six individuals. Antibodies against Pfs230 were detected in 18.9% (7/37) of the individuals at enrolment (table 11.2). Fifteen individuals showed Pfs230 antibodies at least once during the study period, and 46.7% (7/15) of those were seropositive on only one occasion. Two individuals were consistently positive in the Pfs230 ELISA, both had patent asexual parasitaemia and gametocytaemia during the study. The consistent Pfs230 responders were not the same individuals as those who consistently showed antibodies against Pfs48/45 epitope 3. Conversion from Pfs230 seropositive to negative was observed in eleven individuals.

On basis of their prevalence, only antibodies against sexual stage specific Pfs48/45 epitope 3 and Pfs230 were considered useful for more detailed analyses. The prevalence of these sexual stage specific antibodies fluctuated

throughout the study period without a statistically significant association with gametocyte prevalence, season (data not shown) or age (table 11.1).

Table 11.1. The regression coefficient of age as a predictor of parasitaemia, seroreactivity or transmission reducing activity.

	Age			GEE	GEE
	<20 years	20-30 years	≥30 years	Age	Age
Individuals	7	17	19	β (se)	p-value
Parasitology					
Asexual parasite positive, % (n/N)	42.4 (36/85)	15.1 (28/186)	10.9 (24/220)	-0.052 (0.016)	0.001
Asexual parasite density GM (IQR)*	341.3 (160.0-659.1)	213.3 (120.0-406.3)	180.1 (80.0-320.0)	-0.024 (0.0084)	0.004
Gametocyte positive, % (n/N)	5.9 (5/85)	2.6 (5/186)	2.3 (5/220)	-0.016 (0.018)	0.39
Gametocyte density GM (IQR)*	26.3 (16.0-39.2)	20.2 (16.0-32.0)	37.0 (16.0-91.9)	0.013 (0.011)	0.23
Immune responses					
$NANP_5$ prevalence (n/N)	77.8 (28/36)	97.3 (71/73)	100 (90/90)	0.26 (0.10)	0.01
Pfs48/45.3 prevalence (n/N)	19.4 (7/36)	5.5 (4/73)	12.2 (11/90)	0.0067 (0.025)	0.79
Pfs230 prevalence (n/N)	30.0 (9/30)	11.4 (8/70)	21.1 (16/76)	0.028 (0.024)	0.24
TRA >50% prevalence (n/N)	45.5 (5/11)	55.6 (20/36)	42.1 (16/38)	-0.022 (0.014)	0.13

*n = number of observations with a positive result; N = total number of observations. GM = geometric mean; IQR = interquartile range; TRA >50% = transmission reducing activity with >50% reduction; β = coefficient for age in years using a GEE model adjusting for village and season and allowing for within individual correlations; se = standard error. *Geometric mean density for parasite carriers only. Individuals contribute to one age category but multiple observations per individual are included.*

There was also no statistically significant relation between seroreactivity against Pfs48/45 epitope 3 and Pfs230 (OR = 1.66, 95% CI 0.49-5,57). Antibody responses were not related to concurrent presence of asexual parasites or gametocytes. However, antibodies against Pfs48/45 epitope 3 appeared more common in individuals who had patent asexual parasitaemia on the previous visit (OR = 2.27, 95% CI 0.82 – 6.30), although not statistically significant.

Table 11.2. Longitudinal survey of antibody responses to $NANP_5$, Pfs48/45 epitope 3 and Pfs230 antigens in Tanzanian adults.

Donor	*Age*	*Village*	*$NANP_5$*	*Pfs48/4. 3*	*Pfs230*	SMFA
1	56	Ichima	+ . . + . .	− . .+ . .	−	0 . . . . .
2	28	Ichima	+ + + . + +	− − − . − −	− − − . . +	0 0 1 . . 0
3	58	Ichima	+ + + + + .	− − − − − .	+ − − . . .	0 0 1 . . .
4	35	Ichima	+ + + + + +	− − − + − −	+ − − . − −	1 0 2 . . 1
5	25	Ichima	+ + + . + +	− − − . − −	. − − . − −	. 0 1 . . 0
22	36	Ichima	+ + + . + .	− − − . − .	− − − . − .	. 1 2 . . .
23	21	Ichima	+ + + . . .	− − − . . .	− − − . . .	. 1 1 . . .
24	30	Ichima	+ + . + + +	− − . − − +	− − . − − .	. 0 . . . 0
25	54	Ichima	+ + + + + +	− − − − − −	. − − − − −	. 0 0 . . 1
26	22	Ichima	+ + . + + .	− − . − − .	− − . − − .	. 1 . . . .
27	63	Ichima	+ + . + + +	− − . − − −	− − . + + +	. 1 . . . 0
28	23	Ichima	+ + + + + +	− − − − − −	− − − − − −	. 1 1 . . .
29	28	Ichima	+ + . + + +	− − . − + −	− − . − − −	. 0 . . . 0
30	56	Ichima	+ . . . + +	− . . . − −	− . . . − −	. . . . .
31	26	Ichima	+ + + + . +	− − − + . −	− − − − . −	. 0 1 . . .
32	23	Ichima	+ + + . . .	− − − . . .	− − − . . .	. 1 1 . . .
33	20	Ichima	+ + ...	− −	− −	. 1 . . . .
34	24	Ichima	+ + . + + +	− − . − − −	− + . − + +	. 1 . . . .
35	17	Ichima	+ + + . + +	− + − . − −	− − − . − −	. 0 1 . . .
36	58	Ichima	+ . + + + +	− . − − − −	− . − − − −	. . 0 . . .
37	62	Ichima	+ + + + + +	− − − − − +	− − + − − −	. 0 1 . . 0
38	45	Ichima	+ + + + + .	− − − − − .	− −	. 0 0 . . .
39	38	Ichima	+ + ...	− −	− −	. 0 . . . .
40	20	Ichima	+ + + . . .	− − − . . .	− − − . . .	. 1 0 . . .
6	55	Miwangani	+ + . + + +	− − . − − −	− − . − − −	0 0 . . . .
8	28	Miwangani	+ + . + . .	− − . − . .	− − . − . .	1 0 . . . .
9	50	Miwangani	+ + + + + +	− − − − − −	. + + − − +	. 0 . . . .
11	16	Miwangani	+ + + + + +	+ + + + + +	. − + − − −	. 0 . . . 0
12	44	Miwangani	+ + + + + +	− − − − − −	− − − − − .	0 0 0 . . .
13	28	Miwangani	+ + + + . +	− − − − . −	− − − − . −	1 0 1 . . 0
14	24	Miwangani	+ + + + + +	− − − − − −	− − + + − −	1 0 0 . . .
18	18	Miwangani	+	−	−	1 . . . . .
19	19	Miwangani	+ + . + + +	+ + . + + +	+ −	2 1 . . . .
20	27	Miwangani	+ + + + + +	− − − − − −	. − − − − −	. 0 . . . .
21	23	Miwangani	+	−	−	1 . . . . .
41	39	Miwangani	+ + + + + +	− − − − − −	− − + − − −	. 1 . . . .
42	21	Miwangani	− + + + + −	+ + − − − −	+ − − − + −	. 1 1 . . 0
43	18	Miwangani	− − − − − −	− − − − − −	− − − − − −	1 0 1 . . .
44	16	Miwangani	+ − + + + −	− − − − − −	+ + + + + +	. 1 . . . .
45	58	Miwangani	+ + + + + +	− + + − − −	+ − + + + +	1 0 2 . . .
46	16	Miwangani	+ + + + + +	− − − − − −	. −	. . . . .
47	18	Miwangani	+ + + + + +	− − − − − −	+ − − + − −	0 0 . . . .
48	54	Miwangani	+ . + . . .	− . − . . .	− . − . . .	1 . 1 . . .

Each symbol represents a separate serum sample. Samples were collected at three-month intervals, giving a maximum of six observations per individual. + = antibody positive; − = antibody negative; . = missing. Missing values are either missing samples (all assays) or a high background reactivity (Pfs230). For the SMFA, available samples from September 1997, December 1997, March 1998 and December 1998 were analysed, giving a maximum of four observations per individual. 0 = <50%TRA (no reduction), 1 = >50%TRA; 2 = >90% TRA.

Transmission reducing activity

A total of 85 experiments were considered valid in the standard membrane feeding assay. Only 4.7% (4/85) of the sera was capable of blocking transmission (TRA >90%) while TRA >50% was observed for 48.2% (41/85) of the sera. There was no statistically significant relation between TRA and age (figure 11.2, table 11.1). There was also no statistically significant relation between TRA and the presence of gametocytes either at the time of sampling or at the previous time-point. There was no consistency in TRA in sera from the same individuals measured at different time-points (table 11.2). In line with previous findings, TRA was related to antibodies against sexual stage antigens. There was a positive relation between TRA >90% and reactivity to Pfs230 (OR = 6.2; 95% CI 0.79 – 48.5) and Pfs48/45 epitope 3 (OR = 9.13; 95% CI 1.13 – 73.9). We observed no statistically significant association between TRA >50% and reactivity to either Pfs230 (OR = 1.2; 95% CI 0.38 – 4.05) or Pfs48/45 epitope 3 (OR = 0.69; 95% CI 0.18 – 2.63).

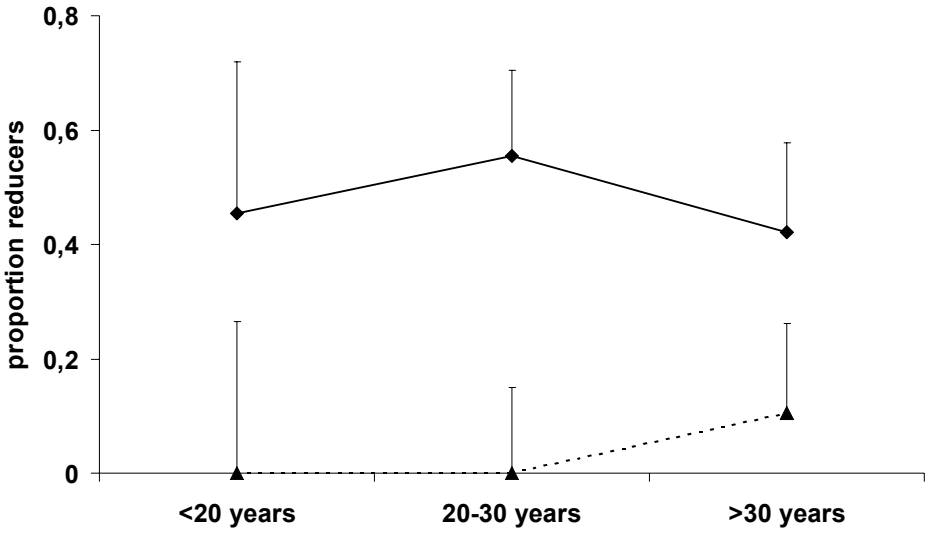

Figure 11.2: Transmission reducing activity against age. The dotted line represents ≥90% reduction, solid line ≥50% reduction in the SMFA. Error bars indicate the upper limit of the 95% confidence interval. Note that more than one observation per individual is included.

Differences between villages

Despite the differences in EIR between villages, asexual parasite prevalence and density did not differ significantly (table 11.3). Gametocyte prevalence and density were non-significantly elevated in Miwangani compared to Ichima (GEE: β = 0.84; se = 0.48; p = 0.08 and GEE: β = 0.65; se = 0.38; p = 0.12,

respectively). Antibodies against circumsporozoite protein $NANP_5$ were common in both villages. The prevalence of anti-Pfs230 antibodies to the sexual stage antigens was significantly higher in Miwangani than in Ichima (GEE: Pfs230 β = 1.31; se = 0.62, p = 0.04). A similar trend was seen for Pfs48/45 epitope 3, albeit not statistically significant (GEE: β = 0.85; se = 0.69, p = 0.22). Although the analyses allowed for clustering of observations from the same individual, it is possible that the difference between Miwangani and Ichima is a consequence of the consistent responders to Pfs230 (n = 2) and Pfs48/45 epitope 3 (n = 2), who all lived in Miwangani (table 11.2). We chose to include these individuals in the analyses because exclusion may dilute the true effect of transmission intensity on sexual stage immune responses. There was no statistically significant difference in the prevalence of TRA between the villages (table 11.3).

Table 11.3. The regression coefficient of village as a predictor of parasitaemia, seroreactivity or transmission reducing activity.

	Village		GEE	
	Ichima	Miwangani	Village	Village
Individuals	24	19	*β (se)*	*p-value*
Parasitology				
Asexual parasite prevalence (n/N)	18.4 (51/226)	17.3 (37/177)	-0.32 (0.36)	0.37
Asexual parasite density, GM (IQR)	212.9 (120.0-440.0)	302.6 (138.6-558.6)	0.060 (0.19)	0.76
Gametocyte prevalence (n/N)	2.2 (6/277)	4.7 (10/214)	0.84 (0.48)	0.08
Gametocyte density, GM (IQR)	21.6 (16.0-35.4)	29.9 (16.0-35.4)	0.65 (0.38)	0.12
Immune responses				
Circumsporozoite protein ($NANP_5$), prevalence (n/N)	100.0 (105/105)	89.4 (84/94)	-	
Pfs48/45.3 prevalence (n/N)	6.7 (7/105)	16.0 (15/94)	0.85 (0.69)	0.22
Pfs230 prevalence (n/N)	10.6 (10/94)	28.0 (23/59)	1.31 (0.62)	0.04
TRA >50% prevalence (n/N)	47.9 (23/48)	48.6 (18/37)	0.029 (0.44)	0.94

n = number of observations with a positive result; N = total number of observations. GM = geometric mean; IQR = interquartile range; TRA >50% = transmission reducing activity with >50% reduction; β = coefficient for village using a GEE model adjusting for age and season and allowing for within individual correlations; se = standard error. Note that there are multiple observations per individual.

Discussion

In this study we assessed functional transmission reducing activity and related factors longitudinally in a cohort of Tanzanian adults. While individuals were exposed to intense malaria transmission, gametocytaemia was uncommon. The prevalence of sexual stage specific immune responses and functional transmission blocking immunity was similarly low. It is likely that these two observations are directly linked. Together they further support the hypothesis that, unlike asexual stage immunity, efficient sexual stage immunity decreases with age (*Chapter 12*) [16].

Unlike the carriage of $NANP_5$ antibodies (*Chapter 10*) [19], sexual stage antibody responses did not become more common with age and were detected in only a minority of our adult population. Immune responses to Pfs48/45 epitope 1, 2b, and 5 were very low in the study group. Antibodies against Pfs48/45 epitope 3 were present in 7% of the individuals at enrolment and the combined seroprevalence of all four epitopes of Pfs48/45 was threefold lower than total Pfs48/45 IgG1 antibodies detected in gametocyte carriers of a similar age group (*Chapter 10*) [19]. This could either be due to the difference in approach, testing per epitope being less sensitive, or by a lower prevalence of antibodies in the absence of concurrent gametocytaemia. Antibodies against Pfs230, that is considered to be more immunogenic [29,30], were similar in this study and in gametocyte carriers from a nearby area (*Chapter 10*) [19].

In this study we observed that sexual stage immune responses were short lived and that there was little consistency in immune response over time. The short lived nature of sexual stage immune responses has been documented previously for *P. vivax* which requires frequent boosting [31,32]. The apparent lack of immunologic memory is possibly explained by a T-cell independent generation of transmission blocking antibodies or antibodies directed against non-protein targets [15,33]. Thus, although some individuals were consistent responders to Pfs230 or Pfs48/45 epitope 3, the majority of responders converted from seropostive to seronegative within the study period. Previous reports from Papua New Guinea, Indonesia (*Chapter 10*) [13,30,34] showed that sexual stage specific immunity increased with age and cumulative exposure. However, these studies were in individuals residing in an area of low malaria transmission intensity or in migrants. Differing levels of asexual immunity between these groups and our study cohort may explain the differences in the sexual stage immunity.

4.7% of the sera from the current study showed TRA >90% and TRA >50% was observed in 48% of the sera. None of the individuals from our cohort of whom multiple samples were tested showed consistent TRA on each time-point. TRA >90% was significantly lower in this cohort than in a cross sectional

group of gametocyte carriers recruited at a nearby clinic (*Chapter 10*) [19]. The lack of consistency of sexual stage immune responses that we observe contrasts with findings from The Gambia where antibody levels against Pfs230 were stable in adults [29]. This difference may be the result of a lower or irregular exposure to sexual stage parasites in our population that was exposed to intense perennial malaria transmission compared to seasonal transmission in The Gambia [29]. Recognition of sexual stage antigens does not appear to be genetically determined [29] and the absence of sexual stage antibodies in a large proportion of the population may therefore be the result of a low recent exposure to sexual stage antigens.

Gametocyte carriage is well known to decrease with age (*Chapter 12*) [16-18,35] and during our 18-month study period, gametocytes were detected in only one-third of the individuals. Although we will have missed some episodes of gametocyte carriage due to monthly sampling and microscopy is a relatively insensitive tool to detect gametocytes [35], exposure to gametocytes was clearly low in our population . Our observation on the kinetics of sexual stage immunity are probably the result of acquired immunity to asexual stages (*Chapter 12*) [16] in this adult population who were exposed to intense malaria transmission. The comparison of the two villages further supports a negative association between transmission intensity and the exposure to gametocytes and sexual stage immunity. We observed a lower exposure to gametocytes in the village exposed to higher transmission intensity, as was previously described [36]. In addition, we find lower sexual stage antibody prevalences at higher transmission intensity. It is logical to assume that these lower antibody responses are a direct result of the lower exposure to sexual stage antigens.

In summary, our findings suggest that sexual stage specific immunity and the resulting TRA depend on recent exposure to gametocytes, which is low in our cohort. Although we observe no age-dependency, functional TRA and sexual stage immune responses were low in our adult population. Together with the recently reported negative age-dependency of TRA in younger gametocyte carriers (*Chapter 10*) [19], our findings fit in well with the hypothesis that sexual stage immunity may be uncommon in older individuals living at high levels of transmission intensity (*Chapter 12*) [16]. Functional transmission blocking immunity may be restricted to those most exposed to gametocytes (*Chapter 12*) [16] and the relative infectivity of older individuals, who have acquired immunity to asexual stage parasites, is less likely to be affected by sexual stage immunity.

Acknowledgements

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Chapter 12

Sexual-stage antibody responses to *P. falciparum* in endemic populations

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Summary

Gametocytes and sporogonic stages are responsible for the spread of malaia disease and drug resistance in the population. Sexual stage immunity affects the infectiousness of gametocytes to mosquitoes and specific antibodies including anti-Pfs48/45 and anti-Pfs230 antibodies are found in individuals with limited prior exposure to malaria. These sexual stage antibodies are rapidly acquired after infection and are relatively prevalent in gametocytaemic individuals. Functional transmission reducing activity (TRA) is found after primary infections and in young children and appears to depend on recent rather than cumulative exposure to gametocytes. Exposure to gametocytes decreases with age most likely as a consequence of the acquisition of asexual-stage immunity that controls asexual parasite density and consequently gametocytaemia. This results in a lower exposure to the antigenic load of gametocytes in semi-immune individuals. Since sexual stage immunity is probably short-lived in the absence of gametocytes, we hypothesize that sexual stage immunity will wane, resulting in low antibody and TRA prevalences in clinically semi-immune carriers.

Introduction

A small proportion of a-sexual parasites differentiates into gametocytes, which represent the sexual forms of the parasite. Gametocytogenesis starts inside the red blood cell when a committed merozoite, develops into a male (micro-) or female (macro-) gametocyte. The events that trigger sexual development are poorly understood [1]. Various stimuli have been implicated such as anaemia, specific antibodies and drugs [2-6]. However, mathematical models [7] and recent work in human experimental infections [8] show that parasites are committed to sexual stages immediately after hepatic schizogony.

The parasites develop through five distinct morphological gametocyte stages (I-V) in 1-2 weeks. Stage I to IV gametocytes sequester from the peripheral blood during maturation [9,10]. Patterns may be distinct from asexual stages and sequestration in organs such as the spleen or immunologically privileged sites like the bone marrow may reduce the risk of immunological clearance [10]. Stage V gametocytes circulate in the bloodstream and become infectious to mosquitoes after 2-3 days of maturation. When mature gametocytes are ingested by a mosquito, transformation of the gametocytes into gametes is initiated and fertilization of female by male gametes takes place in the lumen of the mosquito midgut. Within 24 hours after fertilization, non-motile zygotes transform into motile ookinetes. Ookinetes have to traverse two barriers: the peritropic matrix/membrane and the midgut epithelium. After ookinetes reach the extracellular space, they transform into oocysts between the midgut epithelium and the overlaying basal lamina. Thousands of sporozoites are formed which will migrate to the salivary glands, ready for new human infection. The period in the mosquito midgut is a vulnerable moment in the parasite life cycle. Unprotected by the red blood cell, parasites are exposed for several hours to blood products that have been co-ingested with the blood meal [11]. Specific antibodies, complement and granulocytes have been shown to decrease or abrogate the sporogonic development [12-16].

In this paper, we discuss naturally acquired sexual-stage immune responses to *P falciparum* and their effects on transmission from men to mosquito.

The development of sexual-stage specific immunity

Sexual-stage specific immunity is acquired after exposure to gametocyte antigens. The development of an immune response to gametocytes is unsurprising given that the vast majority of gametocytes are not taken up by mosquitoes but are cleared by the host immune system. Although it has been reported that innate and cellular immune mechanisms may be involved in sexual stage immunity, it is generally well accepted that antibodies are the primary

mediators, either alone or together with complement or white blood cells [12,13,17,18].

Antibody responses against pre-erythrocytic and blood stage specific antigens increase with cumulative exposure. The prevalence of these antibodies is low in children and much higher or almost universal in adults in endemic areas, the rate of acquisition depending on transmission intensity [19,20]. The presence of asexual stage antibodies associates with control of parasite growth, reduces parasitaemia and consequently clinical disease.

The prevalence of gametocytes gradually decreases with age following a similar pattern as asexual parasitaemia, from which they are derived (figure 12.1) [21-25]. Cumulative exposure to malaria, rather than age, is responsible for this decrease in gametocyte prevalence, as was shown by a study comparing endemic population and newly arrived migrants from non-endemic areas [26]. Similar to asexual immunity, one may assume that sexual stage antibodies and functional immunity will increase with age at a rate that depends on transmission intensity. Sexual-stage immunity can be effective against gametocytes inside the human host or against gametocyte-derived target antigens expressed and accessible to antibodies inside the mosquito midgut. The effect of antibodies on infectiousness of gametocytes can be assessed by sporogonic development in mosquitoes after an infectious blood meal.

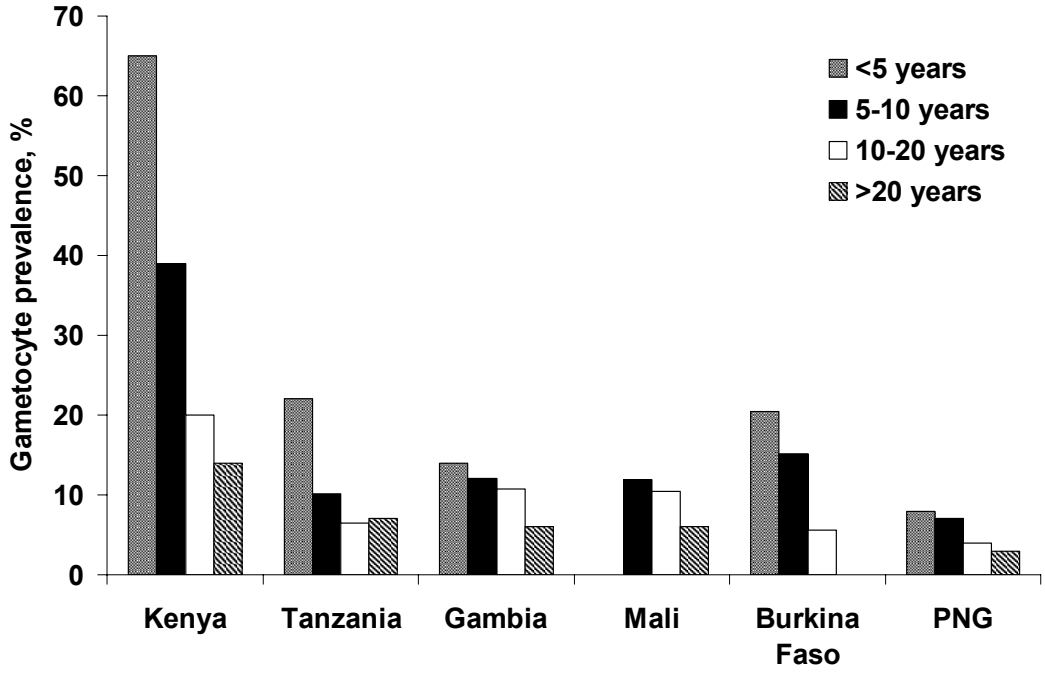

Figure 12.1 The exposure to gametocytes in different age groups.

Gametocyte prevalence per age group in the general population in studies conducted in Kenya [21], Tanzania [22], The Gambia [22], Mali [23], Burkina Faso [24] and Papua New Guinea [25].

Antigens on the membrane of gametocyte-infected erythrocyte

Data are limited on the immune responses directed to gametocytes while inside the human host. It is clear that disruption at any stage during the long gametocyte maturation period could have profound effects on parasite transmission and this area is ripe for investigation. There is some evidence for cross-reactive asexual and sexual-stage immunity [27-29] and specific anti-gametocyte immune mechanisms [26,30] that may clear gametocytes at some stage during their development. Immune responses against gametocytes can be directed against common parasite antigens, which are expressed on the surface of gametocyte infected erythrocytes and include extensive multigene families, *var*, *rif*, *stevor* and more recently *surfin's* [31]. The *var* genes are involved in the expression of PfEMP-1 molecules [32] that are expressed on erythrocytes infected with both trophozoites and early gametocyte stages (I,IIa) [28,33]. PfEMP-1 is the primary mechanism for cytoadherence and sequestration of asexual parasites [34] and PfEMP-1 antibodies may reduce gametocytaemia indirectly by controlling asexual parasitaemia or directly by affecting gametocyte maturation [28,29]. Closely linked to *var* genes is the repetitive interspersed family *(rif)* [35], which is associated with rapid parasite clearance [36]. There is no evidence that *rifin's* are expressed in sexual-stage parasites [37]. *Stevor* (subtelomeric variable open reading frame) gene transcripts [38] are transiently present early in the development of gametocytes and may play a role in gametocyte sequestration and adhesion [37] although their role in reducing gametocytaemia has not been elucidated. Gametocyte prevalence can be influenced by immune responses against asexual parasites [30] and possibly by immune responses directed against gametocytes [26-28,30]. Flow cytometric analysis of early stage gametocytes may provide clues as to which antigens are recognized and whether this recognition correlates with transmission from man to mosquito.[39]

Independent of the rate of gametocyte development or clearance, immune responses may also affect the infectiousness of gametocytes. This type of immunity depends on a different set of antigens.

Gametocyte and gamete antigens

Most serological field data concern antigens that are expressed in gametocytes and gametes in de mosquito midgut shortly after ingestion of the blood meal (Table 12.1). These antigens include Pfs48/45 and Pfs230, which are involved in fertilization and therefore a prime target for transmission blocking antibodies and vaccine development [11].

Pfs48/45 : Pfs48/45 is expressed in gametocytes and on the gamete membrane and essential for fertilization in male gametocytes. The biological function of Pfs48/45 in female gametocyte remains obscure [40-42]. Five distinct B-

cell epitopes have been defined by a panel of anti Pfs48/45 monoclonal antibodies (Mabs) with epitope I and V as best targets for transmission-blocking Mabs [43-47].

Pfs230: Pfs230 expression is similar to that of Pfs48/45, starting from stage II gametocytes onwards. Although expressed on the gamete membrane, Pfs230 does not contain a GPI anchor but associates after proteolytic cleavage with Pfs48/45 [48]. Pfs230 has five distinct epitopes [49], Several studies show that transmission blocking by anti-Pfs230 mAbs is fully dependent on a complement-fixing isotype and the presence of active complement. There is a perfect association between the capacity to block transmission and complement-mediated lysis of *P. falciparum* macrogametes *in vitro* [50,51].

Pfs16: Expression of Pfs16 is the earliest sexual-stage specific antigen in gametocyte development that occurs in committed ring stages.[52]. Expression continues from stage I gametocytes throughout maturation in male and female gametocytes, although at a lower level in stage V gametocytes [53]. Pfs16 is an integral membrane protein present on the parasitophorous vacuole membrane [54] and is not essential but contributes to the generation of gametocytes [55]. The prevalence of antibodies to recombinant Pfs16 or Pfs16 peptides is high in endemic sera (*Chapter 10*) [56,57]; however, the correlation of these responses with antibodies to natural antigens is poor (Roeffen, unpublished data).

Pfg27 is expressed throughout the cytoplasm of sexually committed parasites but not on the surface membrane [53,58]. Disruption of the Pfg27 gene results in a loss of sexual phenotype [59]. 27kD immune responses were detected by immunoprecipitation in a small set of sera from Papua New Guinea[60].

Pfs25 and *Pfs28* are well-known transmission-blocking vaccine candidates, but these antigens are only expressed during late sporogony and consequently naturally acquired antibodies have not been found in endemic sera [23,60]

Table 12.1. The expression of a selection of *P. falciparum* antigens in sexual and sporogonic stages

	Human		Mosquito
	Gametocyte		Gamete / Zygote
	Internal	RBC Membrane	Membrane
PfEMP-1		X	
Pfs48/45	X		X
Pfs230	X		X
Pfs16	X		
Pfg27	X		
Pfs25/Pfs28			X

RBC = red blood cell

Infection from man to mosquito

The infectiousness of gametocytes and the effect of serum factors on the infection from men to mosquito can be measured in bio assays where gametocytes are fed to *Anopheles* mosquitoes. There is a good correlation between infectiousness in these membrane feeding assays and feeding of mosquitoes directly on the skin [61] in microscopically confirmed gametocyte carriers.

Two types of membrane feeding assays are commonly used: i) In the standard membrane feeding assay, cultured gametocytes are fed to mosquitoes in the presence of an (endemic) test sample or non-malaria control serum [62,63]. This assay can detect transmission reducing factors in test samples. ii) In the direct membrane feeding assay, which can be conducted in the field, blood samples from naturally infected gametocyte carriers are fed to mosquitoes in the presence of autologous serum or control serum after a washing step [25,64,65]. This assay can measure the infectiousness of the gametocyte carrier and the presence of plasma factors that may influence the rate of mosquito infection. Transmission reduction in both membrane feeding assays is determined by comparing the number of developed oocysts in mosquitoes fed with test versus control serum.

On average, maximally 40-75% of naturally infected gametocyte carriers is infectious to mosquitoes [21,64-67]. The proportion of infectious gametocyte carriers may decrease with age [22,23,65] but this is closely related to gametocyte density. An effect of age on infectiousness that is independent of gametocyte density, was found in some [21,23] but not all studies [22,25,65].

The possible age dependency and the poor correlation between transmission success and gametocyte density [65,68] suggest that additional factors including immune responses may contribute to the reduced gametocyte infectiousness. A role for the host's immune response was first detected in studies in animal models where immunization with parasite mixtures and purified gametes induced the production of stage specific antibodies capable of blocking the sexual and sporogonic development in the mosquito [69-73]. In addition, several studies have demonstrated the ability of immune human serum to reduce the infectivity of gametocytes to mosquitoes [47,64,74-78].

The degree of transmission reducing activity varies in different studies; more than 50% reduction of oocyst numbers is found with 25-55% of the endemic sera (*Chapter 10*) [56,64,74,75,78]. Antibodies against Pfs48/45 and Pfs230 have been associated with transmission reducing activity and their seroprevalence has been studied in endemic populations.

Antibodies to Pfs48/45 and Pfs230 and transmission reducing activity

Serology

A variety of techniques and antigen sources have been used in sero-epidemiological studies in different endemicities and age groups. Pfs230 antibody prevalence as detected by IgG1 and competition ELISA is 25-60% and more prevalent than anti-Pfs48/45 antibodies (10-40%) in endemic sera [14,64,77,79]. Seroreactivity against both antigens is higher when measured by immunoprecipitation [49,74,78,80]. Almost all these results are obtained with sera from gametocyte carriers. The few studies that compared infected and uninfected individuals show that the seroprevalence in the uninfected population is much lower for both Pfs48/45 and Pfs230 (*Chapter 9*) [81-83]. This suggests that the half life of these antibodies is relatively short in the absence of circulating gametocytes.

Antibodies against Pfs48/45 and Pfs230 are acquired after one or few infections (*Chapter 9*) [81,83,84]**.** Despite the rapid induction of these sexual stage specific responses, prevalences of anti-Pfs48/45 and anti-Pfs230 antibodies are generally low in semi-immune populations when compared to antibodies to asexual stage antigens. Although an effect of cumulative malaria exposure on sexual stage immunity was observed in Papua New Guinea [83], other studies contradict an important influence of cumulative exposure. If cumulative exposure determines seroreactivity, one would expect an increase in seroprevalence with age, as is typically seen for asexual-stage specific antibodies [19,20,85,86]. Although a cross-sectional study from Papua New Guinea indicated that Pfs48/45 seroreactivity may be highest in adults [87], no relation with age has been found in several other studies *(Chapter 10,11)*(vdKolk, unpublished; Ouedraogo, unpublished) [56,77]. Similarly, there is no convincing evidence for an age-dependency of anti-Pfs230 antibodies *(Chapter 10, 11)*(vdKolk, unpublished; Ouedraogo, unpublished) [56,77].

Two studies directly determined the influence of transmission intensity on the prevalence of Pfs48/45 and Pfs230 antibodies. In one study in high endemic areas in Cameroon, a five-fold difference in transmission intensity did not result in a marked difference in antibody prevalence (vdKolk, unpublished). A study in lower and highly seasonal transmission areas in Burkina Faso found a significantly lower prevalence of sexual stage specific antibodies in the higher transmission area (Ouedraogo, unpublished).

Functional assay

Membrane feeding assays can be used to assess transmission reducing activity (TRA) in field sera. The relationship between TRA and sexual stage specific antibodies in these sera has been investigated in several studies. A good

correlation was observed between anti-Pfs230 antibodies in Gambian [77,80] and Papua New Guinean sera (*Chapter 9*) [74,81], but no correlation was found in Cameroonian [47,64] or Sri Lankan [78] sera. The same is true for anti-Pfs48/45 antibodies where a positive relation was found with TRA in some studies (*Chapters 9, 10, 11*) [47,56,64,77,81,82] but not in other studies [74,78,80]. Naturally acquired TRA is found in individuals with recent and/or limited prior exposure to malaria as was shown in young children and transmigrants from a malaria non-endemic to an endemic area (*Chapter 9*) [77-79,81]. In certain epidemiological settings TRA may be negatively related to age (*Chapter 10*) [56] although the data are limited and further work is needed [75,77,88].

In summary, sexual stage antibodies are rapidly acquired after infection and relatively prevalent in gametocytaemic individuals, suggesting the need for the continued presence of antigen. TRA is found after primary infections and in young children and may depend on recent rather than cumulative exposure to gametocytes. The prevalence of sexual stage antibodies seems to be negatively associated with transmission intensity and lacks a convincing association with age. TRA is associated with antibodies against Pfs48/45 and Pfs230 although the strength of the associations differs between populations.

A model for the epidemiology of sexual stage immunity

Although not consistent with all studies, an attempt has been made to incorporate most of the presented data into an epidemiological model of sexual stage immunity (figure 12.2). Our hypothesis is that sexual stage specific immunity is rapidly acquired but, in contrast to asexual immunity, declines with increasing exposure to infection, i.e. in older age groups.

The high prevalence of gametocytes in little exposed individuals induces functional antibodies and, if titers are sufficiently high, transmission reducing activity. The acquisition of asexual-stage immunity controls asexual parasite density and consequently gametocytaemia, resulting in lower exposure to the antigenic load of gametocytes. Sexual-stage specific immunity is considered to be short-lived in the absence of gametocytes and therefore related to recent, rather than cumulative exposure. As a result, sexual stage immunity is highest in young children, where exposure to gametocytes is highest. Exposure to gametocytes will decrease with increasing age and developing asexual stage immunity.

Consequently, sexual stage immunity will wane, resulting in low antibody and TRA prevalences in semi-immune (non-gametocyte) carriers. This profile is likely to be dependent on transmission intensity with a shift to the left in high endemic areas and to the right in low endemic areas.

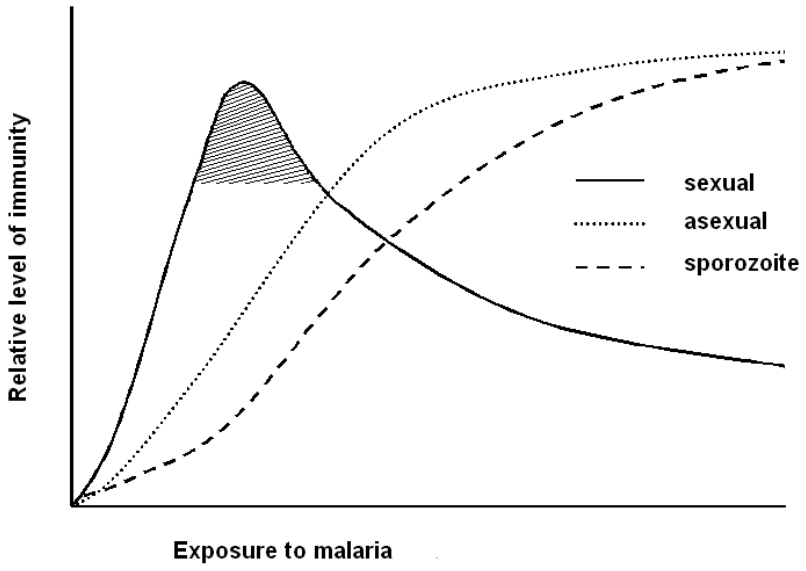

Figure 12.2 Model of sero-epidemiology in relation to exposure

Relative level of immunity against sexual, asexual and sporozoite stages in the population as a function of malaria exposure. The dashed area represents functional transmission reducing activity.

Conclusion

The epidemiology of sexual stage specific immune responses has been relatively little studied compared to pre-erythrocytic and a-sexual stage immunity. The obvious reason is that no direct relationship of this type of immunity can be expected with clinical protection. However, there is a relationship between the presentation of clinical disease and the pattern of transmission, which is determined by the human and mosquito reservoir and which shows an enormous geographical variation. Reduction of malaria transmission can significantly contribute to malaria control [89].

Gametocytes and sporogonic stages are responsible for the spread of disease and drug resistance in the population. Further understanding of the epidemiology of gametocytaemia and related sexual stage immunity can significantly contribute to the implementation and success of interventions and control. This includes all interventions since gametocytes emerge from the asexual parasite pool and a reduction in potential gametocyte carriage would have wide reaching benefits.

It is clear that further studies are needed to test the presented model and to further unravel the complex relationship between transmission and transmission reducing immunity. Among such questions are the functional role in endemic sera of sexual stage antibodies, notably anti-Pfs48/45 and anti-Pfs230, their longevity and their relationship with transmission intensity.

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Chapter 13

General discussion

The work described in this thesis deals with the prevalence of (low density) gametocytes and their infectiousness to mosquitoes, possible ways to reduce gametocytaemia by antimalarial drugs and human immune responses reducing the infectiousness of gametocytes to mosquitoes.

The prevalence of (submicroscopic) gametocytaemia in different populations

Studies on gametocyte development in naturally infected individuals commonly rely on microscopical examination of patients treated with antimalarial drugs [1-10], leaving many question unanswered on the occurrence of gametocytes in the general population. In *Chapter 2* of this thesis, we determined gametocyte dynamics by microscopy in asymptomatic individuals in the absence of treatment [11]. One in five parasitaemic individuals developed gametocytaemia in the month they were followed, often at densities of 16-32 gametocytes/μL (1-2 gametocytes observed while screening 100 microscopic fields). Once detected, gametocytaemia frequently dropped below the microscopic detection limit at the following visit to appear again later. This commonly observed phenomenon of gametocytes circulating at densities close to the microscopic threshold [12-16] illustrates the insensitivity of microscopy for detecting gametocytes. The more sensitive quantitative nuclear acid sequence-based amplification (QT-NASBA) technique was used in this thesis to explore the prevalence and potential importance of low-density gametocytaemia. *Pfs25* QT-NASBA detects a 3-5 times higher proportion of gametocytaemic individuals (*Chapter 5, 6*) [17-19]. One could argue that any molecular gametocyte detection technique will increase the proportion of gametocytaemic individuals and that this increase is neither unexpected nor automatically relevant. The prevalence of submicroscopic gametocytaemia in different populations and its contribution to the human infectious reservoir are key issues in judging the importance of our findings.

We determined submicroscopic gametocytaemia in areas of very different malaria endemicity. In symptomatic children from a high endemic area in western Kenya 80-90% harboured gametocytes at enrolment (*Chapters 5, 6*) [17,18]. The proportion of gametocyte densities that is below the microscopic threshold is illustrated in figure 13.1 that presents their *Pfs*25 QT-NASBA gametocyte densities. Lines indicate the threshold density for detection by microscopy or quantitative buffycoat (QBC). Approximately 75% of the gametocyte carriers detected by *Pfs25* QT-NASBA are below the threshold of microscopic examination of 100 fields (i.e. 5 gametocytes/μL).

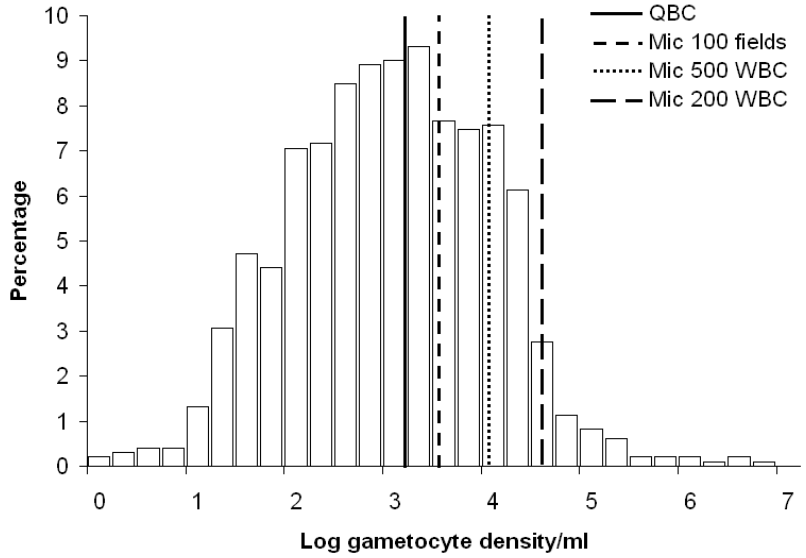

Figure 13.1 Gametocyte detection by different detection techniques. *Bars indicate gametocyte densities by Pfs25 QT-NASBA in symptomatic malaria infections in a high endemic area in western Kenya [17, 18]. Lines indicate the threshold density for detection by quantitative buffycoat (QBC) and microscopic examination of 100 fields, 500 or 200 white blood cells.*

It is important to acknowledge that these results are derived from a highly selected population that is characterised by several known risk factors for gametocytaemia such as young age [20-24], a relatively high density asexual parasitaemia (*Chapter 2*) [3,5,11], and possibly a longer duration of infection [1,2,6] with multiple parasite clones [25]. Although these factors may certainly have contributed, studies conducted in very different settings confirm the abundance of submicroscopic gametocytaemia. In an area of very low transmission intensity in Tanzania where less than 1% of the general asymptomatic population harboured microscopically detectable gametocytes, *Pfs25* QT-NASBA detected gametocytes in 15% of the individuals (*Chapter 8*) [26]. In addition, cross-sectional surveys in area of seasonal malaria in Burkina Faso indicate that a staggering 70% of the general population harbours gametocytes by *Pfs25* QT-NASBA in the peak transmission season compared to 21% by microscopy (Ouédraogo, unpublished observations).

In summary, our findings from three areas of different transmission intensity indicate that 70-93% of all gametocyte carriers harbour gametocytes at densities below the microscopic detection threshold. Unlike previously assumed, gametocyte carriage does not occur in a minority of asexual parasite carriers but appears to accompany asexual parasites in 50-89% of the individuals. Future work should determine the exact relation between transmission intensity, seasonality and submicroscopic gametocytaemia. We nevertheless consider it

likely that in all transmission settings a considerable proportion of gametocyte carriage, probably the majority, occurs below the microscopic detection limit.

The relevance of submicroscopic gametocytaemia for malaria transmission

Importantly, gametocyte carriage does not equal infectiousness to mosquitoes. This depends on gametocyte fitness and density [8,20,27-30]. The latter will of course be of particular importance in determining the relevance of submicroscopic gametocytaemia. Assessing the infectiousness of individuals to mosquitoes is a laborious task. This may be a reason why experiments are often restricted to microscopically confirmed gametocyte carriers despite evidence for the infectiousness of individuals who were gametocyte free according to microscopy [20,27,31-35]. Studies directly relating gametocyte densities with the infectiousness to mosquitoes typically found weak positive associations [24,27,28,30,32,34-39]. In the previous section we concluded that submicroscopic gametocytaemia may be common in all transmission settings. In *Chapter 7* of this thesis, we have shown that there is a positive association between gametocyte density and mosquito infection rates but that mosquito infection is common at submicroscopic gametocyte densities [19]. The infectivity of low concentrations of NF54 gametocytes was assessed under laboratory conditions and mosquito infection became unlikely below a density of 250-300 gametocytes/mL. In contrast, we did not observe a minimum gametocyte density for natural infections and mosquito infection was even observed at densities below 100 gametocytes/mL.

We expected to find a minimum gametocyte density for successful mosquito that would be in agreement with the theoretical minimum density of one male and one female gametocyte per mosquito blood meal of 2μL. The typically female biased gender ratio of gametocytes [40-43] ordinarily resulting in one male gametocyte per 3-4 female gametocytes [44], would give a minimum density in the order of 2-5 gametocytes per mosquito blood meal. Since the uptake of gametocytes by mosquitoes involves a chance element, some successful infections can occur when feeding on samples with lower gametocyte densities. However, when assuming a Poisson distribution with a mean of 0.3 gametocytes/μL, the lowest NF54 gametocyte density at which mosquito infection was observed, suggest that only 0.1% of randomly drawn 2μL samples will contain two or more gametocytes. This contrasts with our finding of 2-5% infected mosquitoes at this density and suggests that a different distribution should be assumed [45]. Biological mechanisms have been suggested that can increase the transmission success at low densities and thereby explain the apparent heterogeneity in gametocyte uptake by mosquitoes. Gametocytes may aggregate to favour the encounter of male and female gametocytes [45,46]. In double gametocyte infections, male and and female gametocytes may even

appear within a single erythrocyte [47]. Low densities of sexual stage antibodies may also enhance the efficiency of gametocytes in infecting mosquitoes [48] and gametocytes may sequester in the small capillaries to maximise the chance of uptake by a feeding mosquito [44,46,49]. These phenomena could play an important role in natural malaria transmission and gametocyte aggregation or double gametocyte infections may also have influenced our results. Nevertheless, they may not adequately explain our findings in the field where mosquito infection was observed at very low densities and where technical explanations should therefore be considered. This is also indicated by QT-NASBA gametocyte densities below 20 gametocytes/mL (*Chapter 7, 8*), which is below one gametocyte per 50μL blood sample.

Parasite nucleic acids were obtained and stored under field conditions. Although a complete loss of RNA or a failure to extract nucleic acids was excluded by the detection of parasitic 18S rRNA in all infectious samples, some degradation may have occurred. If *Pfs25* mRNA was lost, this would have decreased the measured gametocyte density by *Pfs25* QT-NASBA. To prevent similar uncertainties in future studies, a spiking control of exogenous RNA should be added to samples prior to processing. Variation in the *Pfs25* QT-NASBA assay itself could also play a role. Since gametocyte quantification in the *Pfs25* QT-NASBA depends on *Pfs25* RNA expression in field isolates relative to that in cultured gametocytes, any difference in expression between NF54 and field strains would bias results. If *Pfs25* RNA expression is lower in gametocytes from the field, quantification of field samples will underestimate gametocyte densities. Both RNA degradation and a lower *Pfs25* RNA expression could explain the efficient mosquito infection at low measured gametocyte densities. Before this issue is clarified, *Pfs25* QT-NASBA gametocyte densities of individual samples should be considered with some caution.

Despite these possible limitations, it is evident that the infectiousness of submicroscopic gametocyte densities is not a biological rarity but in fact a very common observation (*Chapter 7*) [19]. Submicroscopic gametocytaemia can be crucial in determining the transmission potential of a given population. Although the proportion of infected mosquitoes is lower when feeding on carriers with submicroscopic compared to microscopic gametocyte densities, this can be counterbalanced by the much higher prevalence of submicroscopic gametocytaemia in the population. In symptomatic children in western Kenya, submicroscopic gametocytaemia appeared equally important for post-treatment malaria transmission compared to microscopic gametocytaemia (*Chapter 7*) [19]. Based on the efficiency of mosquito infection at low gametocyte densities, we also hypothesize that carriers harbouring submicroscopic gametocytaemia are responsible for maintaining malaria transmission in an area in northern Tanzania of very low transmission intensity (*Chapter 8*) [26].

The possibility to reduce malaria transmission by antimalarial drugs

Prompt antimalarial drug treatment is an important tool, not only for reducing the burden of disease but also for reducing malaria transmission. While successful clearance of asexual parasites by antimalarial drug treatment will interrupt gametocyte development, the short-term effect of antimalarials on gametocytaemia is not straightforward. In this thesis, we found an initial increase in gametocyte carriage following treatment (*Chapter 2*) [11], as is commonly found for chloroquine (CQ) and sulphadoxine-pyrimethamine (SP) [1-3,8,50-53]. Our longitudinal study directly comparing gametocyte development in the presence and absence of antimalarial treatment suggests that this initial rise in gametocytaemia is a direct result of SP treatment. Treatment outcome can further influence post-treatment gametocytaemia. Evidence is accumulating that treatment failure results in an increased gametocyte production. This was previously shown for treatment with chloroquine (CQ) [1,2,5,10,50] and is now also becoming evident for sulphadoxine-pyrimethamine (SP) [2,9,54] (*Chapter 2, 3*) [11,55]. We observed a higher risk of gametocyte carriage and a higher density of gametocytaemia in individuals experiencing clinical treatment failure (*Chapter 2, 3*) [11,55]. This may simply be the result of unsuccessful clearance of asexual parasites, allowing a continuous production of gametocytes. However, the timing of the peak gametocytaemia suggests that alternative mechanisms also play a role.

The higher prevalence and density of gametocytes on day 7 after unsuccessful treatment is commonly seen [1,3,8,50,51] but this peak comes too early to be the result of newly produced gametocytes from parasites persisting after treatment. The simple reason is that gametocyte development requires 8-12 days [12,40]. It therefore seems plausible that the peak gametocytaemia is stress-induced [40] and is at least partly the result of the efflux of sequestered gametocytes that were present before treatment but whose release was triggered by treatment [8]. Some of these gametocytes may be immature [8,56]. Timing becomes even more intriguing when we realise that the higher gametocytaemia in resistant infections not only occurs too early to allow normal development but an increased gametocyte prevalence of resistant infections may even be apparent before treatment is initiated (*Chapter 4*) [2,57-60]. The most likely explanation is that the higher pre-treatment gametocyte prevalence reflects events that took place prior to enrolment. Unsuccessful treatment of a malaria episode would allow for both gametocyte development and the selection of resistant parasite strains.

Alternatively, resistant parasite strains could have a higher gametocyte production as a result of some intrinsic characteristic of resistant parasites. Although this was not observed in *in vitro* models [53] and is strictly hypothetical,

it seems to be supported by our work in Ghana where we determined gametocytaemia as a function of parasite genotype. We observed a higher gametocyte prevalence in parasites carrying mutations related to SP treatment failure [61], both after and before treatment (*Chapter 4*)[57]. These findings were not explained by the previous use of SP that was determined in the blood of patients at enrolment. Of course, there is always a possibility that mutant parasite strains with gametocytes persist for a longer period than the 3-4 weeks that pyrimethamine is demonstrable in blood (T. Eggelte, personal communication). However, SP was not used as firstline treatment when the study was conducted and SP use was very rare in our study population [62]. One could therefore speculate about possibilities that mutant parasites produce more gametocytes compared to wildtype parasites, even in the absence of drug pressure. Mutations in the *dhfr* gene are unlikely to be directly related to gametocyte production but may infer a certain cost for parasites in the absence of drug pressure [63,64] that may translate in a lower growth rate per parasite lifecycle [65]. Therefore, it could take longer to cross the threshold parasite density before fever occurs [66] in resistant infections. As a consequence, resistant infections may remain untreated for a longer period of time, allowing for gametocyte development [67]. A longer duration of resistant infections before clinic attendance could also play a role in the observation that infections with mutant parasites more often present as severe malaria anaemia [68].

While there is no direct evidence for a higher intrinsic gametocyte production of resistant parasites, a higher infectiousness of resistant parasites to mosquitoes has been observed previously [1,51,69]. A higher gametocyte production and transmission potential of mutant parasite strains would be particularly worrying since it would facilitate the spread of drug resistance.

Artemisinin-based combination therapy

An important tool against the spread of drug resistant parasites is the employment of alternative antimalarial drugs that combine a high therapeutic efficacy with a beneficial effect on gametocytes, such as artemisinin-based combination therapy (ACT) [4,8,70-73]. In our study area in western Kenya where SP mono-therapy fails in ~50% of the infections (*Chapter 2, 3, 6*) [11,17,55], addition of three doses of artesunate (AS) to SP or treatment with Artemether-Lumefantrine (AL) greatly increased therapeutic efficacy (*Chapter 6*) [17]. The impact of ACT on post-treatment gametocytaemia was highly dependent on the method of gametocyte detection. Microscopy showed a dramatic reduction in circulating gametocytes after ACT (SP+AS or AL) [4,8,70-73] while this reduction was gradual and more modest when *Pfs25* QT-NASBA was used (*Chapter 5, 6*) [17,18]. ACT acts against the early stages of gametocytes [8,51,69] and *Pfs25* QT-NASBA gametocytaemia after ACT may simply reflect the carriage of unaffected mature

gametocytes. Many children harboured gametocytes by *Pfs25* QT-NASBA for several weeks after treatment and the duration of gametocyte carriage appeared longer than the 7-10 days that was estimated by microscopy (*Chapter 2*) [11]. The duration of gametocyte carriage needs to be re-assessed in longitudinal studies with a long follow-up period and molecular detection methods for both asexual parasites and gametocytes.

Importantly, the effect of ACT on low-density gametocytaemia was insufficient to prevent malaria transmission or substantially reduce the proportion of infectious individuals on day 14 after treatment (*Chapter 6*) [17]. It is difficult to extrapolate this important finding to the potential impact of wide-scale employment of ACT on malaria transmission in the general population. First of all, many individuals in malaria endemic areas will carry infections asymptomatically, be infectious to mosquitoes but will not receive ACT. Secondly, the influence of ACT on the duration of infectiousness is unknown since post-treatment malaria transmission is often measured on only one time-point [8,51,70]. Although the influence of ACT on malaria transmission shortly after treatment may be modest, its impact on the duration of gametocyte carriage appears substantial. We observed consistently lower gametocyte prevalences by *Pfs25* QT-NASBA after ACT (*Chapter 5, 6*) [17,18]. Gametocytes that were detected after ACT were also circulating at lower densities than those after treatment with SP or SP + amodiaquine. This translates in a lower proportion of infected mosquitoes after ACT treatment (*Chapter 6, 7*) [17,19] that is probably more marked when the entire post-treatment period is considered. Importantly, the higher treatment efficacy in itself will reduce the proportion of individuals harbouring persisting infections and will therefore have a beneficial effect on gametocyte carriage and malaria transmission.

Although there is some first evidence of a reduced susceptibility of some parasite strains for artemisinin [74] and of ACT selecting for mutant re-infecting parasite strains [75], the potency and short half-life of artemisinin [72,76-78] and the evidence that ACT reduces the spread of mutant parasite strains [69,79] indicate that it is the best available tool to counteract drug-resistance. When is comes to reducing overall malaria transmission, ACT alone may not be sufficient. A reduction in malaria incidence following the employment of ACT[79] is likely to be restricted to areas of low transmission intensity where the majority of infections are symptomatic and where ACT coverage is excellent. Any attempt to reduce malaria transmission by mass drug administration with ACT [80,81] needs to take the high prevalence and long duration of post-treatment submicroscopic gametocytaemia into account when considering the coverage and timing of interventions.

Human immune responses reducing malaria transmission

Similar to treatment outcome that depends not only on characteristics of parasites and drugs but also on immunity [82,83], malaria transmission is also influenced by human immune responses that can reduce or block successful parasite development in the mosquito. Although it has been long acknowledged that sexual stage specific immune responses may differ from asexual stage immunity in acquisition and persistence [48,84], there have been few attempts to determine its rate of development or dynamics. Sexual stage antigens appear poorly immunogenic [48,85] but the literature is inconsistent about the relation between (repeated) exposure and sexual stage immunity [48,86] [87,88] and its longevity [48,86,87,89] or age relatedness [48,88]. Little is known about the acquisition of sexual stage specific antibody responses and functional transmission reducing activity (TRA) following natural exposure to asexual parasites or gametocytes. In microscopically confirmed gametocyte carriers, we observed a negative age-dependency of TRA (*Chapter 10*) [90]. This observation did not coincide with a reduced prevalence or density of sexual stage antibodies against Pfs230 or Pfs48/45 and could not be explained by differences in gametocyte density at the time blood samples were taken. Nevertheless, it seems to be confirmed by a higher infectiousness of older individuals in Kenya (*Chapter 7)* [19] and Burkina Faso (Ouédraogo, unpublished observations). The most likely explanation for a lower prevalence of TRA in older individuals is a lower recent exposure to gametocytes. Microscopic gametocyte prevalence decreases with age (*Chapter 2*) [11,20-24] and a lower recent exposure to sexual stage antigens may hinder the maintenance of effective sexual stage immune responses [91]. The study design, however, made it impossible to directly test the validity of this concept. As is commonly done [88,89,92,93], samples were taken from patent gametocyte carriers on one time-point without information on the duration of gametocyte carriage or the previous exposure to malaria infections. The duration and density of gametocyte carriage may influence sexual stage specific immune responses, making it difficult to correlate a single estimate of gametocyte density with concurrent levels of sexual stage specific antibodies. Longitudinal studies are clearly needed to fully understand the rate at which sexual stage immunity is acquired.

In this thesis we describe two longitudinal studies in very different populations. In naïve individuals from Java who were observed for two years following their migration to a hyperendemic area, a rapid acquisition of sexual stage immunity was observed that was independent of age (*Chapter 9)* [94]. Although serological responses were measured only twice per year, a positive relation between the number of experienced infections and sexual stage specific immune responses was apparent. Not only did the prevalence of anti-Pfs230 and anti-Pfs48/45 antibodies increase, functional TRA was also acquired following

malaria exposure. A single infection appeared sufficient to elicit TRA in one-third of the individuals. This proportion increased with cumulative exposure [87], although this may be a sign of the repeated exposure in a short time-period, as was observed for *P. vivax* [91], rather than a long-lasting immune memory. A lack of immune memory was suggested by our second longitudinal study in adults living in a high endemic area in Tanzania (*Chapter 11)*. Despite intense exposure to malaria infections and a high prevalence of anti-sporozoite antibodies, sexual stage immune responses were only present in a minority of the individuals. There appeared to be little consistency in immune responses with many adults converting from seropositive to seronegative within the 18-month study period. Although a substantial proportion of the sera was capable of reducing transmission in the SMFA, complete blockade of transmission was observed in only ~5% of the sera (*Chapter 11*) compared to ~20% in patent gametocyte carriers (*Chapter 10*) [90]. This lower prevalence of functional transmission blocking immunity could be the result of a low exposure to microscopically confirmed gametocytes in adults living in a high endemic area. Gametocyte exposure is inversely related to transmission intensity [7,95], which was also observed in our Tanzanian adults where those exposed to fewer infectious bites more frequently harboured gametocytes and also showed a higher prevalence of sexual stage specific antibodies (*Chapter 11*).

A hypothetical model for the acquisition of sexual stage specific immunity is proposed in *Chapter 12* [96] where we conclude that sexual stage specific immunity is rapidly acquired but depends on recent exposure to gametocytes. This exposure to gametocytes decreases with the acquisition of asexual-stage immunity that controls asexual parasite density and consequently gametocytaemia. Contrary to asexual-stage immunity, sexual stage immunity may therefore be most common in younger age groups or in areas of low transmission intensity. The model in *Chapter 12* is consistent with most of the available literature but relies on microscopy for estimating the antigenic exposure, i.e. gametocyte carriage. Based on our findings with *Pfs25* QT-NASBA, we know this may not be accurate and it is tempting to speculate about a possible role of submicroscopic gametocytaemia in the development or persistence of sexual stage specific immunity. If we assume that most asexual parasite carriers harbour accompanying low levels of gametocytes (*Chapter 5,6,8*) [17,18,26], any malaria infection could theoretically initiate or boost sexual stage immunity. Interestingly, very few individuals from our cohort of Javanese transmigrants were exposed to microscopically detectable levels of gametocytaemia on Irian Jaya although half of them experienced three or more clinical episodes of *P. falciparum* malaria (*Chapter 9*)[94]. We observed a distinct positive association between the number of infections experienced and sexual stage specific immune responses. In line with this, Graves *et al.* found no

difference between the asexual parasite and gametocyte carriers in sexual stage specific immunity although there was a clear difference with those who were malaria negative [87]. In addition, sexual stage immune responses were detected in primary infections in travellers in the absence of patent gametocytaemia [97], a finding that could be explained by our observation that low densities of parasites committed to sexual development can be detected within weeks after primary infection [98]. If a large proportion of the asexual parasite infections is accompanied by submicroscopic gametocytaemia, all these observations suggest that exposure to submicroscopic gametocyte densities can elicit sexual stage immune responses. Clearly, data are too scarce to draw any conclusions and the accumulated evidence that gametocytes are poorly immunogenic [48,85] and sexual stage specific immune responses are relatively uncommon in endemic populations (*Chapter 12*) [96] argue against an important role of submicroscopic gametocytaemia in maintaining sexual stage immunity. Our findings nevertheless suggest that submicroscopic gametocytaemia may play a role in eliciting sexual stage specific immune responses and further studies should define its relative importance for the acquisition and boosting of sexual stage immunity.

Even if submicroscopic gametocyte densities would turn out to be too low to be immunogenic, our knowledge of the prevalence and infectiousness of submicroscopic gametocytaemia does underline the importance of sexual stage specific immunity and transmission blocking vaccines. One important value of transmission blocking vaccines is to protect other vaccines by preventing the spread of resistant parasite strains. Any pre-erythrocytic or blood-stage vaccine with less than 100% efficiency in preventing blood stage infection would benefit enormously from the prevention of the transmission of escaping parasites [99]. With our current knowledge of the omnipresence of low density gametocytaemia and its efficiency in driving malaria transmission, transmission blocking vaccines appear an essential component to ensure the long term efficiency of any multi-stage malaria vaccine.

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Chapter 14

Summary
Samenvatting
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Summary

Plasmodium falciparum is responsible for the majority of malaria attributed morbidity and mortality. Humans are infected with this malaria species through the bite of an infected mosquito. The transmission cycle is completed when a mosquito is in turn infected after feeding on an infectious individual.

Malaria parasites can appear as asexual and sexual forms (gametocytes) in the human peripheral blood. While asexual parasites are responsible for clinical disease, gametocytes do not cause clinical symptoms but ensure the transmission of malaria from man to mosquito. For successful malaria transmission, gametocytes need to be ingested by *Anopheles* mosquitoes that are taking a blood meal on a human host. Gametocytes develop in the mosquito and ultimately render the mosquito infectious to human beings.

Although gametocytes are essential for the spread of malaria, they are only observed in a small proportion of *Plasmodium falciparum* infections. This seems to contrast with the highly efficient transmission of malaria that is observed in many endemic areas. One of the important conclusions of this thesis is that this apparent paradox may simply be the result of the limited sensitivity of commonly used tools to detect gametocytes in the human blood. Mosquitoes appear more successful in finding gametocytes than the human eye.

In this thesis, we show that gametocytes occur in a large proportion of children who are infected with malaria parasites but do not suffer from clinical disease. Gametocytes are most common in the younger age groups, who also seem to harbour gametocytes for a longer period of time (*Chapter 2*). Shortly after treatment with the commonly used antimalarial drug Fansidar®, gametocyte carriage seems to be increased (*Chapter 2*). The magnitude of this increase is related to treatment outcome. Children suffering treatment failure are more likely to carry gametocytes than those whose asexual parasites are cleared by Fansidar® (*Chapter 2, 3*). This is a worrying finding that suggests that the spread of resistant parasites may be enhanced. In *Chapter 4* we provide further evidence for this hypothesis. In a completely different setting we studied genetic markers for resistance to Fansidar®. Children who were infected with resistant parasites were more likely to carry gametocytes than those who were carrying susceptible parasites (*Chapter 4*).

In several studies in this thesis, we used a novel technique for detecting gametocytes. This quantitative molecular technique (QT-NASBA) is based on the detection of genetic material (RNA) of mature gametocytes and is up to 1000 times more sensitive than routine microscopy. Using the QT-NASBA, we found that gametocytes are far more prevalent than suggested by microscopy. While microscopy detects gametocytes in roughly 20% of the children with

uncomplicated malaria, the QT-NASBA detects gametocytes in 80-90% of the same individuals (*Chapter 5, 6*). Although these gametocytes circulate at very low densities, these are sufficient to infect mosquitoes (*Chapter 6, 7*). The proportion of infected mosquitoes decreases with decreasing gametocyte density but mosquito infection is surprisingly efficient at low densities (*Chapter 7*). The relatively lower infectiousness of low density gametocytes is counterbalanced by their commonness. Low density gametocyte carriage is very common in symptomatic patients (*Chapter 5, 6*) and in the general population (*Chapter 8*) and appears to be responsible for maintaining malaria transmission in certain areas (*Chapter 8*).

Even in the light of this effective malaria transmission, there are tools available to reduce malaria transmission. Antimalarial drugs can reduce the occurrence or persistence of gametocytes. Combination therapy with artemisinin (ACT) appears to be the most efficient treatment to effectively cure infected individuals and to reduce their gametocyte carriage (*Chapter 6*). However, the impact of ACT on malaria transmission may be more modest than expected when based on microscopy. After ACT many individuals harbour persisting low density gametocytes (*Chapter 5, 6*) and, as a result, remain infectious to mosquitoes (*Chapter 6*).

In addition to antimalarial drugs, immune responses can also influence malaria transmission. When gametocytes die in the human body, an immune response can be elicited. The resulting human antibodies can be ingested by a mosquito together with the gametocytes when it is taking a blood meal. These co-ingested antibodies can interfere with further parasite development. This type of immunity is called sexual stage immunity and can result in functional transmission reducing activity, a reduction in the infectiousness of gametocytes. This sexual stage immunity is acquired rapidly after several malaria infections (*Chapter 9*) but appears to be short-lived (*Chapter 10, 11*). As a result, sexual stage immunity may require a constant exposure to gametocytes (*Chapter 12*). Since gametocytes are most common in young children (*Chapter 2*) and their prevalence decreases in higher age groups, sexual stage immunity may also wane with age (*Chapter 10-12*).

The findings in this thesis on the abundance of low density gametocytaemia and their role in malaria transmission underline the importance of transmission reducing interventions for malaria control. A transmission-blocking vaccine could be of great value for this purpose. By reducing the efficiency of malaria transmission, a transmission blocking vaccine may also play an important role in preventing the spread of resistant parasite strains.

Samenvatting

De meest gevaarlijke vorm van malaria wordt veroorzaakt door de parasiet *Plasmodium falciparum*. De mens wordt met deze parasiet besmet door de beet van een geïnfecteerde mug. De transmissiecyclus is rond wanneer een mug op haar beurt geïnfecteerd wordt door een infectieus mens.

In het menselijk bloed kan men zowel aseksuele en seksuele stadia van de malaria parasieten aantreffen. Aseksuele parasieten zijn verantwoordelijk voor de met malaria geassocieerde ziekte en de door haar veroorzaakte sterfte. Seksueel stadium parasieten (gametocyten) veroorzaken daarentegen geen klinische symptomen maar zorgen voor de overdracht van malaria van de mens naar de mug. Transmissie van malaria transmissie vindt alleen plaats als volwassen gametocyten opgezogen worden door een *Anopheles* mug die een 'bloedmaal' neemt bij een mens. De gametocyten ontwikkelen zich vervolgens in de mug totdat deze in staat is nieuwe mensen te infecteren.

Hoewel gametocyten in deze zin essentieel zijn voor de verspreiding van malaria, worden ze slechts aangetroffen bij een klein deel van de mensen die geïnfecteerd zijn met *Plasmodium falciparum*. Dit lijkt haaks te staan op de efficiënte transmissie van malaria in veel gebieden in Afrika. Een van de belangrijke conclusies van dit proefschrift is dat deze schijnbare paradox eenvoudigweg het gevolg lijkt te zijn van onze beperkte middelen om de aanwezigheid van gametocyten in het menselijk bloed aan te tonen. Muggen blijken veel beter in staat gametocyten op te pikken dan het menselijk oog.

In dit proefschrift tonen we aan dat gametocyten veel voorkomen bij kinderen die weliswaar geïnfecteerd zijn met malaria maar die hier geen symptomen van ondervinden (*Hoofdstuk 2*). Gametocyten komen het meest voor bij jonge kinderen, die ze ook langer bij zich lijken te dragen dan oudere kinderen (*Hoofdstuk 2*). Na behandeling met het veelgebruikte antimalaria middel Fansidar® lijkt het aantal gametocyt-dragers in eerste instantie toe te nemen. De omvang van deze toename is afhankelijk van de uitkomst van de behandeling. Kinderen bij wie de behandeling zonder succes is, hebben een aanzienlijk grotere kans gametocyten te ontwikkelen dan kinderen bij wie Fansidar® succesvol alle aseksuele parasieten heeft gedood (*Hoofdstuk 2, 3*). Dit is een verontrustende bevinding die suggereert dat kinderen na onsuccesvolle behandeling meer muggen kunnen besmetten en dat parasietenstammen die resistent zijn voor het gebruikte medicijn zich sneller kunnen verspreiden in de populatie. In hoofdstuk 4 leveren we aanvullend bewijs voor deze hypothese. In een volledig andere omgeving onderzochten we mutaties in het genetisch materiaal van parasieten die verantwoordelijk zijn voor Fansidar® resistentie. Kinderen die geïnfecteerd waren met Fansidar resistente stammen hadden een

grotere kans op gametocyt-dragerschap dan kinderen die besmet waren met Fansidar gevoelige stammen.

Bij een aantal studies in dit proefschrift is gebruik gemaakt van een recent ontwikkelde techniek om gametocyten te detecteren. Deze kwantitatieve moleculaire techniek (QT-NASBA) is gebaseerd op het aantonen van genetisch materiaal (RNA) van volwassen gametocyten en is tot 1000 maal gevoeliger dan het routinematig gebruik van de microscoop. Door gebruik te maken van de QT-NASBA vonden we dat gametocyten veel meer voorkomen dan gesuggereerd wordt door microscopie. Terwijl microscopie bij slechts ~20% van de kinderen met symptomatische malaria gametocyten aantoont, vindt de QT-NASBA gametocyten bij 80-90% van dezelfde kinderen (*Hoofdstuk 5, 6*). In veel van de gevallen zijn de gametocyten in lage, submicroscopische, concentraties aanwezig. Toch blijken deze concentraties voldoende om muggen te infecteren (*Hoofdstuk 6, 7*). Het percentage geïnfecteerde muggen daalt weliswaar met een dalende concentratie van gametocyten maar zelfs bij submicroscopische concentraties blijkt de transmissie van malaria verassend efficiënt (*Hoofdstuk 7*). Hun verhoudingsgewijs lagere infectiviteit wordt bovendien ruimschoots gecompenseerd door het frequente voorkomen van submicroscopisch gametocyt-dragerschap in symptomatische patiënten (*Hoofdstuk 5, 6*) en de algemene bevolking (*Hoofdstuk 8*). Personen met submicroscopische gametocyt-concentraties lijken zelfs verantwoordelijk te zijn voor het in stand houden van malaria transmissie in bepaalde gebieden (*Hoofdstuk 8*).

Zelfs in het licht van deze efficiënte transmissie van malaria zijn er manieren om de transmissie te reduceren. Zo kunnen antimalaria middelen het ontstaan of voortduren van het dragerschap van gametocyten verminderen. Combinatietherapie met artemisine (ACT) lijkt de meest efficiënte aanpak om patiënten met succes te bevrijden van de ziekmakende aseksuele parasieten en tegelijk het aantal gametocyten te reduceren (*Hoofdstuk 6*). Helaas lijkt het effect van ACT op de transmissie van malaria bescheidener dan voorheen werd aangenomen. Zelfs na behandeling met ACT houden veel personen gedurende lange tijd lage concentraties gametocyten. (*Hoofdstuk 5,6*). Als gevolg hiervan blijkt een groot deel van de patiënten na behandeling in staat muggen te besmetten (*Hoofdstuk 6*).

Naast antimalaria medicatie kan ook het menselijke immuunsysteem de transmissie van malaria beïnvloeden. Hoewel gametocyten in rode bloedcellen zitten en grotendeels ontoegankelijk zijn voor het menselijk immuunsysteem, kunnen er wel antistoffen gemaakt worden tegen afbraakproducten van gametocyten die in het menselijk lichaam sterven. Deze antistoffen kunnen samen met gametocyten opgenomen worden door een mug die een ‘bloedmaal’

neemt. In de maag van de muggen kunnen de antistoffen vervolgens de ontwikkeling van de parasiet remmen. Dit type immuniteit wordt 'seksueel stadium immuniteit' genoemd en kan resulteren in functionele transmissie reducerende activiteit, een verminderde infectiviteit van gametocyten. Seksueel stadium immuniteit ontstaat in een vroeg stadium na blootstelling aan malaria (*Hoofdstuk 9*) maar lijkt kortdurend te zijn (*Hoofdstuk 10,11*). Als gevolg hiervan heeft seksueel stadium immuniteit wellicht een constante blootstelling aan gametocyten nodig om effectief te zijn (*Hoofdstuk 12*). Aangezien gametocyten het meest voorkomen bij kinderen (*Hoofdstuk 2*) en het percentage gametocyt-dragers afneemt met het vorderen van de leeftijd, zou seksueel stadium immuniteit ook wel eens af kunnen nemen bij het ouder worden (*Hoofdstuk 10-12*).

De in dit proefschrift verkregen kennis over de overvloed aan submicroscopische gametocyten en hun rol in malaria transmissie onderstrepen het belang van transmissie-reducerence interventies voor het bestrijden van malaria. Een transmissie blokkerend vaccin zou hierbij van groot belang kunnen zijn. Door de efficiëntie van malaria transmissie te verminderen zou een dergelijk vaccin ook een belangrijke rol kunnen spelen bij het voorkómen van de verspreiding van resistente parasieten.

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** Authors contributed equally to the manuscript*

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Uiteraard is er meer dan werk alleen, zelfs tijdens een promotietraject. In Kenia vormden de avonden met Vader Tielen een aangename afwisseling met het werk. Uitkijkend over het Victoriameer was er even tijd om over de grotere dingen in het leven na te denken. In Nederland was er dat andere boek, de *Leidraad International Health*, waarvan de voltooiing bijna even lang heeft geduurd als dit proefschrift. Ik dank de auteurs en mederedacteurs voor hun inspanningen. Daarnaast dank ik mijn kameraden van GroenLinks. Hoewel veel vrije avonden gevuld werden met lange vergaderingen op het gemeentehuis, was het een boeiende en leuke periode.

Ten slotte vrienden en familie, voor wie af en toe weinig tijd was. Ik ben blij met mijn grote groep trouwe vrienden. Judith Brouwer en Emile de Roos verdienen speciale dank voor in totaal meer dan honderd brieven richting Afrika. Mijn lieve ouders dank ik. De eerste rit naar Schiphol was zwaar en ik begrijp dat een zoon op afstand nooit helemaal went. Het is bijzonder goed te merken dat onze band sterk is en blijft. Dat is zeker ook het geval voor mijn broer Jaap, zus Annieke, Pim en mijn nichtje Janne. Door een langdurige afwezigheid mis je soms belangrijke dingen in een mensenleven, een vervelend bijproduct van een baan in het buitenland.

Als laatste natuurlijk Alma Tostmann. Mijn toegewijde stagiaire. Een correcter begin van een relatie kan ik me best voorstellen, een beter begin niet. Hoeveel mensen ontmoeten hun grote liefde aan het Victoriameer? Ik zie uit naar onze toekomstige avonturen. In Afrika en in Nederland.

Curriculum Vitae

Teun Bousema, de schrijver van dit proefschrift, werd op 4 september 1977 geboren in Woudsend. Na middelbaar onderwijs genoten te hebben op het St. Andreas college in Drachten deed hij in 1995 eindexamen op het gymnasium van het Florens Radewijns College in Raalte. Aansluitend studeerde hij Biomedische Wetenschappen aan de Radboud Universiteit Nijmegen, waar hij zich specialiseerde in Toxicologie, Epidemiologie en Infectieziekten. Tijdens de laatste jaren van zijn studie deed hij onderzoek naar genetische risicofactoren voor prostaataandoeningen (Experimentele Urologie, RUNMC Nijmegen), dier- en *in vitro* modellen voor *Salmonella* infecties (Rijksinstituut voor Volksgezondheid en Milieu, Bilthoven) en de epidemiologie van HIV-1 en HIV-2 infecties (Medical Research Council Laboratories, Fajara, The Gambia).

Na zijn afstuderen in het jaar 2000 was hij een jaar als vrijwilliger betrokken bij de bouw van een kliniek in Logba Tota, Ghana. In 2001 begon hij zijn professionele carrière aan de Radboud Universiteit Nijmegen. Als deeltijd docent op de afdeling Epidemiologie en Biostatistiek en deeltijd junior onderzoeker op de afdeling Medische Microbiologie schreef hij een individueel project over de invloed van malaria medicatie op de transmissie van *Plasmodium falciparum* malaria. Dit project ontving in 2002 subsidie van NWO-WOTRO en vormt de basis van dit proefschrift. In 2003 en 2004 verzamelde hij gegevens aan het Victoriameer bij het International Centre of Insect Physiology and Ecology (ICIPE) in Mbita, Kenia. In 2005 werd het veldwerk uitgebreid naar het Kilimanjaro Christian Medical Centre (KCMC) in Moshi, Tanzania, waar hij zich sindsdien bezighoudt met malaria transmissie onderzoek in het kader van het Poverty Related Infection Oriented Research Project (PRIOR). Hij was mede-aanvrager van het hierop volgende African-PRIOR-Initiative (APRIORI) dat in 2006 subsidie ontving (NACCAP, NWO-WOTRO). In 2007 ontving Teun Bousema de Young Investigator Award van het Nijmeegs Universitair Centrum voor Infectieziekten.

Sinds het najaar van 2006 is hij samen met Alma Tostmann, met wie hij samenleeft, voor APRIORI werkzaam in Moshi, Tanzania.