Infection dynamics of *Plasmodium* *falciparum* in Papua New Guinea

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Summary

Malaria is one of the leading causes of illness and death in Papua New Guinea (PNG), mainly affecting children under 5 years of age. The current first line treatment for uncomplicated malaria is a combination therapy of sulfadoxine-pyrimethamine and chloroquine (CQ) or amodiaquine, however, frequent treatment failures have been reported shortly after its implementation. Drug resistance has mainly been associated with single nucleotide polymorphisms in five different genes. Different studies have indicated that mutations associated with drug resistance incur fitness costs to the parasite in absence of drug pressure. Among these are reports from different countries where a decreasing prevalence of mutations associated with CQ resistance was observed after CQ has been suspended as first line treatment. As it is experimentally difficult to estimate the fitness of *Plasmodium* parasites, a surrogate marker is needed to quantify fitness costs associated with drug resistance mutations. We hypothesised that the parasites' survival within the human host can be used as surrogate marker for parasite fitness. In a pilot study we determined the drug resistance-associated haplotypes of parasite clones at 25 loci on 4 marker genes, and compared their prevalence between newly acquired and chronic infections. A reduced frequency of a 7-fold mutated haplotype and increased frequency of a 5-fold mutated haplotype in long term persistent infections indicated an impaired survival of highly mutated parasites and suggested that the duration of infections is a promising marker for parasite fitness that deserved further investigation.

A further approach to define duration of infections more precisely was undertaken with samples from a longitudinal field survey in PNG. A cohort of 269 1-4.5 years old children was followed over a period of 16 months. This provided consecutive blood samples collected in 2-monthly follow-up visits plus a blood sample from each morbid episode. All samples were genotyped for the polymorphic marker gene merozoite surface protein 2 (*msp2*) in order to distinguish individual parasite clones within a host. The persistence of genotypes in consecutive blood samples of each child was determined. Unexpected high numbers of antimalarial treatments given in the course of this study led to a high turn-over rate of parasite clones and prevented the establishment of asymptomatic long-term infections. The shortage of long untreated periods in our study participants hampered the determination of a novel molecular parameter termed "duration of infection" for each individual parasite clone. This

parameter could have been useful for modelling the transmission success and fitness of drug resistant versus drug sensitive parasite clones. We concluded that this approach requires a cohort of semi-immune individuals where treatment is given rarely, e.g. older children or adults, where *P. falciparum* infections less frequently cause morbid episodes.

The high incidence of morbidity in our study cohort allowed us to investigate molecular parameters that have an impact on the development of a subsequent clinical episode. We found that in children >3 years, a higher multiplicity of infection (MOI) at baseline reduced the risk of a *P. falciparum* episode. These results are in agreement with previous reports and support the concept of premunition. Furthermore, we studied the impact of co-infecting *Plasmodium* species on MOI and found an increased MOI in the presence of a heterologous species. This can be explained by the observation of reduced parasite densities in mixed-species infections, leading to a decreased need for treatment and thus facilitating the accumulation of multiple clones. Our results provide further evidence for interactions among co-infecting *P. falciparum* clones as well as among co-infecting *Plasmodium* species.

During the 16 months field survey all morbid episodes were treated with Coartem[®]. Analysis of *msp2* genotyping results revealed an unexpected high number of Coartem[®] treatment failures. After confirmation of recrudescent parasites with 2 additional marker genes and exclusion of host genetic factors to be responsible for treatment failures, the most likely explanation for the frequently observed Coartem[®] failures was a combination of poor adherence to the treatment regimen and a lack of fat supplementation which is required for absorption of the drug. Our results disagree with findings from a clinical trial reporting Coartem[®] to be highly effective in PNG. In contrast, our observations from a non-trial setting highlight potential problems of Coartem[®] usage in routine clinical practice.

In the course of this thesis, genotyping techniques for merozoite surface proteins (*msp*) 1 and 2 were optimized and applied. Using these high resolution typing techniques based on capillary electrophoresis, we investigated the effect of transmission intensity on diversity and complexity of *msp1* and *msp2* in samples from PNG and Tanzania. We observed a greater MOI and a greater number of distinct alleles in samples from Tanzania. Genetic diversity was greater for *msp2* than for *msp1*. In both these areas of different malaria endemicity *msp2* was found to be superior for distinguishing individual parasite clones. The probability of two infections carrying by chance the same *msp2* allele was lower than this probability calculated

for *msp1*. Based on the frequency distribution of *msp2* alleles and on the distribution of observed numbers of infections, we estimated the true MOI adjusted for the probability of multiple infections sharing the same allele. For our high resolution typing technique this adjustment made little difference to the estimated mean MOI compared to the observed mean MOI.

A central aim of this thesis was to measure molecular parameters of infection dynamics. These can be determined from genotyping longitudinal sets of samples. Some of these parameters were successfully determined: MOI, force of infection (FOI), and detectability. Other parameters such as natural elimination rates and persistence of infections could not be determined due to frequent treatments. We estimated detectability of parasite clones based on samples collected 24 hours apart and investigated its impact on the MOI and FOI. Imperfect detection of parasites occurs as a consequence of sequestration or when parasite densities fluctuate around the detection limit. We found that in our study participants detectability was high. This was likely an effect of high parasite densities in children of this age. The benefit of short-term sampling on measures of MOI and FOI was marginal. We concluded that in future studies carried out in this age group, taking repeated samples 24 hours apart has limited benefit and does not justify the additional costs, work load and discomfort for the study participants.

This project contributed to our understanding of the infections dynamics of *P. falciparum* and the interactions between parasites clones and *Plasmodium* species. We provided further insights into determinants of malaria episodes and highlighted the potential usefulness of the parameter "duration of infection" as surrogate marker to estimate fitness costs of drug resistance. This thesis provided findings that are relevant for malaria control strategies and treatment guidelines

Zusammenfassung

Malaria ist eine der hauptsächlichen Krankheits- und Todesursachen in Papua Neuguinea (PNG) und betrifft vor allem Kinder unter fünf Jahren. Die Standardbehandlung für unkomplizierte Malaria ist derzeit eine Kombination aus Sulfadoxin-Pyrimethamin plus entweder Chloroquin (CQ) oder Amodiaquin. Schon kurz nach der Einführung dieser Therapie beobachtete man hohe Misserfolgsraten. Medikamentenresistenz ist vor allem auf Punktmutationen in fünf verschiedenen Genen zurückzuführen. Verschiedene Studien haben gezeigt, dass Mutationen, die mit Medikamentenresistenz in Zusammenhang stehen, die Fitness von Parasiten beeinträchtigen. Dies gilt jedoch nur in Abwesenheit von Malariamedikamenten. Einige Länder verzeichneten einen Rückgang der Marker für CQ-Resistenz, nachdem CQ als Standardbehandlung für Malaria abgesetzt wurde. Die Fitness von *Plasmodien* kann experimentell nur sehr aufwändig bestimmt werden. Daher ist es von Vorteil, einen Marker zu finden, der eine Quantifikation der Fitness-Kosten, die durch Punktmutationen verursacht werden, erlaubt. Unserer Arbeitshypothese lag die Annahme zugrunde, dass sich die Überlebensdauer der Parasiten im Wirt als ein solcher Marker für Parasiten-Fitness eignen könnte. Daher haben wir für einzelne Parasitenklone ein genetisches Resistenzprofil erstellt, das sich aus 25 Punktmutationen in vier verschiedenen Markergenen zusammensetzt. Diese so genannten „Haplotypen" wurden zwischen Neuinfektionen und chronischen Infektionen verglichen. Die Anzahl von Parasiten mit sieben Mutationen nahm ab, dagegen stieg die Anzahl fünf-fach mutierter Parasiten in Langzeit-Infektionen an. Dies führte zu dem Schluss, dass die Überlebensdauer von Parasiten mit einer hohen Anzahl an Punktmutationen beeinträchtigt ist. Diese Ergebnisse deuteten darauf hin, dass die Dauer einer Parasiteninfektion im Wirt ein geeignetes Mass für Parasiten-Fitness ist.

In einem weiteren Schritt wurden die Proben einer Langzeitstudie aus PNG verwendet, um die Infektionsdauer präziser zu bestimmen. 250 Kinder zwischen einem und viereinhalb Jahren wurden über einen Zeitraum von 16 Monaten beobachtet. Während dieser Zeit wurden Blutproben in Abständen von 2 Monaten gesammelt. Weitere Proben wurden beim Auftreten von Malariasymptomen bei den Studienteilnehmern gesammelt. Um einzelne, in einem Wirt gleichzeitig vorhandene Parasitenklone zu unterscheiden, wurde bei allen Blutproben, die *P. falciparum* enthielten, das polymorphe Parasitengen „Merozoitenoberflächenprotein 2" (*msp2*) genotypisiert. Die Überlebensdauer aller Genotypen wurde anhand von aufeinanderfolgenden Blutproben aus der Langzeitstudie bestimmt. Eine unerwartet hohe

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Anzahl von Behandlungen führte jedoch zu starker Fluktuation der Parasitenklone, so dass sich fast keine asymptomatischen Langzeitinfektionen etablieren konnten. Die Infektionsdauer wäre sowohl für die Schätzung der Übertragungsrate als auch zum Vergleich der Fitness zwischen medikamentenresistenten und nicht-resistenten Parasiten nützlich. Unsere bei kleinen Kindern gewonnenen Ergebnisse zeigten jedoch, dass für diesen Versuchsansatz semi-immune Studienteilnehmer, wie z.B. ältere Kinder oder Erwachsene, besser geeignet wären, da in diesen Altersgruppen neue *P. falciparum* Infektionen weniger häufig zu klinischen Episoden führen.

Die hohe Inzidenz an Krankheitsfällen in Teilnehmern unserer Studie ermöglichte eine genaue Ermittlung parasitologischer Faktoren, die den Verlauf einer Infektion beeinflussen. Eine hohe Anzahl Infektionen hat bei Kindern über drei Jahren das Risiko einer nachfolgenden klinischen Episode signifikant reduziert. Diese Resultate bestätigen frühere Berichte und stützen die Hypothese, dass bereits bestehende Infektionen vor Neuinfektion schützen („premunition"). Weiterhin untersuchten wir den Einfluss einer Co-Infektion mit weiteren *Plasmodium* Arten auf die Anzahl von *P. falciparum* Klonen pro Wirt und konnten zeigen, dass diese in Anwesenheit weiterer *Plasmodium* Arten erhöht war. Eine mögliche Erklärung hierfür könnte die geringere Parasitendichte in Spezies-Mischinfektionen sein, die den Behandlungsbedarf reduzierte und somit eine Akkumulierung verschiedener Klone erlaubte. Unsere Ergebnisse liefern weitere Hinweis auf eine gegenseitige Beeinflussung mehrerer Parasitenklone als auch mehrerer *Plasmodium* Arten, sofern sie den gleichen Wirt besiedeln.

Während der 16-monatigen Studie wurden alle klinischen Malariaepisoden mit Coartem[®] behandelt. Die Ergebnisse der *msp2*-Genotypisierung zeigten, dass diese Behandlung in vielen Fällen versagte. Behandlungsmisserfolge wurden durch Genotypisierung zweier weiterer Markergene bestätigt und genetische Wirtsfaktoren wurden als Grund für den Behandlungsmisserfolg ausgeschlossen. Die wahrscheinlichste Erklärung für häufiges Fehlschlagen der Coartem[®] Behandlung war, dass Behandlungsvorschriften nicht eingehalten wurden oder dass das Medikament nicht mit einer fettreichen Nahrung verabreicht wurde, welche zur Absorption der Wirkstoffe notwendig ist. Unsere Ergebnisse unterscheiden sich deutlich von den Resultaten einer klinischen Studie, die eine hohe Effizienz von Coartem[®] in PNG verzeichneten. Im Gegensatz zu diesen Berichten verdeutlichen unsere Ergebnisse

potentielle Probleme bei der alltäglichen Anwendung von Coartem® ausserhalb klinischer Studien.

Im Verlauf dieser Doktorarbeit wurden die Methoden zur Genotypisierung von *msp1* und *msp2* optimiert und angewandt. Mit Hilfe hochauflösender Genotypisierungsmethoden, die auf Kapillarelektrophorese beruhen haben wir den Einfluss der Übertragungsintensität auf die Diversität und Komplexität von *msp1* und *msp2* in Proben von PNG und Tanzania (TZ) untersucht. Sowohl die Anzahl verschiedener Klone pro Wirt (Infektionsmultiplizität) als auch die Diversität waren in Proben aus TZ höher und *msp2* wies eine höhere Diversität auf als *msp1*.In beiden Ländern, die sich stark in ihrer Malaria-Übertragungsrate unterscheiden, war *msp2* das geeignetere Markergen um die einzelnen Parasitenklone zu unterscheiden, da die Wahrscheinlichkeit, dass zwei Parasiten durch Zufall das gleiche Allel tragen viel geringer war als für *msp1*. Anhand der Häufigkeitsverteilung der *msp2*-Allele und der Verteilung der Anzahl Klone pro Wirt haben wir die tatsächliche Multiplizität berechnet. Hierbei wurde die Möglichkeit, dass zwei Parasiten durch Zufall das gleiche Allel tragen berücksichtigt. Für die hier angewendete, hochauflösende Genotypisierungsmethode hat sich der berechnete Wert für die Multiplizität nur geringfügig von der gemessenen Anzahl unterschieden.

Ein Hauptziel dieser Studie war es, die Infektionsdynamik mittels molekularer Parameter zu beschreiben. Diese Parameter können durch die Genotypisierung longitudinaler Datensätze ermittelt werden. In dieser Studie wurden einige dieser Parameter erfolgreich bestimmt: die Multiplizität, die Infektionsrate und die Nachweisbarkeit von *P. falciparum* Klonen. Parameter wie die natürliche Eliminierung von Parasiten durch den Wirt oder die Überlebensdauer der Parasiten Klone im Wirt konnten aufgrund häufiger Behandlungen mit Medikamenten nicht bestimmt werden. Anhand aller Proben, die im Abstand von 24 Stunden gesammelt wurden, wurde die Nachweisbarkeit der Klone geschätzt und die Auswirkung einer Datenerhebung in kurzen Zeitabständen auf die Multiplizität und auf die Infektionsrate untersucht. Ein unvollständiger Nachweis von Parasiten wird vor allem durch Sequestration verursacht, oder wenn Parasitendichten nahe bei der Nachweisgrenze des molekularen Tests liegen. Die Nachweisbarkeit von Parasitenklonen war in unseren Studienteilnehmern sehr hoch, was höchst wahrscheinlich auf hohe Parasitendichten in Kindern dieser Altersgruppe zurückzuführen ist. Datenerhebungen in Zeitintervallen von 24 Stunden hatten nur einen geringfügigen Einfluss auf die Anzahl detektierter Klone und die Infektionsrate. Der geringe Informationsgewinn, der durch die zweite Probe innerhalb von 24 Stunden erzielt wurde rechtfertigt weder die zusätzlichen Kosten, für die Durchführung und den zusätzlichen

Arbeitsaufwand noch die zusätzlichen Unannehmlichkeiten für die Studienteilnehmer. Deshalb raten wir für zukünftige Studien, die in dieser Altersgruppe durchgeführt werden davon ab zwei Proben innerhalb von 24 Stunden zu sammeln.

Diese Arbeit hat zum Verständnis sowohl der Infektionsdynamik als auch der Interaktion zwischen Parasitenklonen und *Plasmodium* Arten beigetragen. Weitere Erkenntnisse wurden über Faktoren gewonnen, die die Entwicklung einer klinischen Episode beeinflussen. Wir konnten aufzeigen, dass der Parameter „Infektionsdauer" von Nutzen sein kann, um Fitness-Kosten zu messen. In dieser Arbeit wurden Erkenntnisse gewonnen, die für Kontrollstrategien zur Bekämpfung von Malaria als auch für Richtlinien zur Behandlung von Malaria von Bedeutung sind.

Abbreviations

ACT	Artemisinin-based combination therapy
AQ	Amodiaquine
CQ	Chloroquine
DNA	Deoxyribonucleic acid
FOI	force of infection
FUB	follow-up bleed
Hb	Haemoglobin level
LDR-FMA	Ligase detection reaction-fluorescent microsphere assay
LR	likelihood ratio
MOI	multiplicity of infection
msp1	merozoite surface protein 1
msp2	merozoite surface protein 2
OR	odds ratio
PCR	polymerase chain reaction
pfcrt	*Plasmodium falciparum* chloroquine resistance transporter
pfdhfr	*Plasmodium falciparum* dihydrofolate reductase
pfdhps	*Plasmodium falciparum* dihydropteroate synthase
pfmdr1	*Plasmodium falciparum* multidrug resistance gene 1
PNG	Papua New Guinea
RDT	rapid diagnostic test
RFLP	restriction fragment length polymorphism
SNP	single nucleotide polymorphism
SP	Sulfadoxine-pyrimethamine
STI	Swiss Tropical Institute
TRS	treatment re-infection study
TZ	Tanzania
WHO	World Health Organization

CHAPTER 1: Introduction

Malaria situation in Papua New Guinea

Malaria is one of the leading causes of morbidity and mortality in Papua New Guinea (PNG) [1]. Its distribution within the country is very heterogeneous, ranging from highly endemic areas in the costal lowland [2-4] to absence of malaria with sporadic epidemic outbreaks in the highlands [5-7]. These regional differences observed within PNG are mainly attributable to the difference in temperature depending on altitude. In most lowland areas malaria transmission is perennial and shows limited seasonal variations [2-4], whereas endemicity decreases with increasing altitude [8-12]. In the areas of permanent transmission in PNG the main burden of malaria is carried by young children, and both, the prevalence of infection as well as the incidence of morbidity peak in this age group [2,3,13-16]. No clear age relationship is observed in areas of low endemicity or of occasional epidemics, where most infections result in a clinical episodes even in adults [7].

Severe malaria and malaria mortality are less frequent in PNG than in areas of comparable endemicity in Africa [17]. This could be related to the co-existence of all four *Plasmodium* species that affect humans which is a characteristic feature of the malaria situation in PNG. *P. falciparum* is the predominant species in most areas followed by *P. vivax,* and mixed species infections are common [2,3,8,15,18,19]. PNG therefore provides an optimal setting to study interactions between different *Plasmodium* species. So far, mechanisms of species interactions and the impact of mixed species on the epidemiology and morbidity of malaria are poorly understood and studies investigating these topics have reported inconsistent results [19-21].

The first line treatment against uncomplicated malaria in PNG has been chloroquine (CQ) or amodiaquine (AQ) monotherapy for a long time. A steadily declining efficacy of these drugs in PNG [22-25] has prompted health authorities to change the first line treatment to a combination therapy of CQ or AQ plus sulfadoxine-pyrimethamine (SP). However, a recent surveillance trial in three different provinces of PNG has reported treatment failure rates up to 28% [26]. This highlights the rapid decrease in efficacy of this combination therapy only shortly after its implementation. In view of rapid failure of current first line drugs there is an urgent need for alternative treatment regimens. Artemisinin based combination therapies (ACT) are recommended by the World Health Organization (WHO) for countries where

conventional drugs are ineffective [27]. A recent drug trial in PNG has shown that the combination therapy of artemether and lumefantrine (Coartem®) was highly efficacious against *P. falciparum* infections [28] which gives a strong argument for the implementation of Coartem® as first line treatment in PNG.

Genotyping and infection dynamics

In the past two decades advances in the development of molecular genotyping techniques have made it possible to distinguish individual parasite clones within a host and numerous molecular epidemiological studies have increased our understanding of the parasite population structure in endemic countries. A hallmark of *Plasmodium* infections in highly endemic areas is the presence of concurrently infecting parasite clones within a host. The molecular markers most often used to discriminate *P. falciparum* clones are the genes of merozoite surface proteins 1 and 2 (*msp1* and *msp2*) which are both highly polymorphic. *Msp2* alleles can be grouped into 2 allelic families (3D7 and Fc27) according to a dimorphic non-repetitive region flanking a highly polymorphic domain of tandem repeats [29]. The size polymorphism of *msp2* is generated by differences in copy number and length of the repeat units. The *msp1* gene falls in three distinct allelic families (K1, MAD20 or RO33). Similar to *msp2*, the central repeat regions of MAD20-type and K1-type alleles give rise to size polymorphism and are flanked by family-specific sequences. The RO33 sequence does not contain any repeats [30]. Several genotyping techniques have been devised for studying genetic diversity in these two genes encoding surface antigens. All assays are based on PCR amplification of the central polymorphic region. Two commonly applied methods are: (i) sizing of PCR fragments on agarose gels. Identification of the allelic family is achieved by either use of family-specific primers [31] or by hybridization with family-specific probes [32], and (ii) restriction digestion of the amplified PCR products [33,34]. A more recent genotyping approach for *msp2* is based on capillary electrophoresis of fluorescently labelled family-specific PCR fragments [35,36]. The major advantage of this technique lies in the accuracy of discriminating distinct parasite clones, even in complex mixtures, and in facilitating high throughput genotyping.

Genotyping studies have shown that *P. falciparum* infected individuals from endemic areas generally harbour multi-clonal infections. Multiplicity of infection (MOI) describes the number of parasite genotypes simultaneously infecting one host. MOI does not only vary by

transmission intensity [37,38] but also by age. In endemic areas mean MOI generally increases up to the age of about 10 years and decreases thereafter during adolescence and adulthood [32,35,38,39].

Genotyping samples collected in short term intervals revealed large fluctuations of parasite densities and genotype patters even from one day to another, suggesting that the detectable parasite clones are not stable over time, but rather exhibit highly complex daily dynamics [40,41]. These studies have emphasised that a single blood sample might not be representative for the complex parasite population present in an individual host. Important insights in the dynamics of *Plasmodium* infections can be gained by describing genotypes of parasite infections over time. Longitudinal studies have made it possible to track individual parasite clones over time and to determine acquisition and clearance rates. They also allow estimating the persistence of individual clones, and to study these parameters of infection dynamics with respect to age, seasonal variation or as an outcome measure of an intervention. Thus, molecular typing provides novel molecular malariological parameters.

Acquired immunity affects the dynamics of clonal infections and it is therefore informative to conduct longitudinal genotyping surveys in different age groups. This will likely add to our understanding of developing immunity against malaria. In infants and young children in endemic areas, high MOI is a risk factor for clinical malaria [42,43], whereas in older children a high MOI was associated with protection against subsequent morbidity. The latter effect could be due to cross-protection against super-infecting parasites [13,44,45].

Genotyping studies can greatly contribute to the understanding of interdependence between different *Plasmodium* species and between multiple clones of each species co-infecting one carrier. Previous studies reported that in areas sympatric for several *Plasmodium* species, mixed species infections are less common than expected by chance [46]. This implies some kind of interaction between species. How such effects may act upon a population of parasites composed of multiple competing clones has never been investigated in detail. *Plasmodium* species interactions within the human host were proposed to occur as a consequence of density dependent regulation. This was suggested by Bruce et al., (reviewed in [47]) from a study conducted in PNG, where the total parasite density within a host was found to fluctuate around a threshold, while densities of each species changed over time in a sequential pattern. It was proposed that density dependent regulation gets activated once the total parasite

population in a host reaches a certain density threshold, and that this regulation acts in a species transcending manner and therefore reduces total parasitaemia in a host.

Few studies have reported a protective effect of co-infections with other species against severe malaria [48] or against a subsequent *P. falciparum* episode [21], whereas others failed to confirm these findings [19,20]. More prospective studies are needed to provide further evidence for *Plasmodium* species interactions in humans and ways these interactions operate.

Fitness cost of drug resistance

Advances in molecular typing have led to identification of a number of genetic events involved in or responsible for parasite resistance to antimalarial drugs. Among these are single nucleotide polymorphisms (SNP) or gene amplifications in genes encoding drug targets. Polymorphism in the *P. falciparum* chloroquine resistance transporter (*pfcrt*) is central to CQ resistance [49] with the mutation K76T playing a major role in determining the outcome of CQ treatment [50,51]. The role of *P. falciparum* multidrug resistance gene 1 (*pfmdr1*) in drug resistance is controversial. Some authors found an association of certain SNPs, in particular the mutation N86Y, with chloroquine resistance (e.g. [51-53]) whereas others did not (e.g. [54,55]). Transfection experiments [56] showed that SNPs in the *pfmdr1* gene were involved in modulating the susceptibility to CQ and a joined action of the two genes *pfcrt* and *pfmdr1* was suggested to be involved in high level CQ resistance [50]. No conclusive evidence was found so far for the importance of variations in copy number of the *pfmdr1* gene on treatment outcome. Resistance to SP was found to be associated with polymorphism in the *P. falciparum* dihydrofolate reductase gene (*Pfdhfr*) (pyrimethamine resistance) [57-60] and the dihydropteroate synthase gene (*Pfdhps*) (sulfadoxine resistance) [61-63]. The mutation S108N in *pfdhfr* and A437G in *pfdhps* are considered the key mutations that confer resistance against SP, whereas the accumulation of additional mutations in these genes increases the level of resistance [59,61,64,65]. The calcium dependent ATPase6 is considered a target of Artemisinin drug action [66-68], however, field data on the involvement of ATPase6 mutations in drug resistance to Artemisinin are controversial.

Mutations are thought to be costly for parasites in absence of drug pressure. Before the widespread use of antimalarial drugs, the frequency of resistant parasites was generally low, and only rose in response to drug pressure [69]. The frequency at which drug resistance alleles occur in a population is determined by a balance between the selective advantage

conferred by the mutated alleles in the presence of the drug and natural purifying selection eliminating mutations that incur fitness cost in absence of the drug [70]. There are a number of observations indicating that mutations associated with drug resistance indeed reduce the parasites' fitness in absence of drug pressure. Experimental support comes from an *in vitro* competition experiment showing a reduced survival of drug resistant compared to sensitive parasites, when drug pressure was removed [71]. Similar results were observed in competition experiments in mice where a pyrimethamine resistant *Toxoplasma gondii* strain was found to have a fitness defect over a sensitive strain [72] or where a drug resistant *P. chabaudi* clone was completely suppressed by a sensitive clone in absence of drug pressure [73].

Evidence from field studies comes from longitudinal surveys reporting temporal fluctuations in the frequency of mutations in the genes *pfcrt* and *pfmdr1* [74-76]. Inter-seasonal analysis revealed in all studies an increased frequency of the mutant alleles towards the end of the rainy and beginning of the subsequent dry season and a decrease in prevalence of mutant alleles over the time of the dry season resulting in an increased proportion of sensitive strains at the beginning of the ensuing wet season. A likely explanation for the observed pattern is a seasonal variation in drug pressure which is high during the wet season, but virtually absent during the dry season. The increased number of sensitive strains in absence of drug pressure towards the end of the dry season is likely the result of an impaired fitness of resistant parasites compared to sensitive ones.

A study in Hainan, China, found the prevalence of chloroquine-resistant *P. falciparum* strains to decrease after abolishment of CQ as first-line treatment [77]. The frequency of the resistance marker *pfcrt* 76T decreased from 90% in 1978 to 54% in 2001. Similarly, re-emergence of sensitive parasites after suspension of CQ as first line treatment has also been reported from Malawi [78], where a progressive decline in the prevalence of *pfcrt* 76T was observed from 85% to 13% between 1992 and 2000. Mita et al. [79] gave evidence that the recovery of CQ sensitive parasites in Malawi was due to expansion of the wild type allele rather than due to back mutation in the *pfcrt* gene.

Recent studies have reported a selection of the *pfcrt* K76 wild type allele [80] and the *pfmdr1* wild type alleles N86, F184 and D1246 following treatment with the artemisinin combination therapy Coartem® (artemether-lumefantrine) [80-85]. Therefore, Coartem® seems to be an ideal replacement for CQ as a first line-treatment in areas with high levels of CQ resistance.

It's likely that a faster reversal of drug resistance against CQ is achieved through Coartem[®] treatment compared to the situation in absence of treatment.

Estimating the reduction in parasite fitness is desirable as it remains a crucial parameter for epidemiological models that aim at predicting the transmission dynamics of malaria and the spread of drug resistance. Gaining further knowledge on the extent of fitness loss in drug resistant parasites will increase the prediction power of mathematical models [70,86-88]. In the future, models predicting the spread or elimination of drug resistance genotypes could be applied for guidance of treatment policies and strategies for control of drug resistance. The estimation of costs of resistance can also play a major role in making predictions about the re-emergence of sensitive parasite strains after a particular drug had been suspended.

The fitness of parasites determines their survival in the host or vector and their reproductive success. One key factor of fitness is thus the transmission rate to a new host, which is represented by the basic reproductive number (R_0). In the case of malaria several parameters need to be measured in order to determine R_0. Among these is for example the infectiousness from humans to mosquitoes, the duration of infection in the human host, the probability that the mosquito survives the development of the parasite, the infectiousness from mosquitoes to humans etc. (summarized in [87]). However, measuring the actual transmission from one host to another is experimentally very difficult. Thus, surrogate markers for parasite fitness are needed in order to estimate fitness costs of drug resistance. Since experimental infections are impossible for ethical reasons and symptomatic infections require immediate treatment, all studies of fitness costs in the human host must be restricted to chronic, asymptomatic infections in semi-immune hosts in a malaria endemic setting. Parameters of infection dynamics such as the duration of infection or the clearance rate might be suitable markers as they are considered important parameters to contribute to transmission success of parasites.

This PhD project attempted to identify and measure malariological parameters that might be useful for estimating the transmission success of drug resistant parasites. The molecular typing data used for determination of these parameters derived from two longitudinal studies conducted in the years 2003- 2005 and 2006-2007 in PNG.

Rationale

I. Determination of molecular parameters of infection dynamics by longitudinal sampling

The present study sought to assess the infection dynamics of *P. falciparum* clones in a prospective longitudinal survey in children between 1 and 4.5 years of age that were subject to repeated blood sampling over a period of 16 months. Molecular genotyping of consecutive blood samples from individual hosts is thought to provide important measures of infection dynamics such as the infection and clearance rate or the duration of individual parasite clones which might lead to further insights into the acquisition of natural immunity.

Following up our cohort of 269 children provided numerous samples from each individual to describe the infection and clearance rate or the duration of infection as baseline measures for further analysis. Optimization of sampling strategies was attempted because previous studies had reported considerable fluctuations of genotypes in samples collected in daily or short term intervals [89,90], which implicates that parasite detection in a single sample is imperfect and only partly represents the total parasite population in a host. In order to overcome this limitation, we collected 2 consecutive blood samples taken 24 hours apart from each individual at regular follow-up visits. This study design allowed estimating the detectability of parasite clones and made it possible to assess the benefit obtained by collecting short term samples in a large cohort study.

II: Infection dynamics parameters used as surrogate markers for parasite fitness.

In absence of drug pressure, mutations are thought to incur fitness costs to the parasite. There is some evidence from *in vitro*, but also *in vivo* studies that mutations associated with drug resistance are costly when the usage of specific drugs is abolished. Recently the first attempt to quantify the loss of fitness *in vivo* in the human host was published [91]. This approach was based on the reduction of *pfcrt*76 mutant allele frequency after the abolishment of CQ treatment over a period of 13 years. Our assumption is that fitness costs can be measured as effects on parasite survival in the host. Therefore parameters of infection dynamics such as the 'duration of infection' or the 'clearance rate' might be useful surrogate markers to estimate the loss of fitness caused by drug resistance mutation. If these assumptions were true one would expect to find a different genetic make-up of markers associated with drug resistance between long-lasting infections and infections that are rapidly cleared by the human host. A first indication whether this approach is suitable can be obtained by comparing newly

acquired infections and infections from adults, which have previously been shown to carry long lasting, chronic infections [35]. This approach will be followed in samples from a "treatment to reinfection study" conducted in 2004/2005 where new genotypes can be easily identified.

In a further step, more accurate estimates can be gained using a similar approach based on the measures of infection dynamics from our longitudinal cohort, as detailed information on the persistence of individual parasite clones are available from these data. A great advantage of our study site in PNG compared to highly endemic areas in Africa is the overall low multiplicity of infection. In PNG most individuals harbour single clone infections ([92] and own data) which makes it possible to clearly assign a drug resistance haplotype to an infecting parasite clone and therefore to study the effect of drug resistance mutations on the parasite survival within the host.

III Infection dynamics parameters used to determine risk factors for clinical episodes
Since the age group studied in our longitudinal survey is most susceptible to clinical malaria episodes, our study provides optimal conditions to investigate changes in the actually infecting genotypes when children develop symptomatic episodes. As indicated previously, morbid episodes occur when children acquire new parasite clones [89,90]. Our study design made it possible to compare genotypes of a sample set collected from clinical cases and compare these to samples collected in the preceding asymptomatic phase of each child. This study design also allows the definition of risk factors for a clinical malaria episode such as effects of concurrent infections, i.e. a different *Plasmodium* species or multiplicity of *P. falciparum* infections.

Objectives

I. Describe the infection dynamics of *P. falciparum* by molecular typing in our longitudinal survey of 269 children in Papua New Guinea.

II. Determine and apply parameters of infection dynamics to estimate the fitness costs incurred by mutations associate with drug resistance, *in vivo*.

III. Investigate the genetic profile of samples derived from clinical episodes to define risk factors for malaria morbidity.

The specific objectives of this project were as follows:

I.1.   To estimate the detectability of infection in a large set of samples collected 24 hours apart, and to investigate the benefit obtained by collecting 24 hour bleeds for our longitudinal study.

I.2.   To describe the parasite population of a longitudinal cohort in children from Papua New Guinea and to determine acquisition rates, clearance rates and duration of infections as essential parameters describing infections dynamics.

I.3.   To develop a new protocol for high resolution genotyping of the polymorphic marker gene merozoite surface protein 1 (*msp1*) by capillary electrophoresis in order to improve discrimination of individual parasite clones during longitudinal tracking of an infection.

II.1.   To compare the genetic profile of known markers of drug resistance between new infections after radical cure and infections from adults from a cross sectional study where measurements of clone persistence are absent, assuming that adults harbour chronic infections.

II.2.   To compare the genetic profile of known markers of drug resistance between new infections and persisting infections of known duration.

III.1.   To determine the genetic profile in samples from clinical episodes as compared to samples from asymptomatic carriage in order to investigate whether symptoms are caused by newly acquired parasites strains and whether co-infections with other strains or *Plasmodium* species protect against morbidity.

Study design and population

Under the auspices of the Institute of Medical Research in Papua New Guinea (PNG IMR) and in collaboration with Case Western Reserve University (USA) a prospective longitudinal survey was conducted in the Maprik district, East Sepik Province, PNG. A cohort of 269 children 12 to 36 months (± 1 month) of age at enrolment was followed-up over 16 months from March 2006 until July 2007. All study participants were recruited from 10 different villages in the vicinity of Ilahita health centre (HC) (Figure 1).
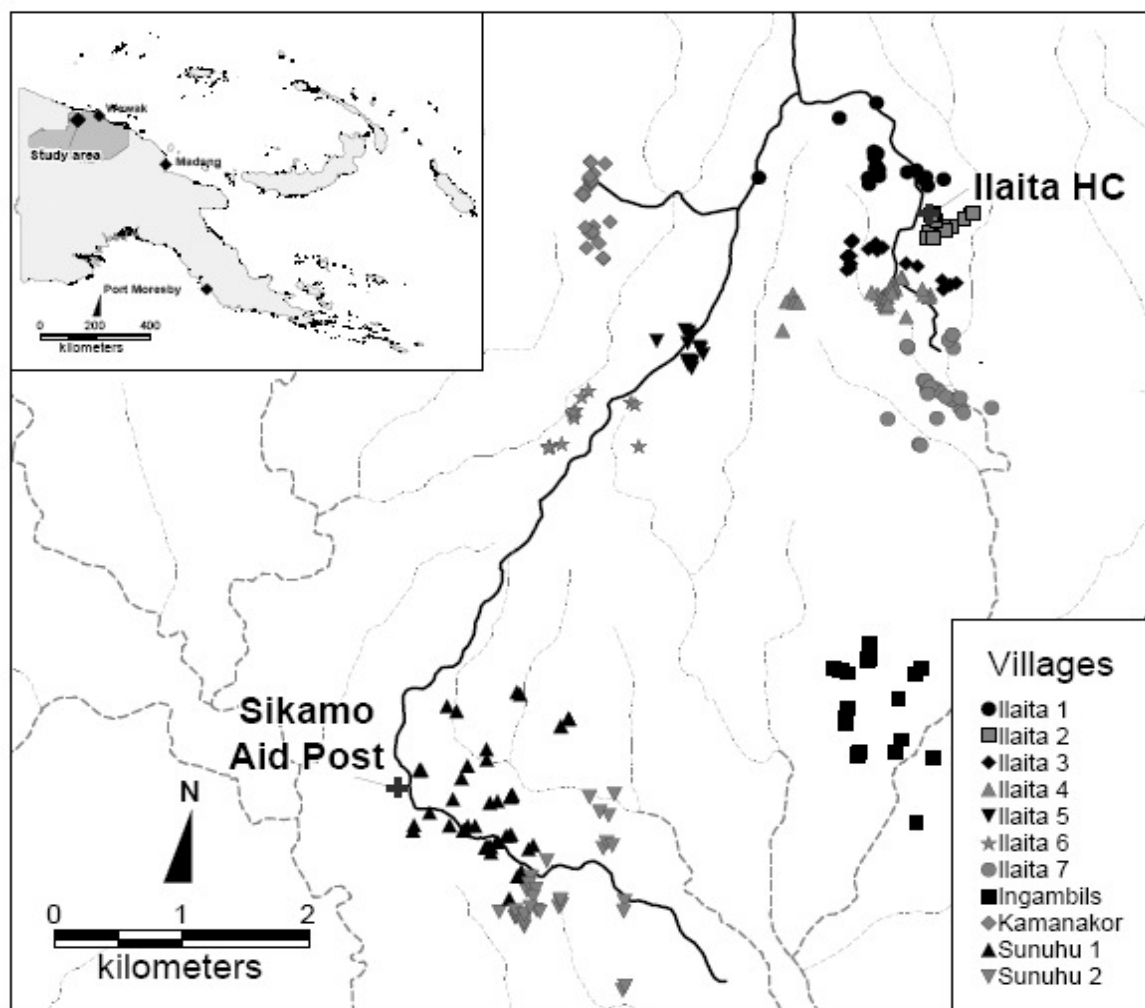
Figure 1: Study location – The Ilahita area, East Sepik Province, Papua New Guinea. This map was kindly provided by Ivo Müller.

Enrolment was conducted during a period of two weeks. A clinical examination was performed for each child after obtaining written informed consent from parents or legal guardians and each child was assessed for inclusion / exclusion criteria. A 5ml venous blood sample was collected and a case report form was completed to document a unique study identification number, demographic information as well as reported history of illness, antimalarial treatments and bednet usage of each child. Since the expected number of children could not be reached during the first 2 weeks of enrolment, further children were still included into the study at later follow-up visits until the number of participants we aimed at was reached.

Study visits	□ ● ● ● ⋀⋀ ● ● ● ⋀⋀ ● ● ● ⋀⋀ ● ● ● ⋀⋀ ● ● ● ⋀⋀ ● ● ● ⋀⋀ ● ● ● ⋀⋀ ● ● ● □
HC surveillance	▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬▬
Venous bleed	✓ ✓
250µl blood (24h apart)	✓ ✓ ✓ ✓ ✓ ✓ ✓
Blood slide (24h apart)	✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓
Hb	✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓
Spleen size*	✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓
Weight	✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓ ✓
Temperature	✓ ✓
Clinical examination	✓ ✓
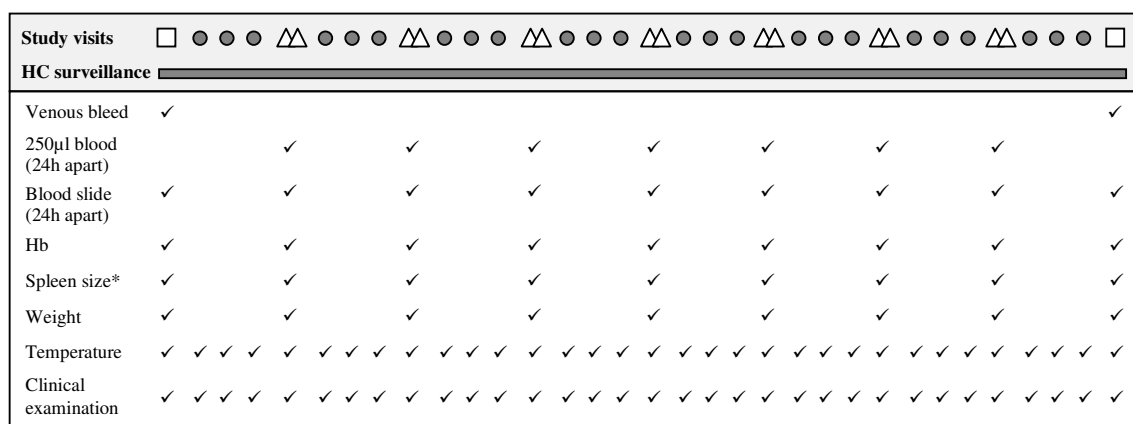
Figure 2: Schematic presentation of the study design

□   Enrolment visit

⋀⋀   Follow-up bleed (FUB) visits on two consecutive days

●   Clinical follow-up visit in two-weekly intervals

HC   health centre

Hb   haemoglobin

* Spleen size was determined according to Hacket's grading.

Active follow-up consisted of biweekly clinical follow-up visits and 8 weekly follow-up bleed (FUB) visits. The design of the study is demonstrated schematically in Figure 2. At the two-weekly morbidity surveillance visits, a short physical examination was performed and the history of symptoms in the preceding 2 weeks was recorded. In case a study participant presented with a presumptive malaria infection, a blood slide and a finger-prick blood sample (250µl) were collected and a rapid diagnostic test (RDT) was performed. Upon positive RDT and/or blood slide, antimalarial treatment with Coartem® (Novartis, Switzerland) was initiated

by a medically trained staff. At the 8-weekly FUB visits the study team collected 2 finger-prick blood samples 24 hours apart and recorded the spleen size and haemoglobin (Hb) level of each child. In case of malaria symptoms and/or a positive RDT or in case of moderate-to-severe anaemia (Hb <7.5 g/dL), antimalarial treatment with Coartam® was initiated. At the final study visit another 5ml venous blood sample was collected. A passive case detection system was set up at the Ilahita Health Centre, where a team nurse was based during the entire period of the study. Whenever a participating child presented at the local HC with a febrile illness, a case report form was completed, a finger-prick blood sample and a blood slide collected, a RDT performed and the Hb level was measured. Similar to the regular follow-up visits, antimalarial treatment was administered upon positive RDT and/or blood slide or in case of moderate-to-severe anaemia.

Ethical considerations

This study was conducted according to the Declaration of Helsinki and in compliance with Good Clinical Practice (ICH GCP E6) regulations and guidelines. The study was approved by the Institutional Review Boards of the PNGIMR (PNG Medical Research Advisory Committee) and Cleveland Hospital IRB and the Ethikkommission beider Basel (EKBB).

Prior to recruitment of study participants, community information meetings were held where the purpose of the study and the detailed study procedures were explained to the village population. Written informed consent was sought from parents or legal guardians prior to first sample collection. Participants were informed about their right to withdraw from the study at any time point. In order to protect confidentiality participant information were identified by a unique study identification number and/or blood sampling code and serial number. Databases were made anonymous to eliminate any variables that allowed identification of individual participants.

Children which were discontinued from the study due to development of chronic illness were offered continuous participation in the biweekly morbidity surveillance visits and medical advice from the study team.

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Thesis outline

The following 5 chapters correspond to individual publications that are already published or will be submitted to the mentioned scientific journals:

Chapter 2: "Heterogeneous distribution of *Plasmodium falciparum* drug resistance haplotypes in subsets of the host population"; Malar J. 2008 May 6;7:78.

Chapter 3: "Comparison of *Plasmodium falciparum* allelic frequency distributions in different endemic settings by high resolution genotyping"; to be submitted to the Malaria Journal.

Chapter 4: "Treatment with Coartem® (artemether-lumefantrine) in Papua New Guinea"; submitted to the American Journal of Tropical Medicine and Hygiene.

Chapter 5: "Estimates of Detectability of *P. falciparum* and Force of Infection based on molecular data"; to be submitted to PLoS ONE.

Chapter 6: "Parasitological risk factors for *Plasmodium falciparum* episodes in Papua New Guinean children"; to be submitted to the Malaria Journal after further revisions.

Annex papers

In the course of this thesis contributions were made to additional publications which are not directly linked to the main part of this thesis.

Malaria Journal

Research

Heterogeneous distribution of *Plasmodium falciparum* drug resistance haplotypes in subsets of the host population

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Abstract

Background: The emergence of drug resistance is a major problem in malaria control. For mathematical modelling of the transmission and spread of drug resistance the determinant parameters need to be identified and measured. The underlying hypothesis is that mutations associated with drug resistance incur fitness costs to the parasite in absence of drug pressure. The distribution of drug resistance haplotypes in different subsets of the host population was investigated. In particular newly acquired haplotypes after radical cure were characterized and compared to haplotypes from persistent infections.

Methods: Mutations associated with antimalarial drug resistance were analysed in parasites from children, adults, and new infections occurring after treatment. Twenty-five known single nucleotide polymorphisms from four *Plasmodium falciparum* genes associated with drug resistance were genotyped by DNA chip technology.

Results: Haplotypes were found to differ between subsets of the host population. A seven-fold mutated haplotype was significantly reduced in adults compared to children and new infections, whereas parasites harbouring fewer mutations were more frequent in adults.

Conclusion: The reduced frequency of highly mutated parasites in chronic infections in adults is likely a result of fitness costs of drug resistance that increases with number of mutations and is responsible for reduced survival of mutant parasites.

Background

The emergence of drug resistance poses a major problem for malaria control. Research has focused mainly on elucidating the mode of action of antimalarial drugs and on the molecular mechanisms leading to drug resistance. The development of drug resistance mostly involves single nucleotide polymorphisms (SNPs) in genes encoding the drug targets, such as metabolic enzymes or transmembrane transporters. Molecular studies have identified a number of SNPs in the *Plasmodium falciparum* multi-drug resistance gene 1 (*pfmdr1*), the chloroquine resistance transporter (*pfcrt*), the dihydrofolate reductase (*pfdhfr*) and the dihydropteroate synthase (*pfdhps*) that were associated with drug resistance against the most commonly

used antimalarials chloroquine (CQ) and the combination of antifolate sulphadoxine/pyrimethamine (SP). Allelic exchange experiments did provide direct evidence for the role of *pfdhfr* in resistance to pyrimethamine [1], *pfdhps* in resistance to sulphadoxine [2] and the role of *pfcrt* in resistance to CQ [3]. Reed *et al* [4] could show that mutations in *pfmdr1* are not essential, but can modulate the level of drug resistance against CQ. Evidence from molecular epidemiological field studies confirmed associations of the mutations K76T [5-7] and N86Y [8-10] with *in vivo* drug resistance against CQ, whereas different combinations of mutations in *dhfr* and *dhps* were associated with *in vivo* drug resistance against SP [for example [11-14]].

A number of mathematical models have been designed to predict the transmission and spread of drug resistance [15-20]. Questions addressed include the role of transmission intensity on the spread of drug resistance or the possibility of a preferential transmission of resistant versus sensitive parasites. The effect of transmission intensity on the spread of drug resistance was discussed controversially [19,21]. A more recent review by Hastings & Watkins [15] proposed that transmission intensity does not have a direct impact on the evolution of drug resistance, but directly determines the dynamics of resistance via "effectors", such as intra-host competition, level of drug use in the population, extent of sexual recombination, proportion of malaria infections treated, or number of parasites in a human host.

Different authors studied the preferential transmission of resistant versus sensitive parasites. It has been proposed that moderately mutated parasites that result in high levels of parasitological failures following SP treatment, have a greater transmission potential than highly mutated parasites, because the latter are more likely to cause clinical failure within a short time after treatment and are therefore more often subject to rescue treatment [22]. A number of field studies have reported increased gametocyte carriage in blood of CQ or SP treated individuals [23-27] or in individuals carrying drug resistant strains [23,25]. However, these findings have been questioned by lacking evidence that parasites post SP-treatment fully develop to infective stages in the mosquito [28].

The field study presented here was conducted in Papua New Guinea (PNG) and investigated the distribution of drug resistance genotypes in subsets of the host population. In particular, the actually transmitted genotypes were characterized. This was possible by following individuals after radical cure treatment. The first *P. falciparum* positive blood samples of these individuals were genotyped for all known markers for drug resistance.

The underlying hypothesis of this study was that new infections following radical cure are not yet subject to major selective constraints, because these newly arriving infections do not have to compete against already persisting infections. Here all transmitted genotypes irrespective of their fitness are expected to be found. In contrast, parasites in asymptomatic chronic infections from community samples have been subject to within-host competition and differential fitness is likely to affect their long-term survival in the host. Asymptomatic individuals are therefore expected to carry a lower prevalence of mutated genotypes as a result of decreasing fitness with increasing number of mutations.

By molecular epidemiological studies in Madang, PNG, detailed information has been provided on the currently high levels of mutation rates in *Pfcrt* K76T (97%), *Pfmdr1* N86Y (96%) and *Pfdhfr* S108N (82%) and C59R (74%)[29]. Another study in PNG observed a significant increase in the *dhfr* double mutant C59R + S108N over a period of two years (83% to 96%). This study was performed in 2002 and 2003, a few years after the introduction of SP as first line treatment in combination with 4-aminoquinolines [30]. They also found an increase in the quadruple mutant *dhfr* C59R + S108N + *dhps* A437G + K540E from 0 % to 8.2% and even though this was not significant, these results suggest that resistance to SP is rapidly developing in PNG. The genotype most frequently found in treatment failures contained a quadruple mutant in *pfcrt* (K76T + N326D + I356L + A220S) in combination with the *pfmdr1* mutant N86Y and the double *pfdhfr* mutant S108N+C59R.

For the present investigation, which aims primarily at multi-loci haplotypes, the PNG field site provides optimal conditions. A haplotype, which is here defined as the genetic make up of an individual parasite clone at 25 loci from four marker genes of drug resistance, can be directly deduced after genotyping a single clone infection. As multiclonal infections are rare in PNG, with a mean multiplicity of infection (MOI) between 1.3 and 1.8 [31-33], the high prevalence of single-clone infections greatly facilitated the study of drug resistance haplotypes.

Materials and methods

The present study made use of two sets of blood samples: (i) cross-sectional surveys including Papua New Guinean individuals of all ages, and (ii) first *P. falciparum* positive samples from a follow up after radical cure with artesunate in five to 14 year-old children. Both sets of samples were collected in the same villages in PNG, in the years 2003 – 2005.

In the treatment to reinfection study (TRS) in 2004/2005 [for details see [34]] a total of 206 children from five to 14

years of age were enrolled at the Mugil and Megiar elementary schools which are situated about 50 km north of Madang town, PNG. After obtaining written informed consent from the parents or guardians each child was clinically examined, two blood slides (thick and thin films) were prepared for microscopical determination of malaria infections and a venous blood sample was collected at baseline. Subsequently, all children were treated with a seven-day course of artesunate monotherapy according to PNG national treatment guidelines (i.e. 4 mg/kg at day 1, 2 mg at days 2–7). After treatment, two-weekly active follow ups were conducted at the schools to check for new malaria infections and presence of febrile illness. Therefore, each child was clinically assessed, a rapid diagnostic test (RDT) (ICT Diagnostics, South Africa) was performed, blood slides were prepared and 250 µl of blood were collected by finger prick every two weeks.

Positive samples were identified by microscopy and LDR-FMA [35] as described in more detail in Michon *et al* [34]. All baseline and first PCR positive samples after treatment were genotyped for the highly polymorphic marker gene merozoite surface protein 2 (*msp2*) and compared by PCR-RFLP [36,37] in order to distinguish new from recrudescent infections. Samples that were typed as recrudescent infection, but were collected as late as 10 weeks or more after the baseline survey, were additionally genotyped for a second marker gene (*msp1*) [38], because recrudescence seemed to be unlikely after an interval of this length.

In the same villages two household-based cross sectional surveys were conducted in 2003 and 2004 which included participants of all age groups. Upon receiving informed consent a questionnaire was completed for each participant, blood slides prepared for microscopical examination and a venous blood samples was collected for further laboratory analysis. All samples were *msp2*-genotyped using PCR-RFLP as described above to determine the multiplicity of infection (MOI).

All samples that were determined to be single or double infections by *msp2* genotyping were further analysed for mutations in drug resistance genes by DNA chip technology [39]. This method allows parallel identification of 25 single nucleotide polymorphisms (SNPs) that were found to be associated with drug resistance against a number of different antimalarial drugs. It is based on PCR amplification of target sequences within the genes *Pfmdr1*, *Pfcrt*, *Pfdhfr* and *Pfdhps*. A primer extension reaction with fluorescent labelled ddNTPs follows this PCR step. The extended primers are subsequently hybridized on a microarray carrying the antisense DNA of the extension primers and scanned at different wavelength using an Axon 4100A fluorescent scanner to determine the incorporated ddNTP. Pictures were acquired and analysed using the Axon GenePix® Pro (version 6.0) software. The codons investigated with this method include N86Y, Y184F, S1034C, N1042D and D1246Y in *Pfmdr1*, the codons K76T, H97Q, T152A, S163R, A220S, Q271E, N326D/S, I356L/T and R371I in *Pfcrt*, the codons A16V, N51I, C59R, S108N/T and I164L in *Pfdhfr* and the codons S436A, A437G, K540E, A581G, I640F and H645P in *Pfdhps*.

The dataset for statistical analysis consisted of all cross-sectional samples that had been genotyped as single-clone infections and all new infections (irrespective of malaria symptoms) plus baseline samples with MOI = 1 from TRS study. Samples with MOI = 2 were taken into consideration, if it was possible to unequivocally determine the haplotype of these samples, i.e. samples showing a mixed infection for more than two loci were not included. Samples from the cross sectional surveys were grouped into adults (> 14 years of age) and children five -14 years of age. All individuals from the household surveys who had received any antimalarial treatment during the last two months prior to the survey were excluded.

For simplified presentation of haplotypes, only SNPs of which the mutated alleles were actually detected in this study area are itemized in the haplotype descriptions, whereas SNPs found only in the wild type form are not listed. Thus, the presented haplotype provides molecular typing information on alleles at the following 11 positions: *dhfr*59, *dhfr*108, *dhps*437, *dhps*540, *mdr*86, *mdr*184, *mdr*1042, *crt*76, *crt*220, *crt*326 and *crt*356. Only samples with a complete set of these 11 polymorphic SNPs were taken into consideration.

To compare the haplotype frequencies between datasets logistic regression statistics was applied.

Results

Blood samples from radically cured individuals were genotyped for *msp2* in order to distinguish new infections from recrudescent ones and to determine multiplicity of infection (MOI). All new infections with MOI = 1 or 2 were analysed by DNA Chip to identify SNPs in genes associated with drug resistance. In addition, genotyping on Chip was performed for all single and double infections found in baseline samples of these individuals prior to treatment. Complete haplotype data were obtained for 144 new infections and 109 baseline samples.

From cross sectional surveys, 61 samples with a complete haplotype were grouped as adults > 14 years and 63 samples derived from children aged five-14 years. Individuals that were treated with antimalarial drugs in the two months prior to the survey were excluded.

Genotyping was performed using DNA Chip technology [39]. Among the 25 analysed SNPs in the four different genes *Pfmdr*, *Pfcrt*, *Pfdhfr* and *Pfdhps* some occurred only as the wild type allele, whereas some mutant alleles had already reached fixation at this field site. For 8 SNPs both the wild type and mutant allele was detected in the samples set.

At this study site a total of 13 different haplotypes were found, all listed in table 1. The most frequent haplotype in all subsets of the host population was the 7-fold mutated haplotype '*crt*76T-*crt*356L-*crt*326D-*crt*220S-*dhfr*59R-*dhfr*108N-*mdr*86Y'. The wild type allele was fixed at the remaining 4 polymorphic codons.

In the samples set, a number of haplotypes occurred at very low frequency (one or two observations). The detection of rare haplotypes depends on sample size and presence or absence of these haplotypes in a population comparison is likely due to chance.

Effect of host age on haplotype frequency

Haplotype frequencies in children and adults of the cross sectional surveys were compared in order to test for age-specific effects. The 7-fold mutated haplotype '*crt*76T-*crt*356L-*crt*326D-*crt*220S-*dhfr*59R-*dhfr*108N-*mdr*86Y' was the most frequent haplotype in adults and children with a prevalence of 65.57 % and 84.13 %, respectively (Table 1). The frequency of this haplotype was significantly lower in adults compared to children by more than 20% (OR =

2.78, p = 0.02) (Figure 1). In addition to the seven-fold mutated haplotype, seven additional haplotypes were found in adults, most of them occurring at very low frequency with the exception of the 6-fold mutant '*crt*76T-*crt*356L-*crt*326D-*dhfr*59R-*dhfr*108N-*mdr*86Y' which showed an increased frequency in adults compared to children (13.11% vs. 6.35%). This difference was not statistically significant due to the very few observations.

Haplotypes in new infections

Since new infections best reflect the haplotype frequencies that are actually transmitted, first infections after radical cure with artesunate monotherapy were genotyped. By genotyping the highly polymorphic *msp1* and *msp2* locus all recrudescent parasites due to treatment failure were excluded. Baseline samples prior to radical cure which had a single or double-clone infection were also genotyped. Figure 2 shows that haplotype frequencies agreed well in baseline samples and new infections, with the exception of some rare types which are probably fluctuating randomly. In addition, haplotype frequencies from age-matched cross sectional samples are indicated and also show good agreement with frequencies found in new infections. The fact that haplotypes in new infections appearing after radical cure, which likely reflect the transmitted parasite population, do not differ from haplotype frequencies in age matched children, suggests a high rate of clone acquisition in children. The concordance between baseline samples and samples from age-matched children proves that the data sets from the cross sectional

Table 1: Frequency of haplotypes in different subsets of the host population

Haplotype[1]	Adults cross section[2] (n = 61)		Children cross section[2] (n = 63)		TRS[3]baseline samples (n = 109)		TRS[3]new infections (n = 144)		adults vs. children			adults vs. new infections		
	n	%	n	%	n	%	n	%	OR	p^4	95% CI	OR	p^4	95% CI
crt76 crt326 crt356	0	0	0	0	0	0	1	0.69	-	-	-	-	-	-
crt76 crt326 crt356 crt220	1	1.64	1	1.59	0	0	0	0	0.97	0.98	0.06 – 15.83	-	-	-
crt76 crt326 crt356 dhfr108 dhfr59	0	0	0	0	3	2.75	0	0	-	-	-	-	-	-
crt76 crt326 crt356 crt220 dhps540	0	0	1	1.59			0	0	-	-	-	-	-	-
crt76 crt326 crt356 crt220 mdr86	4	6.56	2	3.17	0	0	1	0.69	0.47	0.39	0.08 – 2.65	**0.1**	**0.04**	0.01 – 0.91
crt76 crt326 crt356 crt220 dhfr108 dhfr59	3	4.92	0	0	4	3.67	1	0.69	-	-	-	0.14	0.09	0.01 – 1.33
crt76 crt326 crt356 dhfr108 dhfr59 mdr86	8	13.11	4	6.35	15	13.76	8	5.56	0.45	0.21	0.13 – 1.58	0.39	0.07	0.14 – 1.09
crt76 crt326 crt356 crt220 dhfr108 mdr86	1	1.64	2	3.17	2	1.83	2	1.39	1.97	0.59	0.17 – 22.27	0.85	0.89	0.75 – 9.5
crt76 crt326 crt356 dhfr108 dhfr59 mdr86 dhps540	0	0	0	0	0	0	1	0.69	-	-	-	-	-	-
crt76 crt326 crt356 crt220 dhfr108 dhfr59 mdr86	40	65.57	53	84.13	85	77.98	126	87.5	**2.78**	**0.02**	1.18 – 6.56	**3.68**	**0.001**	1.78 – 7.57
crt76 crt326 crt356 crt220 dhfr108 dhfr59 mdr86 dhps540	2	3.28	0	0	0	0	4	2.78	-	-	-	0.84	0.85	0.15 – 4.73
crt76 crt326 crt356 crt220 dhfr108 dhfr59 mdr1042 mdr184	1	1.64	0	0	0	0	0	0	-	-	-	-	-	-
crt76 crt326 crt356 crt220 dhfr108 dhfr59 mdr86 dhps437	1	1.64	0	0	0	0	0	0	-	-	-	-	-	-

[1] Haplotypes represent 11 single nucleotide polymorphisms (SNPs) in 4 different genes. The remaining 14 SNPs tested but not listed equal to wildtype.
[2] Cross-sectional samples include only individuals that have not been treated with antimalarial drugs in the 2 months prior to the survey.
[3] TRS, treatment to reinfection study
[4] Bold indicates p < 0.05
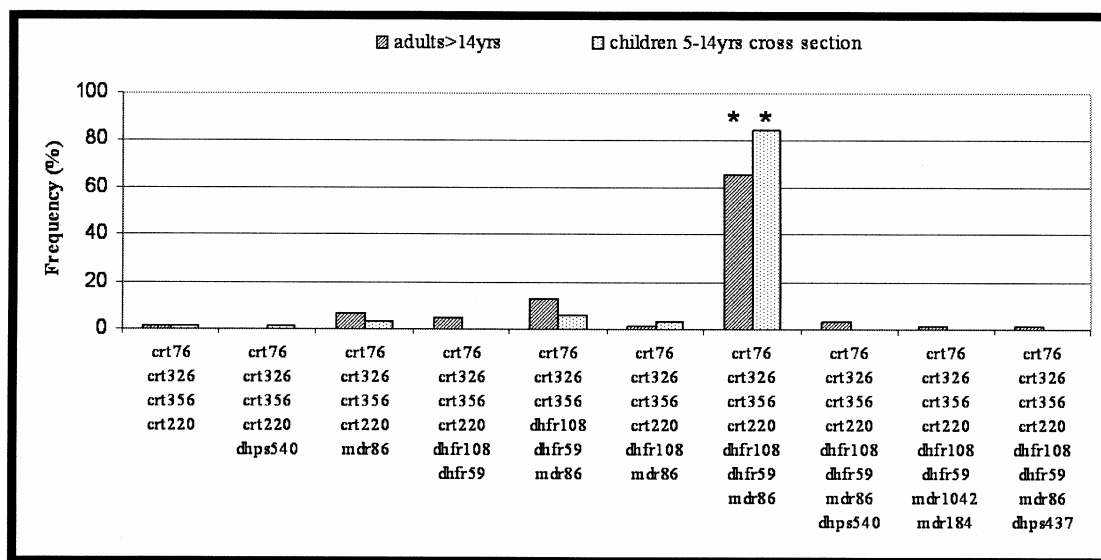
Figure 1
Comparison of haplotypes between adults and children of the cross-sectional surveys. * indicates significant difference of haplotype frequency between the two compared groups.

survey and from the radical cure are comparable and unbiased. In all three subsets of the host population from Figure 2, the frequency of the dominant haplotype agreed well and showed no statistically significant difference.

New infections versus persisting infections in adults
In order to determine whether all haplotypes persist in the host equally well, the haplotype distribution in newly arriving infections in relation to persistent parasites from long term infections were compared. Previously, it was
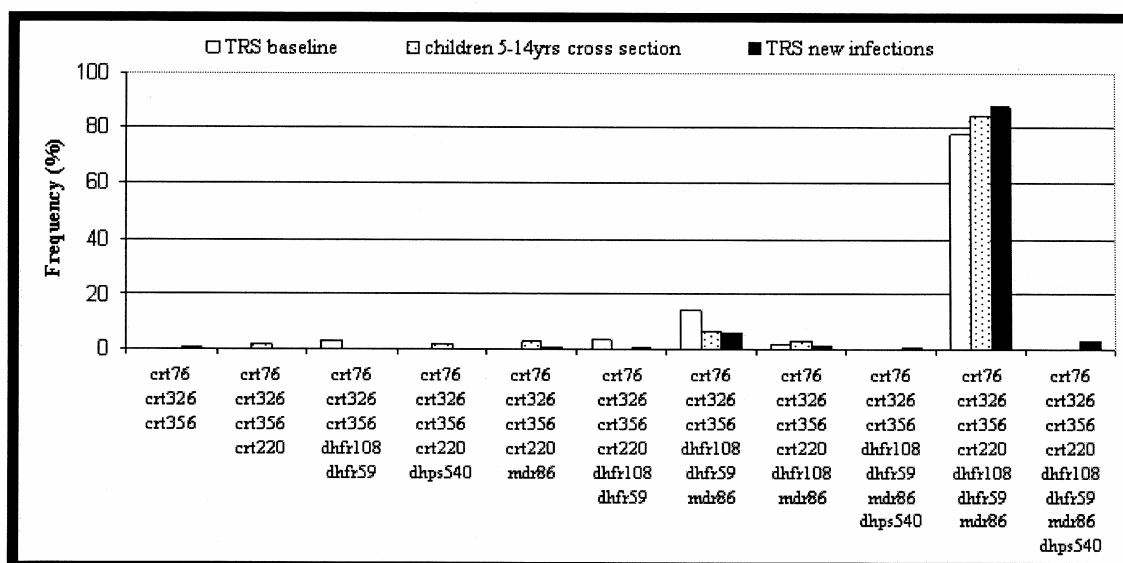
Figure 2
Comparison of haplotypes between children of the cross sectional surveys and baseline samples and new infections from the treatment to reinfection study (TRS). There are no significant differences in haplotype frequencies between groups.

shown by genotyping *P. falciparum* clones from a longitudinal study that individual parasite clones can persist over very long time in a semi immune host at very low densities fluctuating around the detection level [40,41]. To select long term persistent infections from this data set, infections from adults > 14 years were chosen (mean age = 31 years; SD = 13.67). To avoid any influence of previous drug intake on the prevalence of certain haplotypes, all patients treated with antimalarials two months prior to sampling were omitted from the analysis.

Figure 3 compares new infections and persisting infections in adults. A significantly higher number of sevenfold mutated haplotype was found in new infections compared to frequencies in adults with a difference between the groups of 21.92% (OR = 3.68, p < 0.001; Table 1). In contrast, the frequency of the five-fold mutated haplotype '*crt*76T-*crt*356L-*crt*326D-*crt*220S-*mdr*86Y' was significantly lower in new infections (OR = 0.1, p = 0.04; Table 1). The frequency of 6-fold mutant '*crt*76T-*crt*356L-*crt*326D-*dhfr*59R-*dhfr*108N-*mdr*86Y' was also reduced in new infections, although this difference did not reach statistical significance. This suggests for adults a higher clearance rate for clones carrying seven-fold mutations and as a consequence, accumulation of five- and six-fold mutations.

Discussion

The most frequent haplotype in the study area showed the following seven point mutations: *crt*76T *crt*326D *crt*356L *crt*220S *dhfr*59R *dhfr*108N *mdr*86Y. The high frequencies of these mutations were concordant with findings from previous studies in PNG [29,30]. Frequent mutations in *mdr*1 and *crt* reflect the long history of 4-aminoquinoline usage in the country and the high levels of resistance against these drugs [42]. High frequencies of *dhfr* mutations suggest that resistance to pyrimethamine is common and sulfadoxine, as indicated by the low level of mutations in *dhps*, is probably the only effective component in the locally used first line treatment combination of CQ or Amodiaquine with SP.

The study presented here demonstrated different haplotype frequencies in subsets of the host population. Children five to 14 years of age harboured more seven-fold mutated haplotypes compared to adults. The same frequency of this haplotype was observed in new infections as in children. Assuming that new infections, as they were observed after radical cure, reflect the actually transmitted haplotypes via the Anopheline vector, these findings suggest three interpretations: (i) transmission of highly mutated and therefore probably more resistant haplotypes is modulated by drug pressure or other environmental factors so far undetected in this study; (ii) transmission of drug resistance markers occurs in an age-dependent mode by which mutant parasites accumulate in children;

Figure 3
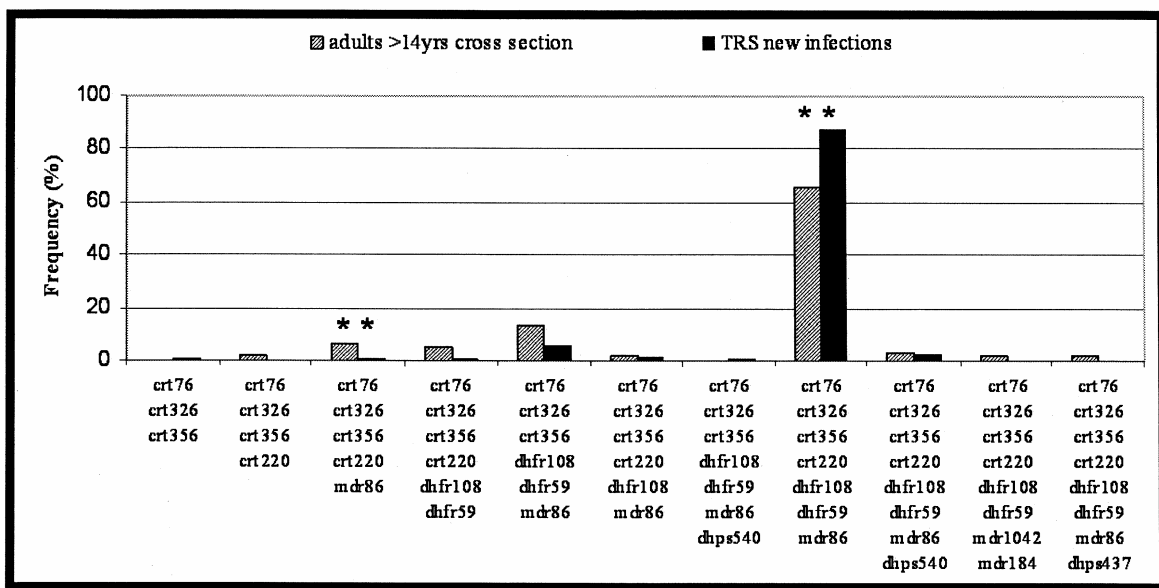Comparison of haplotypes between adults (cross section) and new infections of the treatment to reinfection study. * indicates significant difference of haplotype frequency between the two compared groups.

or (iii), the parasite population found in children differs intrinsically from that in adults.

The first possible interpretation of the present findings implies that previous treatment effects the transmission of particular haplotypes. This is supported by the finding of higher prevalence and density of gametocytes following treatment with SP or CQ which was reported by a number of different authors [24-27], but is contradicted by a report from Dunyo *et al* [43] who could not find such an effect of SP treatment on subsequent transmission gametocyte carriage or density. In this study an altered transmission potential as a consequence to antimalarial treatment [44] was ruled out by excluding all individuals that had received antimalarial treatment two months prior to blood collection. Thus, effects of SP which is known to have a long elimination half life (4–9 days for Sulfadoxine and ca. 4 days for pyrimethamine) [45] should have waned. However, long lasting effects of SP treatment on transmission need to be further investigated.

An alternative explanation of the present findings implies that children and adults differ in their infectivity to mosquitoes. The age effect on transmission has been investigated by Graves and colleagues [46] in Madang, PNG, who performed direct mosquito feeding experiments on human blood. They found that the 1–20 year old individuals are more infectious to the mosquitoes than older age groups. However, a mathematical model developed by Ross *et al* [47] proposed that also infected adults are likely to make a substantial contribution to the infectious reservoir. The concordance of haplotype frequencies between new infections and children but not adults could suggest that children contribute more to transmission than adults in our study area. But such differential infectivity to mosquitoes is not the only explanation for the fact that frequencies in new infections do not differ from frequencies in age matched children. This could also be due to high turnover of infecting parasite clones in children. If in a particular age group clone acquisition rate is high, radically cured as well as non-cured individuals will all have predominantly recent infection and as a consequence will share the same haplotype frequencies of drug resistance markers.

As third possible explanation for heterogeneity in haplotype distribution other malariological parameters or host factors that have the potential to determine survival of the haplotypes in the host have to be considered. For example, *P. falciparum* infections in children differ from those in adults by a higher mean number of multiple infections [40,48,49] and higher parasite densities [49,50]. A significantly reduced parasite density has been associated with resistance patterns [51]. In the data presented here, a reduced parasite density in mutant samples compared to wild type could not be confirmed. Also the densities of the two most common haplotypes did not differ between adults and children (data not shown). As further determinant of parasite survival in the host, some authors have proposed that mutations associated with drug resistance will incur fitness costs to the parasite in absence of drug pressure [51-55]. Since parasite fitness cannot be measured directly, a surrogate marker for fitness is required. The parameter "persistence of a clonal infection", measured as duration of an infection in a given host, would serve this purpose and can be measured experimentally in a longitudinal set of samples. This leads to the speculation that long-term persistence of a clonal infection in a host indicates better survival and thus could be used as a surrogate marker for parasite fitness.

Such fitness costs of drug resistance mutations can obviously only be studied in the absence of treatment. The effect of previous treatment on infections of particular haplotypes was ruled out in this dataset by excluding all individuals that had received antimalarial treatment 2 months prior to blood collection.

A recent paper of Ord and colleagues [56] reported that the prevalence of two mutations associated with chloroquine resistance declined during the dry season. The authors suggest fitness costs of drug resistance to be responsible for reduced survival of mutant parasites. A similar seasonal fluctuation has been suggested for Sudan [57]. These findings from a longitudinal study in a seasonal setting are perfectly in line with our findings from an area of perennial transmission where a higher frequency of mutated haplotypes was detected in new infections as compared to long lasting chronic infections.

In malaria endemic areas where transmission is perennial, most adults carry asymptomatic infections. These infections largely remain untreated and reflect chronic infections that persist over long periods of time (about 150 days) [40]. In case of long-term survival within a host, less mutated parasites would be expected to be more frequent due to their higher fitness. The present observation of a reduced frequency of the seven-fold mutant in adults compared to new infections is in support of reduced fitness of this particular haplotype. The opposite is true for less mutated haplotypes with increased prevalence in adults who frequently carry chronic subpatent parasitaemia.

When looking at each SNP separately, it was found that the frequency of the four most prevalent mutant and non-fixed SNPs (*crt*220S, *dhfr*59R, *dhfr*108N and *mdr*86Y) was lower in chronic infections than in children or new infections. This also supports the hypothesis that infections harbouring fewer point mutations are fitter and can there-

fore persist in the absence of drug pressure. The frequency of other SNPs was very low and does not allow for any interpretation.

The host's acquired immunity is an important parameter that needs to be considered when using the persistence of infection as a measure of parasite fitness. Immunity is a major determinant of duration of infection. However, the hosts acquired immunity is unlikely linked with a specific drug resistance haplotype as lack of linkage disequilibrium and sufficient outbreeding has been shown previously for the parasite population in the study area [33]. Therefore the immune response is expected to act on parasites irrespective of their number of drug resistance mutations.

Fitness costs of drug resistance is one important parameter for mathematical models that remains to be quantified in order to make more precise predictions on the spread of drug resistance. If point mutations incur fitness costs to the parasite in the absence of drug pressure, natural selection might lead to a decline in the prevalence of these mutations once the use of a specific drug is abolished. This might then result in the drug becoming efficacious again as has been observed in Malawi in the 12 years since CQ was removed from standard treatment [58]. Longitudinal studies are needed in order to estimate and quantify the reduction in survival of mutated versus wildtype genotypes in the host. This could provide more precise fitness measurements for parasites harbouring point mutations that are associated with drug resistance.

Authors' contributions
SS carried out the molecular genetic work and the statistical analysis. JM carried out the field survey and participated in the molecular genetic work. MG and MB carried out field work. IM was responsible for the treatment to reinfection study and participated in data analysis. IF was responsible for the study design and contributed to data analysis. All authors contributed to writing the manuscript.

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CHAPTER 3

Comparison of *Plasmodium falciparum* allelic frequency distributions in different endemic settings by high resolution genotyping

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ABSTRACT

Diversity and complexity of the merozoite surface proteins 1 and 2 (*msp1* and *msp2*) of *P. falciparum* infections were investigated in different malaria transmission settings. We applied a high resolution capillary electrophoresis based technique to genotype samples from Papua New Guinea and Tanzania. We compared the results to previous work based on low resolution genotyping of PCR fragments on agarose gels to investigate whether more accurate sizing generates different results. Our results confirmed previous reports of a higher mean multiplicity of infection for both marker genes and increased genetic diversity as estimated by the total number of distinct alleles for *msp2* in areas of higher endemicity. For *msp1* alone a minor increase in diversity was observed. Measures of between population variance in allele frequencies (F_{ST}) indicated little genetic differentiation for both marker genes between the two populations in different endemic settings. The mean multiplicity of infection (MOI) adjusted for the probability of multiple infections sharing the same allele was estimated by using the *msp2* allele frequency distribution and the distribution of observed numbers of infections. For the high resolution typing technique applied in this study, this adjustment made little difference to the estimated mean MOI compared to the observed mean MOI.

INTRODUCTION

Plasmodium falciparum populations are highly diverse and individual hosts are often simultaneously infected by multiple parasite clones. In order to discriminate parasite clones within one host PCR-based genotyping of a number of different marker genes has been established. Among these are the merozoite surface proteins 1 and 2 (*msp1* and *msp2*), and the glutamate-rich protein (*glurp*), which show extensive length polymorphism and can therefore be distinguished by electrophoresis on agarose or polyacrylamide gels.

Recently, capillary electrophoresis (CE) based genotyping of *msp2* was shown to have a much higher discriminatory power than previously applied techniques (Falk et al. 2006). This technique has been proven useful in areas of high endemicity where individuals are simultaneously infected with many different parasite clones. It has also been useful for distinguishing recrudescent from new infections after antimalarial treatment (Jafari et al. 2004) and in tracking individual parasite clones over time in longitudinal surveys, where consecutive blood samples were collected from each individual (Falk et al. 2006). Similar to the CE based *msp2* genotyping, we have applied a CE protocol for genotyping the polymorphic block 2 of the *msp1*gene

In this study we investigated the impact of transmission intensity on the genetic diversity of the two marker genes *msp1* and *msp2*. Most previous studies have shown a greater diversity for *msp2* in high endemic settings (e.g. Babiker et al. 1997;Hoffmann et al. 2001;Konate et al. 1999;Paul & Day 1998), but for *msp1* conflicting results were reported (e.g. Bendixen et al. 2001;da Silveira et al. 1999;Ferreira et al. 1998a;Ferreira et al. 1998b;Kaneko et al. 1997;Konate et al. 1999;Paul & Day 1998;Silva et al. 2000).

These data all relied on low resolution genotyping consisting of fragment sizing on agarose gels with individual genotypes defined as bins of ≥ 20 base pairs. Using this approach several distinct fragments may be combined within one genotype bin. Greenhouse et al. (2007) studied the impact of transmission intensity on the accuracy of genotyping and found that with increasing transmission intensity and therefore increasing complexity of infection, genotyping becomes less accurate. These finding are not only important for recrudescent-reinfection typing in antimalarial drug trials but also for assessing the impact of transmission intensity on genetic diversity. The authors concluded that a genotyping technique with higher discriminatory power is needed for genotyping samples from areas of high transmission intensity. We have now applied an improved high resolution genotyping approach to *P.*

falciparum infected samples from Papua New Guinea (PNG) and Tanzania (TZ). These study sites differ extensively in their transmission intensity as previously shown by Arnot et al. (1998). We sought to assess whether the previously observed difference in *msp1* and *mps2* diversity in different endemic settings is independent of technical approaches or whether more sensitive detection of allelic size differences would alter these previous results. For direct standardized comparison the same genotyping technique was applied for all samples.

METHODS

The *msp1* and *msp2* loci were subject to high resolution genotyping in 2 sets of DNA samples that had been PCR positive in previous tests. 108 samples from PNG were derived from a longitudinal field survey in 1-4 year old children conducted in an area near Maprik, East Sepik Province (Lin et al., in preparation). Scientific approval and ethical clearance for the study was obtained from the Medical Research and Advisory Committee (MRAC) of the Ministry of Health in PNG and from the Ethikkommission beider Basel in Switzerland. Informed consent was sought from all parents or guardians prior to recruitment of each child. 115 Tanzanian samples were derived from the placebo group of a vaccine efficacy trial conducted from 1993-1994 in children <5 years of age in the village Idete, which is located in the Kilombero District of Morogoro Region, Tanzania (Alonso et al. 1994;Beck et al. 1997)

Prior to PCR amplification of the *msp1* and *msp2* target sequence, DNA was extracted from cell pellets using QIAamp® 96 DNA Blood Kit (Qiagen, Australia) according to the manufacturer's instructions. *Msp2* genotyping was performed as previously described by Falk et al. (2006) with some minor changes and adaptations of PCR conditions for highly purified DNA. In brief, primary PCR reaction conditions were adjusted to a final volume of 50µl including 2µl of extracted DNA. Cycle conditions for primary PCR were 2 min at 94°C followed by 25 cycles of 30 s at 94°C, 45 s at 45°C, 90 s at 70°C, and a final extension at 70°C for 10min. In order to reduce the carry over of primary PCR primers into the nested PCR, only one µl of primary PCR product was amplified in the nested PCR reaction with the following cycle conditions: 2 min at 94°C followed by 25 cycles of 30 s at 94°C, 45 s at 50°C, 90 s at 70°C and a final extension at 70°C for 10 min.

Msp1 occurs as one of three distinct allelic families: K1, MAD20 and RO33. The unique family specific sequences K1 and MAD20 flank intragenic repeat units that give rise to extensive size polymorphisms, whereas RO33 is not polymorphic. For amplification of the polymorphic region of *msp1* block 2 (Miller et al. 1993), a nested PCR approach was used. Primary PCR was performed in a total volume of 50µl containing 5µl of 10xBufferB (0.8M Tris-HCl, 0.2M $(NH_4)_2SO_4$, 0.2% w/v Tween-20), 2mM $MgCl_2$, 200µM dNTPs, 2.5U Taq DNA polymerase (FirePol, Solis BioDyne). Primary PCR primers which are located in the conserved sequence spanning the *msp1* block2 (M1-OF 5'-CTAGAAGCTTTAGAAGATGCAGTATTG-3' and M1-OR 5'-

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CTTAAATAGATTCTAATTCAAGTGGATCA-3' (Snounou et al. 1999)) were used at a final concentration of 300nM each. Two µl of DNA was used as template for this PCR reaction. An initial denaturation step of 94°C for 2 min was followed by 30 amplification cycles of 30 s at 94°C, 1 min at 54°C, 1 min at 72°C and a final extension for 5 min at 72°C.

In the nested PCR reaction specific primer pairs were used to amplify the allelic families K1, MAD20 and RO33 of *msp1* block2. In order to distinguish the size of PCR products by capillary electrophoresis, one of the primers for each PCR was labelled with the fluorescent dyes VIC, NED or 6-FAM, respectively (Applied Biosystems). Size variations due to sporadic addition of adenine by the Taq polymerase at the 3' end were avoided by adding a 7bp tail (Applied Biosystems) to the 5' end of the other primer promoting the additional adenine incorporation. Primer sequences for nPCR have previously been published (Snounou et al. 1999), but have been modified by fluorescent dyes and 7bp tails: M1-KF 5'-Tail-AAATGAAGAAGAAATTACTACAAAAGGTGC-3'; M1-KR 5'-NED-GCTTGCATCAGCTGGAGGGCTTGCACCAGA-3'; M1-MF 5'-Tail-AAATGAAGGAACAAGTGGAACAGCTGTTAC-3'; M1-MR 5'-6-FAM-ATCTGAAGGATTTGTACGTCTTGAATTACC-3'. K1 and MAD20 allelic sequences were amplified in a duplex nPCR in a total volume of 50µl, containing a final primer concentration of 100nM for each primer, 5µl of 10xBufferB (0.8mM Tris-HCl, 0.2M $(NH_4)_2SO_4$, 0.2% w/v Tween-29), 2mM $MgCl_2$, 200µM dNTPs and1.5U Taq DNA polymerase (FirePol, Solis BioDyne). 1µl of primary PCR product was used as template for the nested PCR with the following conditions: initial denaturation for 2 min at 94°C followed by 35 cycles of 30 s at 94°C, 1 min at 59°C, 1 min at 72°C and a final extension for 10 min at 72°C. RO33 allelic sequences were amplified with primers M1-RF 5' VIC-TAAAGGATGGAGCAAATACTCAAGTTGTTG-3' and the reverse primer M1-R2 5' Tail-CAAGTAATTTTGAACTCTATGTTTTAAATCAGCGTA-3' which is located in the conserved region of *msp1* block3 and is not family specific. Therefore, RO33 nested PCR was run as a separate reaction under the same conditions as described for K1 and MAD20 nested PCR. All amplifications were performed on a PTC-100 thermocycler (MJ Research Inc.).

Nested PCR products were analysed on a 1.5% agarose gel. Depending on the intensity of the band, PCR products were diluted 1:5 – 1:40 in H_2O. 2.5µl of diluted PCR product was mixed with 0.3 µl GeneScan®-500 LIZ® size standard (Applied Biosystems) and 12µl HiDi (highly deionized) formamide. The mixture was heated for 5 min at 95°C to separate the double strands and then immediately chilled on ice for a few minutes before capillary electrophoresis was performed on an AB3130 Sequencer (Applied Biosystems).

Data were analysed using the GeneMapper[®] v3.7 Software (Applied Biosystems). The results of size calling were exported as a tab delimited file and imported into an in-house generated software which calculated for each sample a cut-off based on the mean height of the size standard peaks and grouped all alleles into 3bp bins. For the cut-off, an empirically defined constant factor was multiplied by the mean size standard for each sample. This constant factor was manually defined after inspecting the background level in single infections. For both marker genes the total number of alleles as well as their frequency distributions were analysed. The theoretical probability of being infected by two parasites with the same allele was calculated as $P=\sum p_i^2$ where p_i is the frequency of allele i (Gatton & Cheng 2008). The combined probability that two independent clones share the same genotype for both marker genes was calculated by multiplying the probabilities P for both marker genes. As a measure for genetic diversity, the expected heterozygosity was calculated by use of the formula $H_E=[n/(n-1)] \times [(1-\sum p_i^2)]$ (Nei 1987), where n is the number of samples and p_i the frequency of allele i. H_E is the probability that two alleles randomly drawn from the population sample are different. The mean multiplicity of infection (MOI) was calculated as the total number of clones divided by the number of positive samples for each marker gene. Allele frequencies were further compared between populations from PNG and TZ using Wright's F statistics to calculate the fixation index F_{ST}. F_{ST} is a measure of between population variance and gives the proportion of overall diversity which is attributable to differences between populations (Nei & Chesser 1983). H_E and F_{ST} values were calculated by Arlequin ver3.1 software (Excoffier, Laval, & Schneider 2005).

We estimated both the frequency distribution and the mean MOI adjusted for the probability of multiple infections sharing the same allele using the *msp2* allele frequency distribution and the distribution of observed numbers of infections according to the method of Ross et al (in preparation).

RESULTS AND DISCUSSION

The distribution of *msp1* and *msp2* allele frequencies for 108 Papua New Guinean samples is shown in Figure 1. The total number of alleles detected in this sample set is greater for *msp2* than *msp1*, with 35 vs. 24 differently sized alleles, respectively. The overall genetic diversity is also slightly greater for *msp2* than *msp1* (P=0.07; H_E=0.933 for *msp2* and P=0.084; H_E=0.918 for *msp1*). By combining the two marker genes, the probability that two samples share the same genotype by chance can be reduced to 0.0058. Figure 2 shows the allelic frequency distribution of *msp1* and *msp2* in 115 samples from Tanzania. In the Tanzanian samples, the total number of *msp2* alleles detected by CE was much greater than for *msp1* with 76 vs. 29 alleles discriminated, respectively. The probability P of being infected by two parasites with the same allele and the expected heterozygosity values H_E showed a greater overall diversity for *msp2* compared to *msp1* (P=0.036; H_E=0.965 for *msp2* and P=0.085; H_E=0.917 for *msp1*) in Tanzanian samples. Combining the two marker genes reduced the probability that two samples share the same genotype by chance to 0.003.

In both countries, *msp1* K1 type alleles were the most polymorphic, followed by MAD20 type alleles, whereas RO33 was non-polymorphic. For *msp2*, alleles belonging to the 3D7 family showed much more polymorphism than Fc27 type alleles, in both countries (Table 2). For *msp1*, the most dominant alleles were similar between PNG and TZ. For *msp2* the most frequent allele was the same between the two sites whereas the remaining alleles showed a different order of frequency. We compared the variance in allele frequencies between the two study sites using Wright's F statistics. The F_{ST} values for pairwise comparisons of allele frequencies between population samples were 0.041 for *msp1* and 0.017 for *msp2*, respectively (p<0.001 for both markers). These low F_{ST} values indicate that allele frequencies are highly similar and that there is little genetic differentiation between the investigated geographic populations for both marker genes. As previously suggested, such low F_{ST} values may indicate that the *msp1* and *msp2* loci are under balancing selection (Conway 1997), increasing the possibility that observed patterns of allele frequencies are the result of balancing selection rather than of variation in transmission dynamics.

The quality of genotyping, i.e. optimal differentiation of parasite clones within a blood sample and between samples depends on a number of parameters. It is essential that the chosen marker gene is highly polymorphic. Many genotyping applications require minimization of the probability that two parasites share the same allele by chance. Minimizing this probability

is achieved by choosing the most polymorphic marker, but also the allelic frequency distribution plays a crucial role and should be homogeneous. Non-uniform distribution of allele frequencies increases the number of distinct alleles required (Gatton & Cheng 2008).

Discrimination can be enhanced by using a second marker gene, which further reduces the probability that two samples share the same genotype by chance. In fact, the number of marker genes that should be used to adequately discriminate parasites can be different between study sites and it has been recommended to choose the number of genes in a way that this probability is below 0.05 (Gatton & Cheng 2008). We have shown that in Tanzania, the *msp2* gene is highly diverse with a total of 76 differently sized alleles, and that the probability P of being infected with two parasites sharing the same *msp2* genotype is only 3.6%. In contrast, the frequency distribution of the *msp1* gene in Tanzania is less favourable, since only 29 alleles could be distinguished and the most common allele has a frequency of >20% . Our results suggest that in a high transmission region in Tanzania, where our study was conducted, CE genotyping for *msp2* alone provided sufficient discrimination power to adequately differentiate parasites, since the probability P that two parasites share the same genotype was 3.6%. For *msp1* however, the genetic diversity was not sufficient for use as a single genotyping marker. The number of alleles was greater for both marker genes in Tanzanian samples than in samples from Papua New Guinea (*msp1*: 24 vs. 29; *msp2*: 35vs. 76 in PNG vs. TZ, respectively, table 1). Although the overall genetic diversity in PNG was slightly greater for *msp2* than for *msp1* with 35 vs. 24 alleles, the diversity of *msp2* in this area was not high enough for *msp2* to be used as a single marker for genotyping. This is due to a high probability (7%) of being infected with two parasites sharing the same allele by chance which is above the 5% threshold suggested by Gatton & Cheng (2008). Therefore, genotyping of both, *msp2* and *msp1* is required in order to increase discriminatory power in this area. Combining the two marker genes reduced the probability that two samples share the same genotype by chance to 0.6%. In an area of lower transmission, genotyping two or more markers is clearly an option for single clone infections as *msp1-msp2* haplotypes are evident. But in the case of frequent multiple clone infections this strategy is less beneficial as both markers are unlinked and *msp1-msp2* haplotypes cannot be determined. This shortfall poses a serious handicap if an individual parasite clone needs to be followed up over time in a longitudinal series of samples. Other applications, e.g identification of new infections, are well suited for combining several markers.

Mean MOI was significantly greater in Tanzania than in PNG for both marker genes (*msp1*: 1.99 vs. 3.04; p<0.001 and *msp2*: 1.84 vs. 3.72, p<0.001 for PNG vs. TZ, respectively, table 1) which confirms previous observations of an increased complexity of infection with increasing endemicity (Babiker et al. 1997;Paul et al. 1995). In Tanzanian samples, the mean MOI was significantly greater for *msp2* than for *msp1* (p<0.001), which reflects the great difference in the number of distinct alleles between the two allelic families. Despite the fact that the number of alleles was also greater for *msp2* than for *msp1* in PNG, the mean multiplicity of infection was slightly higher for *msp1*, however, this difference was not statistically significant. This minor difference in mean MOI might be due to various technical reasons like differences in PCR efficiency for the two marker genes, which results in different detection limits. Another parameter that influences the number of genotypes per sample is the cut-off that is determined for each sample, which is dependent on the internal size standard used to control for unequal loading of the PCR product onto the automated sequencer. The use of two markers obviously produces minor discrepancies due to technical differences but does not affect the overall MOI result.

The results we obtained by applying our high resolution genotyping technique revealed similar results to previous reports on allelic diversities in three areas of different malaria endemicity in Brazil, Vietnam and Tanzania where the extent of allelic diversity of *msp2* as estimated by the total number of distinct alleles increased with increasing endemicity (Hoffmann et al. 2001). The same populations were also investigated for genetic diversity in the *msp1* gene. In contrast to *msp2*, the extent of *msp1* diversity did not seem to correlate with the level of malaria transmission in these regions (da Silveira et al. 1999;Ferreira et al. 1998a;Ferreira et al. 1998b;Kaneko et al. 1997;Silva et al. 2000). Our observations of only a minor effect of transmission intensity on diversity of *msp1* confirmed these previous findings. An increased diversity of *msp2* in areas of higher endemicity was also reported previously (Babiker et al. 1997;Haddad et al. 1999;Konate et al. 1999;Paul & Day 1998), however, most of these studies also reported a correlation between endemicity and the number of distinct alleles for *msp1*, which was not the case for the comparison of diversity between Brazil, Vietnam and Tanzania and in our study. There were also some studies that did not find a correlation between transmission intensity and genetic diversity for both marker genes (Bendixen et al. 2001;Montoya et al. 2003;Peyerl-Hoffmann et al. 2001).

Our findings might have implications for genotyping samples from drug efficacy trials where recrudescence must be reliably distinguished from new infections and the probability of new infecting parasites having the same allele as the initial infecting parasite should be as low as possible. Our data suggest that for this purpose *msp2* is the more suitable marker gene than *msp1* in both study sites. Genotyping only *msp1* would not provide adequate discriminatory power according to the standards suggested by Gatton and Cheng (2008) as the probability of being reinfected with the same genotype is 8.5%. In areas of lower transmission intensity, such as in PNG, genotyping only one marker gene (either *msp1* or *msp2*) will not provide enough discriminatory power and two markers are necessary to improve discrimination power. In highly endemic areas like Tanzania, the resolution obtained with *msp2* might be sufficient to discriminate all concurrent clones within an individual, as the probability of multiple parasites sharing the same allele is low. However, the high mean MOI in this area is likely to lower the discriminatory power. This effect of high MOI on the resolution of genotyping markers has so far been ignored in genotyping studies. It is of relevance in particular for genotyping of recrudescences in highly endemic areas, because high MOI increases the probability of concurrent parasite clones carrying the same genotype. We therefore made an attempt to estimate the true number of alleles present in a host, allowing for this probability. Details of the estimation approach are being published elsewhere (Ross et al. in preparation). Table 3 lists the probability of observed infections conditional on the true number of infections based on the PNG allelic frequencies. Adjustment for the probability of multiple infections sharing the same *msp2* allele made little difference to the estimated MOI (estimated mean MOI was 1.84 in PNG, 3.99 in Tanzania compared to the unadjusted values of 1.84 in PNG and 3.72 in TZ, respectively). This reflects the low probability of being infected with two parasites sharing the same allele with such a high resolution typing system.

In conclusion, our CE based genotyping, which provides highly accurate fragment sizing data is in line with previous findings on genetic diversity in different geographic locations. A higher mean MOI for both marker genes was found in an area of more intense malaria transmission. *msp2* diversity was higher in the high transmission area, but *msp1* only showed a minor increase in diversity. The between population variance in allele frequencies, as estimated by Wright's F statistics, was found to be very low for both marker genes. This indicates that there was little genetic differentiation between the two sites of different endemicity and suggests that the observed patterns of allele frequencies are independent of transmission intensity.

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Figure1: Allelic frequencies of *msp1* and *msp2* in samples from Papua New Guinea
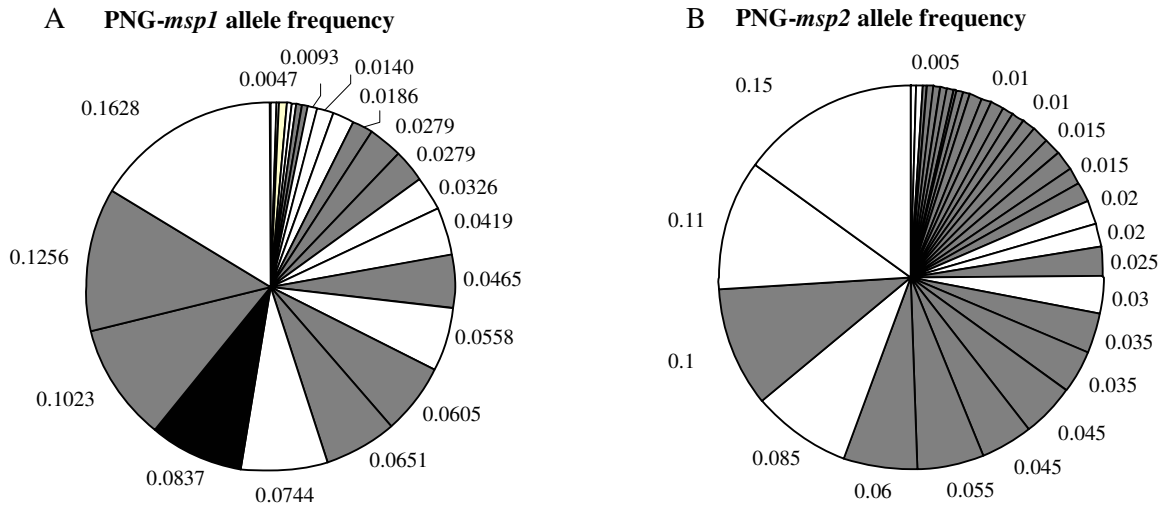
A. White, grey and black areas indicate frequencies of K1, MAD20 and RO33 alleles, respectively. **B.** White and grey areas indicate Fc27 and 3D7 allele frequencies, respectively.

Figure 2: Allelic frequencies of *msp1* and *msp2* in samples from Tanzania
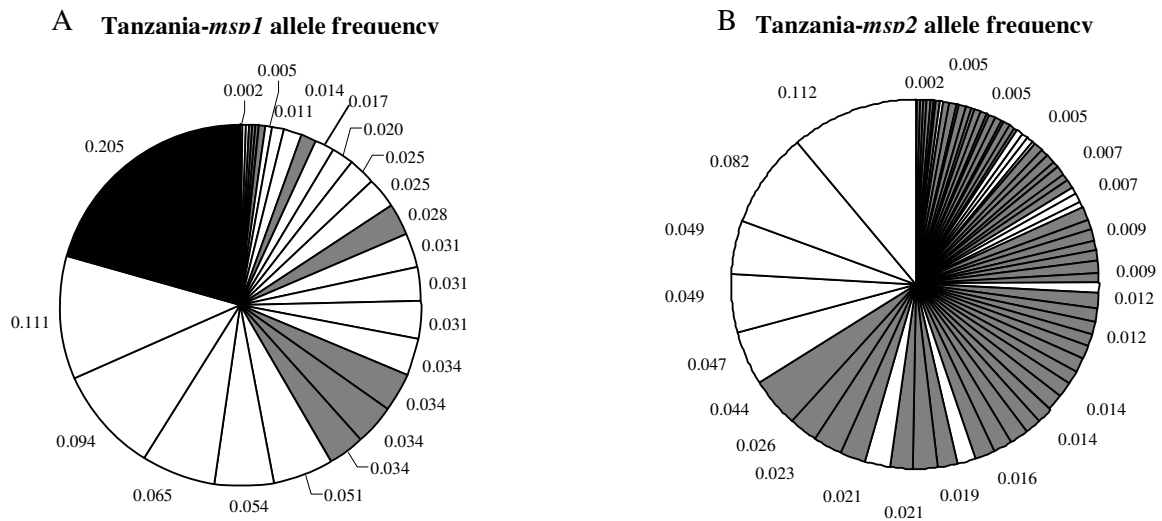
A. White, grey and black areas indicate frequencies of K1, MAD20 and RO33 alleles, respectively. **B.** White and grey areas indicate Fc27 and 3D7 allele frequencies, respectively.

Table1: Diversity of *msp1* and *msp2* in samples from Papua New Guinea and Tanzania

	Papua New Guinea		Tanzania	
	msp2	*msp1*	*msp2*	*msp1*
Number of samples (n)	108	108	115	115
Number of alleles	35	24	76	29
Frequency of most common allele	15.08 %	16.28 %	11.21 %	20.57 %
Number of clones	199	215	428	350
Mean MOI[*]	1.84	1.99	$3.72^{\S}$	$3.04^{\S}$
H_E[**]	0.933	0.918	0.965	0.917
$P=\sum p_i^2$	0.07	0.084	0.036	0.085
Combined probability[***]	0.0058		0.003	

[*] MOI = mean multiplicity of infection

[**]H_E = expected heterozygosity. This is defined as the probability that two randomly chosen alleles are different in the population.

[***] The combined probability that two independent clones share the same genotype for both marker genes was calculated by multiplying the probabilities P for both marker genes.

[§] Indicates significant difference between mean MOI of *msp1* and *msp2* ($p<0.001$)

Table 2: Number of different *msp1* and *msp2* alleles detected in samples from Papua New Guinea and Tanzania

	Papua New Guinea	Tanzania
Number of different *msp1*-K1 alleles	13	19
Number of different *msp1*-MAD20 alleles	10	9
Number of different *msp1*-RO33 alleles	1	1
Number of different *msp2*-3D7 alleles	27	59
Number of different *msp2*-Fc27 alleles	8	17

Table 3: Probability of each number of observed infections (rows), conditional on the true number of infections (columns). Results are shown for MOI≤5.

	1	2	3	4	5
1	1	0.068	0.007	0.0008	9.38E-05
2		0.931	0.185	0.0358	0.007077
3			0.808	0.3108	0.097212
4				0.6526	0.404946
5					0.490671

CHAPTER 4

Treatment with Coartem® (artemether-lumefantrine) in Papua New Guinea

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ABSTRACT

Standard treatment for uncomplicated malaria in Papua New Guinea (PNG) is currently a combination of chloroquine or amodiaquine plus sulfadoxine-pyrimethamine. High levels of resistance against this treatment regimen give strong argument for the implementation of artemisinin based combination therapy (ACT) in the country as recommended by the WHO. A recent drug efficacy trial has reported the combination therapy Coartem[®] (artemether-lumefantrine) to be highly effective against *P. falciparum* in children less than 5 years in PNG. In contrast, we have observed high levels of treatment failures in non-trial conditions in a longitudinal cohort study in the same age group in PNG. Recrudescences were confirmed by molecular genotyping of 3 different marker genes using a high resolution technique which provided optimal discrimination power between parasite clones. After excluding genetic host factors by genotyping potentially relevant cytochrome P450 loci, the high number of treatment failures in our study is best explained by poor adherence to complex dosing regimens in combination with insufficient fat supplementation, which are both crucial parameters for the outcome of Coartem[®] treatment. Opposed to the situation in classical drug trials with ideal treatment conditions, our field survey highlights potential problems with unsupervised usage of Coartem[®] in routine clinical practice.

INTRODUCTION

With respect to increasing drug resistance, the World Health Organisation currently recommends a switch of first line treatment against uncomplicated malaria to artemisinin based combination therapies (ACT) for countries where conventional antimalarial treatments such as chloroquine (CQ), amodiaquine (AQ) or sulphadoxine-pyrimethamine (SP) have become ineffective [1].

First line treatment for uncomplicated malaria in Papua New Guinea (PNG) has been chloroquine or amodiaquine plus sulphadoxine-pyrimethamine since the year 2000. However, the efficacy of this treatment regimen was found to be low only three years after its implementation [2], which gives a strong argument for the introduction of artemisinin-based combination therapies. A recent study conducted in Papua New Guinea [3] has tested the efficacy of different artemisinin-combination therapies in children <5 years with *P. falciparum* or *P. vivax* malaria. Artemether-lumefantrine was found to be the most effective combination therapy against *P. falciparum* with an adequate clinical and parasitological response of 97.3% at day 28 and 95.2% at day 42 after treatment.

While the effectiveness of Coartem® (artemether-lumefantrine) under ideal trial conditions was very promising, the situation may look different in less controlled conditions in routine practice. It is likely that adherence to the recommended treatment is sub-optimal under non-study conditions, because the rapid relief of symptoms might tempt patients to early interrupt treatment.

We undertook a longitudinal study in PNG during which children between 1 and 5 years of age were followed up in 8-weekly intervals with the aim to investigate the infection dynamics of *P. falciparum* clones. All children presenting with a clinical malaria episode were treated with Coartem®. In our study, we observed an unexpected high number of *P. falciparum* recurrent infections which were confirmed by molecular genotyping of three different markers. Our observations are in contrast to recently published results of a drug efficacy trial in the study area [3]. We discuss our findings with respect to the forthcoming introduction of Coartem® as first line treatment in the country [4].

METHODS

Study design and site. The study was carried out in an area near Maprik, East Sepik Province in Papua New Guinea which is endemic for malaria. There is little seasonal variation in temperature or rainfall and malaria transmission is perennial [5]. A detailed description of the study design is given elsewhere (Lin et al, in preparation). Briefly, 269 children 1-3 years of age at enrolment were followed-up in 8 weekly intervals from March 2006 until July 2007. At each visit the children were clinically assessed and a 250µl blood sample was collected from each participant by finger prick. In between these regular follow-up bleeds, each child was clinically examined every 2 weeks, and in case of malaria symptoms a 250µl blood sample was collected. Furthermore, a blood sample was collected at the local health centre whenever a study participant presented with malaria symptoms. During anytime of the study, symptomatic children that were confirmed to carry malaria parasites by rapid diagnostic test and/or microscopy were treated with a 6-dose regimen of Coartem® (Novartis Pharma, Switzerland) which was mainly administered unsupervised. Caretakers were advised to complete the full treatment regimen and to supplement the drugs with a fatty diet.

Scientific approval and ethical clearance for the study was obtained from the Medical Research and Advisory Committee (MRAC) of the Ministry of Health in PNG and from the Ethikkommission beider Basel (EKBB) in Switzerland. Informed consent was sought from all parents or guardians prior to recruitment of each child.

DNA isolation and *msp2* genotyping. All finger prick blood samples were separated into plasma and cells. DNA was extracted from cell pellets using QIAamp® 96 DNA Blood Kit (Qiagen, Australia) according to the manufacturer's instructions. All samples were genotyped for the highly polymorphic marker gene merozoite surface protein 2 (*msp2*) by capillary electrophoresis as previously described by Falk et al. [6] with some minor changes and adaptations of PCR conditions for highly purified DNA as described in Schoepflin et al (in preparation).

Determination of treatment failures and *msp1* genotyping. The current protocol of the WHO to assess drug efficacy recommends a follow-up of 28 days for most antimalarial drugs and an extended follow-up to 42 days for Coartem® [7]. In consideration of these guidelines,

the genotype dataset was screened for *msp2* alleles that reappeared in the same patient within 7-42 days after treatment with Coartem®. Since most of the study participants were treated several times throughout the course of the study, there were a total of 89 individuals that accounted for more than one entry in the database, but from different treatment time points during the survey. An outcome was defined as recrudescence if the first *P. falciparum* positive sample after treatment contained at least one *msp2* allele that was identical to any of the alleles present in the treatment sample. An infection was defined as new infection when the first positive sample after treatment only contained new *msp2* alleles. If the first positive sample contained both, alleles present in the treatment sample and new alleles, the outcome was considered recrudescent. As suggested by Mugittu et al. [8] we used a stepwise approach to discriminate recrudescent from new infections. All *msp2* treatment failures were further genotyped for the polymorphic marker gene, merozoite surface protein 1 (*msp1*). For the amplification of the *msp1* block 2 [9], a nested PCR approach followed by capillary gel electrophoresis was used (details see Schoepflin et al., 2009; in preparation). Classification as recrudescent or new infection was done in the same way as for *msp2*.

Genotyping of microsatellite TA81. Samples identified as treatment failure by *msp2* and *msp1* and those samples for which *msp1* PCR had failed were further genotyped for the polymorphic microsatellite TA81. Genotyping of microsatellite TA81 was only performed for follow-up samples collected within 28 days after treatment. Samples between day 28 and day 42 were not further genotyped for TA81. Amplification of the TA81 locus was performed using primers and conditions previously described [10]. One of the amplification primers was 5' end-labeled with Cy5. PCR products were mixed 3:1 (vol/vol) with denaturing loading dye buffer and analysed on a 6% denaturing polyacrylamide gel as previously described by DaRe et al. [11].

Sequencing of cytochrome P450 and UDP-glucuronosyltransferase. Amplification of *CYP3A4* was performed in a total volume of 50µl containing 5µl of 10xBufferB (0.8M Tris-HCl, 0.2M $(NH_4)_2SO_4$, 0.2% w/v Tween-20), 2mM $MgCl_2$, 200µM dNTPs, 2.5U Taq DNA polymerase (FirePol, Solis BioDyne). PCR primers (forward: 5'CTCACCTCTGTTCAGGGAAAC 3'; reverse: 5'ATGGCCAAGTCTGGGATGAG 3') were used at a final concentration of 1µM each. 2.5µl of purified DNA was used as template

for this reaction. An initial denaturation step of 96°C for 3 min was followed by 40 amplification cycles of 30 s at 96°C, 1 min at 64°C, 1 min at 72°C and a final extension for 10 min at 72°C. PCR products were purified using NucleoSpin Extract II Kit (Clontech Laboratories, Inc.) and sequenced. Sequences were analysed using the ABI Prism AutoAssembler version 1.4.0 (Applied Biosystems) for assembly. Amplification and SNP-genotyping of *CYP2B6* 516G>T and *UGT2B7* 802C>T was performed as previously described [12,13].

RESULTS

During the 16 months follow-up of our field survey a total of 793 Coartem® treatment regimens were administered to symptomatic children infected with *P. falciparum*, as diagnosed by *msp2* PCR. 671 of these children were also positive by microscopy for *P. falciparum* and 188 carried mixed species infections at the time of treatment. Clinical and parasitological failure rates were determined in samples selected according to the inclusion criteria depicted in Figure 1. For determination of the parasitological treatment failure rate, only treated episodes occurring within 42 days prior to a regular follow-up visit were taken into account (Figure 1a). For clinical failure rate, two consecutive *P. falciparum* episodes of an individual were taken into account that both had occurred within an interval of 7 to 42 days (Figure 1b). The recurrent episode was detected either at regular follow up bleed or by active or passive case detection.

Figure 2 summarizes genotyping results generated with three marker genes. Out of the 793 Coartem® treated children, only 182 had a consecutive sample collected within the next 28 days after treatment and 299 within the next 42 days. To estimate the parasitological treatment failure rate, all sample pairs were genotyped sequentially for 3 markers starting with the most polymorphic marker, *msp2*. For the remaining 494 Coartem® treated children, no consecutive blood sample was available within 42 days and these samples were excluded from further analysis.

The PCR-corrected parasitological treatment failure rates were 36/182 (19.78 %) and 48/299 (16.05 %) at day 28 and 42, respectively. Samples collected between day 28 and day 42 after treatment were only genotyped for *msp2* and *msp1* and final classification of infections occurring during this time period were therefore only based on these two marker genes.

Out of the 793 *P. falciparum* episodes treated with Coartem®, 141 cases experiencing a further episode within 7-42 days after treatment. PCR-corrected clinical failure rates were 26/793 (3.27 %) and 42/793 (5.3 %) at day 28 and 42 respectively.

Cytochrome P450 genotypes. 64 different individuals representing persistent or new infections were genotyped for the cytochrome P450 3A4 392A>G and 2B6 516G>T and for the UDP-glucuronosyltransferase *UGT2B7* 802C>T which are all involved in the metabolism

of artemisinin drugs and/or lumefantrine [14-17]. Sequencing of cytochrome P450 3A4 was successfully performed for 56/64 participants. Results from sequencing of cytochrome P450 3A4 showed homozygote wild type carriers at position 392, i.e. adenosine, for all 56 individuals. The P450 loci *CYP2B6* 516G>T and *UGT2B7* 802C>T were found to be polymorphic in the 64 individuals from our study population which was in agreement with previous results from the same population in PNG [12,13]. It has been proposed by these authors that the outcome of treatment with artemisinin drugs might be associated with a combined effect of polymorphisms in both *CYP2B6* and *UGT2B7* genes. In our study population, the combination of *CYP2B6* 516T and *UGT2B7* 802T was observed in individuals carrying new infections as well as in individuals showing treatment failures. There was a trend towards increased frequency of double mutants in individuals carrying recrudescent infections (30.3% of all *CYP2B6-UGT2B7* double mutants were observed in individuals carrying new infections and 69.7% in treatment failures), however, the association between treatment outcome and the presence of double mutants in *CYP2B6* and *UGT2B7* was not statistically significant (p=0.79).

DISCUSSION

A number of different drug trials were conducted worldwide to test the efficacy of artemether-lumefantrine. This combination treatment has proven highly efficacious in studies conducted in Ghana [18], Tanzania [19,20], Thailand [21,22] or Papua New Guinea [3] with >97% PCR adjusted cure rates at day 28. In contrast, we observed an unexpected high number of recurrent infections after treatment with Coartem® in our longitudinal survey in children under 5 years of age in Papua New Guinea. Treatment failures were confirmed by molecular typing of three different marker genes using a high resolution technique with optimal discrimination power between genotypes. Using 3 different marker genes for discrimination of recrudescent from new infections provides confident identification of treatment failures. In the drug trial by Karunajeewa et al. [3] only microscopy positive recurrent infections were genotyped. In our analysis, all samples were genotyped irrespective of microscopy results. Since sensitivity of PCR is considerably higher than that of microscopy, we most likely have detected recrudescences of low parasite density that would have remained undetected by microscopy. This might have contributed to the higher treatment failure rate in our study.

Our study was not designed as a classical drug efficacy trial, but as a cohort study with longitudinal follow-up in 8-weekly intervals. This study design does not allow estimating in a standardized way the true rate of parasitological treatment failures, because recrudescent infections were only detected when the treated episode occurred within 28 or 42 days prior to a regular follow-up visit. If the interval between the episode and the next regular follow up bleed was longer than 42 days, any recrudescent or newly occurring parasitaemia remained undetected. Thus, with our study design we certainly underestimated the number of both, recrudescences and new infections. However, the specific advantage of our study consists in the fact that treatment success was viewed outside a clinical trial setting with outcomes reflecting the local situation in village health care. Our finding of a substantial number of recrudescences in non-trial conditions highlights potential problems of unsupervised Coartem® usage.

In principle, several factors could have reduced the effectiveness of Coartem® treatment in our study: (i) reduced sensitivity of parasites to Coartem®; (ii) host factors accounting for differences in metabolism of the drug; (iii) inadequate adherence to treatment regimen; (iiii) suboptimal absorption of drugs.

A reduced sensitivity to artemether-lumefantrine has been associated with an increased copy number of *pfmdr1 in vitro* and *in vivo* [22]. Similar findings of a reduced sensitivity to lumefantrine *in vitro* were described by Lim et al. [23], however, in that study the correlation could not be confirmed *in vivo*. Recently, it has been shown that *pfmdr1* gene amplifications were absent from different study sites in PNG, including an area adjacent to our study site [24]. These results, together with the fact that an efficacy trial [3] had confirmed Coartem® to be highly effective in our study area indicate that reasons other than reduced drug sensitivity due to an increase in *pfmdr1* copy numbers are more likely to account for the high frequency of recrudescent infections.

Artemisinin derivatives are mainly metabolized by the human cytochrome P450 3A4 [15] and 2B6 [17] and also by the metabolic enzyme UDP-glucuronosyltransferase *UGT2B7* [16]. The lumefantrine component of Coartem® is also metabolised by *CYP3A4* [14]. So far functional significance of polymorphisms in *CYP2B6* and *UGT2B7* on the treatment outcome of artemisinin drugs is lacking. However, previous *in vitro* studies have shown that these polymorphisms caused significant reduction in enzyme activity or expression [12,13] which generally elevated the plasma drug concentration of a antiretroviral drugs [12]. It is likely that these polymorphisms act in a similar way on artemisinin plasma levels, and it has been proposed by Mehlotra and others [12,13], that the large inter-individual variability in the pharmacokinetics of artemisinin drugs which has frequently been observed might partly be a result of a joint contribution of both polymorphisms in *CYP2B6* and *UGT2B7*. In order to exclude the possibility that host genetic factors account for the frequent treatment failures in our study population, polymorphisms in the human cytochrome 3A4, 2B6 and in *UGT2B7* were investigated. For the *CYP2B6* and *UGT2B7* we only focused on the two polymorphisms *CYP2B6* 516G>T and *UGT2B7* 802C>T which have been observed at high frequency in a previous study in PNG [12,13]. We found mutant alleles in both, patients carrying new infections as well as in recrudescent infections, but treatment outcome and the presence of double mutants were not significantly associated. Thus the double mutant *CYP2B6/UGT2B7* seems not to influence the outcome of Coartem® treatment in this population. However, the trend towards increased proportion of double mutants in patients with treatment failures suggests that the correlation between treatment outcome and presence of double mutant should be further investigated in a bigger study population under standard drug trial conditions. The mutant allele *CYP3A4* 392A>G has previously been associated with a significant decrease in *CYP3A4* activity [25] and therefore leads to an increased exposure to

lumefantrine. Our sequencing results did not reveal any polymorphisms at position 392. Homozygous carriers of the wild type SNP were found in individuals harbouring recurrent or new infections. These results indicate that differences in metabolism of lumefantrine due to mutations in *CYP3A4* do not seem to account for the frequent treatment failures observed.

In most drug efficacy studies, treatment regimens are administered under supervision of a team nurse and therefore optimal compliance is mostly guaranteed or at least information on the adherence to the recommended treatment intervals or early interruption of treatment are available and can be accounted for in the analysis. In our study, treatment was mostly administered unsupervised. Parents were encouraged to complete all treatment doses, but in fact, no information is available from our study participants whether or not the complete treatment regimen was taken. Coartem® rapidly alleviates malaria symptoms, which might frequently tempt patients to stop treatment earlier and keep the remaining tablets for a later malaria attack. Incomplete adherence to the full 6-dose regimen might have contributed to the observed frequency of treatment failures.

After initial reduction of the parasite biomass by the fast acting artemether, clearance of the residual parasites greatly depends on the longer lasting partner drug lumefantrine. Previous studies have shown that the plasma level of lumefantrine is a critical factor for treatment success [22,26]. Food has a significant effect on the bioavailability of both components of the drug with an increased absorption when supplemented with fatty food [27]. An increase in treatment success by 15% was observed in a study in Thailand when a fatty diet was co-administered with Coartem® [28]. In the present study, parents or guardians of the study participants were advised to administer treatment with a fatty diet, however, no information are available to what extent these recommendations were followed. Inadequate plasma concentrations of lumefantrine might therefore have contributed to the high rate of treatment failures.

The high frequency of parasitological and clinical treatment failures in our study are contrary to findings from a recent drug efficacy trial which was conducted in an area adjacent to our study site. In this previous study Coartem® was reported to be highly effective in clearing *P. falciparum* in children under 5 years of age [3]. An important difference between the two studies was that in the drug efficacy trial at least half of the doses were dispensed under supervision and supplemented with milk. This implies that incomplete adherence to the

treatment regimen in combination with a lack of fatty diet might have contributed to the observed differences in treatment outcome. Furthermore, in our study Coartem® was also given to *P. falciparum* infected children who presented with anaemia (Hb<7.5), but did not show any other sign of symptoms. It is likely that these children were even less adherent to treatment, because they were without noticeable signs of disease.

Rather low levels of drug efficacy had also been reported from two studies in Ghana [29,30], where PCR corrected cure rates at day 28 were only 86.2% and 88.3%, respectively. In one of these studies only the first dose was given under supervision, whereas in the other study the administration of all 6 tablets was supervised. In both studies, no fatty diet was provided by the study team, but caretakers were encouraged to give fatty food at the time of drug administration. It was not checked by the study team whether these recommendations were followed. In a study in Uganda, no difference in treatment efficacy was observed between supervised and unsupervised administration of Coartem® [31]. In the latter study, detailed explanations on intake of tablets and treatment schedule were provided to the patients. Such information may have resulted in adequate adherence.

In summary, our observations highlight the importance of strict adherence to the complex dosing regimens of Coartem® and the need to supplement the treatment with a fatty diet. The study by Kuranajeewa et al. [3] had shown that under optimal treatment conditions Coartem® was highly effective in Papua New Guinea, whereas our results indicate that these high success rates might be difficult to achieve under routine clinical practice. Thus, it is of great importance that the introduction of Coartem® as first line treatment in PNG is accompanied by provision of training and education for health workers to guarantee accurate treatment and compliance to the recommended guidelines. As pointed out by Piola et al. [31] a great effort has to be made to convince patients and caretakers to complete the full 6-dose regimen despite the fast relief of symptoms.

ACKNOWLEDGEMENTS

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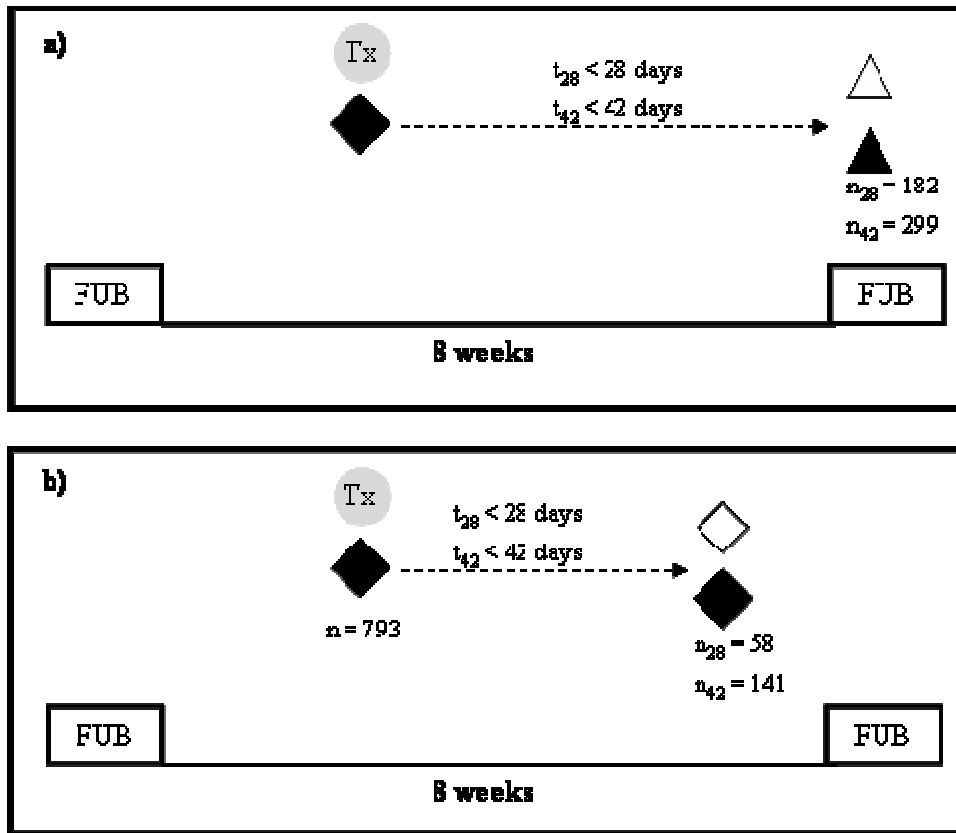
Figure 1. Schematic representation of inclusion criteria for the analysis of parasitological treatment failures (a) and clinical failures (b).

☐ FUB regular 8-weekly follow-up bleed, ◆ clinical episode sample; Tx treatment with Coartem®; ▲ FUB sample PCR positive for recrudescent allele, △ FUB sample PCR negative for recrudescent allele
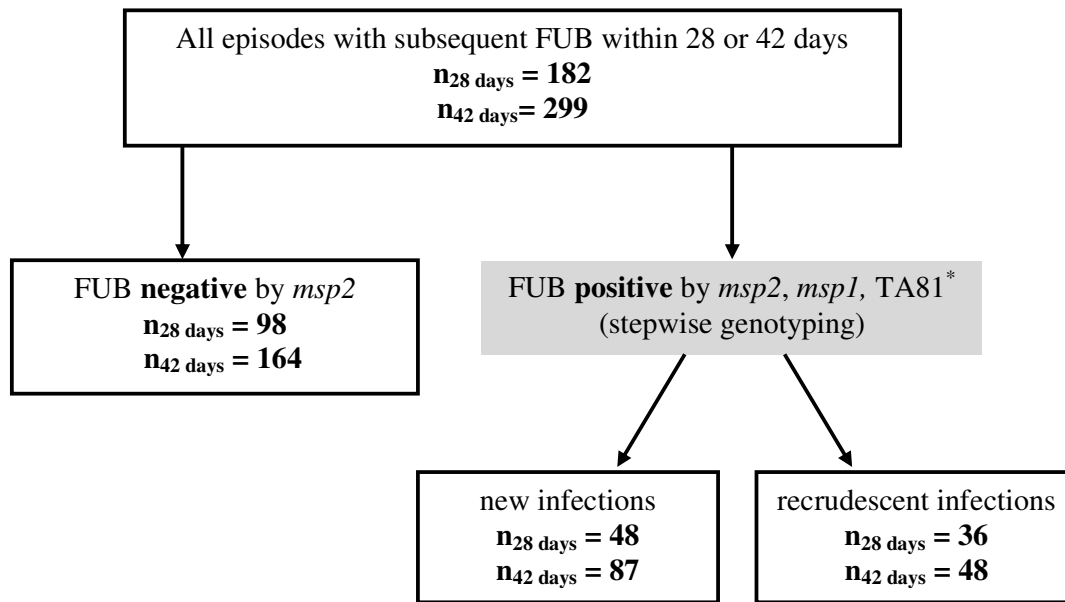
Figure 2. Summary of final treatment outcome based on *msp2*, *msp1* and TA81 genotyping
FUB= follow-up bleed;* Genotyping of the microsatellite TA81 was only performed for
infections occurring within 28 days after Coartem® treatment. Samples collected between day
28 and day 42 after treatment were genotyped only for *msp2* and *msp1*.

CHAPTER 5

Estimates of detectability of *P. falciparum* and force of infection based on molecular data

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ABSTRACT

Background: In areas endemic for malaria, most people are simultaneously infected by different *P. falciparum* clones. Detection of all clones concurrently present in a host is complicated by sequestration of parasites during part of their life cycle, or by densities fluctuating around the detection limit of molecular typing. This shortfall has implications for basic measures of epidemiology or for outcome measurements of clinical trials. To quantifying the proportion of undetected parasite clones, we estimated detectability by repeated sampling and determined its effect on multiplicity of infection (MOI) and force of infection (FOI)

Methods: A longitudinal field survey was conducted in 1-4.5 years old children from Papua New Guinea over 16 months with follow-up visits in 2-monthly intervals. At each visit two blood samples were collected 24 hours apart from each child. Samples were genotyped for the polymorphic marker gene *msp2*. Detectability was estimated from the presence of genotypes at either both days or only one day. MOI and FOI were determined for single or repeated blood sampling.

Findings and Conclusion: FOI was defined as the number of parasite clones acquired per time. Different approaches to measure FOI were tested. When new clones infected a parasite-free host after treatment, FOI was found to be substantially higher compared to estimates of FOI in absence of treatment. Without interference of antimalarials, competition between newly acquired and already persisting infections could lead to the low FOI observed. Detectability was 0.79 in our data set and we found that conducting 24h bleeds had only a marginal impact on measures of MOI and FOI. This led us to conclude that the additional efforts and costs of such a study design do not justify short term sampling in future studies of this age group.

INTRODUCTION

Development of PCR based genotyping techniques has greatly increased knowledge about infection dynamics of *Plasmodium falciparum*. Studies on multiplicity of infections, incidence and clearance rates or duration of infections became possible by genotyping longitudinal samples. Molecular genotyping has also been used to improve classification of treatment outcome in drug trials. Irrespective of the application of these techniques, researchers always face the problem of imperfect detectability of parasites due to the fact that *P. falciparum* is periodically absent from the peripheral blood. The parasite sequesters in blood vessels for part of its 48 hour life cycle. In addition, detectability is influenced by the detection limit of the applied technique. Highly complex dynamics of *P. falciparum* were previously observed in children followed-up on a daily basis and the composition of infections was found to be unstable over time and even changing from one day to another [1]. This implies that a single blood sample only partly represents the true parasite population present in a host. Several publications have addressed the issue of imperfect detectability and described mathematical models to estimate infection dynamics of *P. falciparum* under conditions of imperfect detection of parasites [2-4]. The model by Sama et al. [2] was applied for a longitudinal field study in Ghana, where consecutive blood samples were collected from each participant in 2 monthly intervals. From this dataset, the detectability by PCR-RFLP was estimated as approximately 0.45, meaning that on average only 45% of the parasites present in a host are actually detected. Bretscher et al. (in preparation) have outlined a method to estimate the detectability of infection for *P. falciparum* based on pairs of samples collected in such small time intervals that re-infection with a new parasite clone can be excluded. Detectability for sample pairs that were collected between 1 and 7 days apart were estimated to take values between 0.45 and 0.63. We have now applied the same method to a large set of paired samples that were collected 24 hours apart.

Imperfect detectability might also have important implications for estimating the incidence of infection. This was observed in a treatment to re-infection study in Papua New Guinea [5], where two weeks after antimalarial treatment a remarkable proportion of microscopy-negative children were positive for any *Plasmodium spp.* by PCR based detection. Compared to the standard method of detecting parasites by microscopy, PCR based detection of parasites has the advantage of being more sensitive and therefore has the capacity of detecting infections at an earlier time point when parasite density is still under the detection limit of microscopy.

More precise measures for calculating incidence rates can be obtained by genotyping techniques that distinguish co-infecting parasite clones within one host. However, despite improved detection through molecular techniques parasite clones remain undetected due to sequestration or densities fluctuating around the detection limit of PCR. Thus, imperfect detectability of *P. falciparum* infections may lead to underestimates of the force of infection (FOI).

In the study presented here, a large number of samples collected 24 hours apart from a cohort of Papua New Guinean children were genotyped to calculate the detectability of infection and to investigate the benefit of collecting 24h bleeds on basic measures of epidemiology such as the multiplicity of infection or the force of infection.

METHODS

Field survey and patients

This study was conducted in a rural area near Maprik, East Sepik Province, Papua New Guinea. A detailed description of the study is given elsewhere (Lin et al., in preparation). Briefly, 269 study participants were enrolled at an age of one to three years starting in March 2006. Since during the first round of sampling the expected number of participants could not be reached, late enrolments were conducted for the remaining children in the consecutive sampling rounds. Regular follow-up visits were conducted in 8-weekly intervals over a period of 16 months until July 2007. Except for the first and last round of sample collection, two consecutive blood samples were collected by finger prick at intervals of 24 hours for each study participant at each follow-up visit (in the following termed: 24h bleed). Each individual contributed up to 16 samples, 14 of which were paired samples with 24 hour intervals. Active and passive case detection was performed between the regular 8-weekly follow-up visits and a blood sample was collected from all participants with suspected malaria infection and a rapid diagnostic test (RDT) was performed. Antimalarial treatment with Coartem® (Novartis, Switzerland) was administered upon a positive RDT or if haemoglobin levels were <7.5 g/dl. Informed consent was sought from all parents or guardians prior to recruitment of each child. Scientific approval and ethical clearance for the study was obtained from the Medical Research and Advisory Committee (MRAC) of the Ministry of Health in PNG and from the Ethikkommission beider Basel in Switzerland.

Laboratory procedures

All finger prick blood samples were separated into plasma and cells. DNA was extracted from cell pellets using QIAamp® 96 DNA Blood Kit (Qiagen, Australia) according to the manufacturer's instructions. All samples were genotyped for the polymorphic marker gene merozoite surface protein 2 (*msp2*) by use of capillary electrophoresis for fragment sizing as previously described by Falk et al. [6] with some minor changes and adaptations of PCR conditions for highly purified DNA as described by Schoepflin et al. (manuscript in preparation).

Data analysis

Analysis of 24h interval bleeds. For data analysis pairs of samples that were collected 24 hours apart were considered from each individual. Sample pairs were excluded from the analysis if antimalarial treatment was given on the first day. For each genotype, each sample pair was classified by positivity on each of the two days, leading to three categories for each genotype, negatives (where the genotype was not observed, with frequency n_0), singles (where it was observed in one sample, n_1) and double positives (n_2). An estimate, $\tilde{q}$, of the detectability, q, is to equate it to the proportion of positive observations among all observations where the genotype is present, which gives, allowing for the negatives, the following:

$\tilde{q} = \dfrac{2n_2}{n_1 + 2n_2}$ (Bretscher et al., in preparation). An approximate confidence interval was calculated as follows: CI [$q \pm$ 1.96 se(q)], where the standard error is :

$se(q) = \dfrac{2\sqrt{n_1 n_2 (n_1 + n_2)}}{(n_1 + 2n_2)^2}$.

The age on day 1 of each 24h bleed was calculated for each individual and the detectability was calculated for different age groups. Comparison of proportions between day 1 and day 2 samples was done by McNemar's exact test for paired data.

Force of infection. For estimating the force of infection (FOI) the time period between an antimalarial treatment and first re-infections after treatment was calculated. Since the study design only allowed for sample collection at predefined dates (8-weekly intervals) or when a participant had malaria symptoms, this time period can only be determined approximately. Recrudescent infections as determined by *msp2* genotyping that emerged within 15 days after Coartem$^®$ treatment were excluded from the analysis. This corresponds to approximately 3 times the elimination half life of lumefantrine. New infections occurring within this time period were also excluded. The age was calculated for each participant at the time of treatment and 3 age categories were made (1-2 years; 2-3 years; >3 years). Kaplan-Meier failure estimates were calculated and plotted for different age groups, and a log-rank test for equality of survivor functions across groups was performed at a 5% significance level.

As a second approach to estimate FOI we observed the number of newly acquired *P. falciparum* clones occurring between any two consecutive Coartem® treatments. Poisson regression was used to examine effects of different covariates on the number of acquired clones per time and the FOI was modelled on $\ln(\frac{n}{t})$ as a linear function of the covariates, where n is the number of newly acquired infections, and t is the length of the interval between treatments. Random effect models were examined to correct for potential clustering.

Furthermore, FOI in absence of antimalarial treatment was estimated. For this analysis, an interval was defined by two blood slides from the same individual at successive 8-weekly follow-up bleeds (FUB). The number of newly acquired clones was observed for each interval. Intervals were excluded for this analysis if treatment was given at start or during the interval. Intake of antimalarials within 15 days prior to start of the interval was also an exclusion criterion for this analysis. A total of 605 intervals of 8 weeks were eligible for this analysis. 3 samples were genotyped per interval. The samples at start and end of an interval were monitored, plus the last sample collected prior to the interval. The latter was required to classify a parasite clone as new or persistent infection. The genotype sample triplets were recorded as 3 digit codes, 001, 011, 010, 110, 101, 111, where the first position indicated the presence or absence of a specific genotype in the sample prior to the interval, and the second and third position represented this genotype in the first and last sample of the interval:

111, denoted the presence of an allele in all three samples, which was indicative for persistence; 011, denoted the presence of a genotype already at the beginning of an interval and was therefore classified as persistent infection; 001, stands for a new acquisition of a genotype during the interval monitored. 010, denoted the loss of a genotype during the interval monitored; 101, denoted the detection of an allele prior to interval start and at the end of the interval, and failure to observe an allele at the start of the interval, which was indicative for imperfect detection at interval start. This sequence was considered a persistent infection. With this strategy, the number of newly acquired clones during 605 8-weekly intervals was identified. FOI was estimated in different age groups by fitting a Poisson regression model, as in the previous approach.

All statistical analysis was performed using STATA® 9.1 statistical analysis software (Stata Corporation, College Station, TX). Models were compared using AIC (Akaike's Information Criterion) as measure of goodness of fit. Lower values of AIC indicate a better fit to the data.

RESULTS

Effect of repeated sampling

For the analysis of paired samples collected in 24h intervals a total of 1016 pairs were eligible. Of these, 311 pairs were positive at least on one day. Table 1 summarizes the *msp2* PCR results on day 1 and day 2. Overall the prevalence of *P. falciparum* infection did not differ significantly between the two days (27.85% on day 1 vs. 28.54% on day 2; Mc Nemars test: χ^2=1.0, p=0.39), but a slight increase in prevalence up to 30.61% was observed, when results from both days were combined.

When the presence of *msp2* alleles was compared between samples from 2 consecutive days, considerable variation in the presence of alleles was observed: 35.33% of all genotypes were only observed on either day. Table 2 summarizes the presence of genotypes in all paired samples collected in 24h intervals. Combining the genotyping results from both days led to a small increase in mean MOI to 1.68 compared to mean MOI of 1.52 on day 1 and 1.47 on day 2 determined for sampling only once (ttest for paired data: t=-8.4990, p<0.001).

Detectability

We estimated a very high detectability of 0.79 for all pairs of samples collected 24 hours apart. Table 3 lists the detectability calculated for different age groups. No significant difference was observed between the 3 groups.

Force of infection after treatment with Coartem®

The time to occurrence of the first new infection after antimalarial treatment is a measure of FOI. In Kaplan-Meier survival analysis, increasing FOI is leading to steeper slopes of the failure curves. The log-rank test for comparison of time to first infection between children of different age groups showed a significant difference between survivor functions (p<0.001). The Kaplan-Meier failure estimates for the three different age groups show a faster re-infection of children with increasing age (Figure 1). The median number of days to first infection was 49 days (inter quartile range (IQR) [31, 69].

In order to obtain a numerical value for FOI from our data, we assumed that times until re-infection follow an exponential distribution with rate λ, corresponding to FOI, with $\lambda = \dfrac{r}{\sum t}$, where $\sum t$ is the total time at risk in the population and r is the total number of new infections observed. The assumption of an exponential distribution of times to re-infection was verified by comparison of the cumulative hazard obtained from our data with the theoretical values. The two curves showed a good fit (data not shown) indicating that our data can justifiably be assumed to follow an exponential distribution. The overall force of infection (λ) for the time to first infection was estimated 3.29/child/year.

A further estimate for FOI was calculated by measuring the number of newly acquired *P. falciparum* clones between any two consecutive treatments with Coartem®. A Poisson regression model was used to determine the relation between age and the number of clones acquired per time. Heterogeneity in FOI among villages was accounted for by random effect. An increased FOI was observed with increasing age of the children (Figure 2). This analysis was also performed for genotypes observed at the first day 1 of any 24 hour sample pair. As expected, the number of clones detected was lower and this led to a lower estimate of FOI. The average FOI was 4.85/child/year when genotypes from both paired samples taken 24 hours apart were considered and 4.49/child/year when only genotypes present on the first day were taken into account. The trend of an increased FOI with increasing age was also seen when only the first sample of each 24h sample pair was used to estimate FOI (Figure 2).

Force of infection in absence of antimalarial treatment

To estimate FOI in absence of treatment a Poisson regression model was fitted to determine the relationship between age and the number of newly acquired clones per 8-weekly interval. Similar to the previous analysis, the model was adjusted for the village of residence. The age relationship was similar to the previous model and also showed an increased FOI with increasing age (Figure 2). FOI after antimalarial treatment was on average 2.91/child/year, which was overall approximately 1.7 times lower compared to the FOI estimated after antimalarial treatment.

DISCUSSION

Imperfect detectability of *P. falciparum* in blood samples occurs when parasites sequester in the deep blood vessels for part of their life cycle or when parasite densities fluctuate around the detection limit of PCR. Therefore a single blood sample might not be representative for the entire parasite population in a host. Imperfect detection is a major constraint for molecular epidemiology of *P. falciparum*. Parameters such as prevalence by PCR, MOI or duration of infection are compromised.

The focus of this work was to investigate changes in the genotypic profile of parasite populations within a host over a period of 24 hours. We aimed at quantifying for our study site the daily fluctuations reported from studies made in Africa, that had shown considerable fluctuations in the presence of alleles from one day to another [1,7]. Our analysis of PCR positivity in samples collected 24 hours apart revealed limited day to day fluctuations and showed that short term sample collection has only a marginal impact on the outcome of the prevalence when results from 24h bleeds were combined. Combining the *msp2* genotyping results from both days resulted only in a small increase in the mean MOI compared to results obtained from a single sample (1.52 vs. 1.68). This is reflected in the very high detectability of 79% estimated in our analysis. Children <5 years most likely have not yet developed a strong immunity and therefore carry high parasite densities (mean parasite density: 9503.23, 95% CI [5438.71, 13567.75]), which leads to a better chance to detect most of the parasites present by PCR. In comparison, a previous study conducted in a highly endemic area in Ghana had estimated an overall detectability of only 35% [6] and reported an age-dependency of detectability [3]. However, this low value for detectability reported form Ghana was derived from individuals of all age groups. Furthermore, a less sensitive genotyping technique, PCR-RFLP, was used and detectability was estimated by a different approach using samples collected in 2 monthly intervals. Despite these obvious differences, our estimate of detectability of 79% in 1-4.5 years old children compares well with the estimate of about 60% in children of the same age from Ghana [3]. The overall very low detectability of 35% in all ages in the Ghana study is due to the extremely low detectability of about 10% in adults [3]. Such reduced detectability in older individuals from endemic areas is thought to depend on their low parasite densities controlled by acquired immunity. The strong age trend seen in the Ghana study was not found in our study. The reason for this is likely the narrow

age range in our study and the focus on children harbouring high parasite densities due to not yet or only partly developed immunity.

The differences in endemicity between our study site and the survey area in Ghana might also have influenced the differences in detectability. In high transmission areas like Ghana, where individuals frequently harbour high MOI, the chance to amplify only the dominant clone in a PCR reaction and to miss minority clones due to template competition is higher than in areas, where infections are less complex.

We investigated the impact of repeated sampling on estimates of FOI. When the number of newly acquired clones between any two consecutive Coartem® treatments was used to estimate FOI, we observed an increasing number of clone acquisition with increasing age. When only the first sample of each 24h bleed was used for the same analysis, the total number of clones observed was lower leading to an overall lower estimate of FOI. It is note worthy that the observed trend of an increased FOI with increasing age was not altered when taking into account either a single sample or both paired samples collected 24 hours apart. Short term consecutive blood sampling therefore provided an improved picture of the parasite population present in a host and led to more precise epidemiological measures, such as prevalence of *P. falciparum*, FOI, or MOI.

The major disadvantages of sampling on consecutive days were a considerable increase in efforts in the field and laboratory, added costs, and additional discomfort for study participants. As children were visited in their villages, a duplication of samples collected and processed inflicted a substantially increased workload on the field team. The limited benefit for more precise estimates of malariological parameters does not justify routine 24h bleeds for future studies. The considerable logistical efforts required for this sampling scheme seems prohibitive and not justified in this age group. However, in older children and adults such increased efforts might well be justifiable or even required, because in endemic areas older individuals often carry lower density infections as a result of acquired immunity. Accordingly, detection in these age groups will be impaired. Our conclusion on a single sample being sufficient might therefore not apply to studies conducted in older individuals.

In a second approach, we used the time between antimalarial treatment and the appearance of the first new *P. falciparum* clone to measure FOI. Similar to the estimates of FOI described above, the age distribution showed that children older than 3 years of age were re-infected

faster and that there was a significant trend towards shorter time to re-infection with increasing age. Incidence rates of *P. falciparum* estimated by microscopy in an endemic area in Nigeria, Garki, increased up to 5-8 years and decreased thereafter [8]. A study conducted in a highly endemic area in Ghana using a more sensitive technique to detect new infections reported an increase in the number of newly acquired infections up to the age of 5-9 [3,6]. Even though we looked at a very narrow age range, our results confirmed these previous findings.

In an alternative approach FOI was estimated in absence of antimalarial treatment by measuring the number of newly acquired clones between any two consecutive follow-up visits. The age relationship of FOI was similar to the first approach in showing an increased FOI in older children, but estimates were overall approximately 1.7 times lower. The advantage of this analysis is that it provides an estimate of FOI under natural conditions. The natural FOI is reflected more closely in absence of antimalarial treatment, where newly acquired parasite clones have to establish themselves in an already infected host and compete against persisting infections. Several studies indicated that established infections do protect against superinfections with new *P. falciparum* clones [9-11]. Artificial infections of mice have also shown that already present parasite clones have a competitive advantage over newly incoming clones [12]. The lower number of clone acquisition per time in absence of antimalarial treatment might therefore be a result of competitive interactions between clones which might affect the ability of newly incoming parasites to establish in the host. Therefore, the estimates of FOI, obtained from our alternative approach that does not study clone acquisition after treatment, most likely represent more precise values. The major reason for the observed discrepancy in FOI determined either after treatment or in absence of treatment is likely due to overestimation of FOI between two treatments. Overestimates are caused by the fact that each interval surveyed was terminated by a morbid episode which in turn is associated with clone acquisition in most of our observations ([13,14] Schoepflin et al., in preparation). Thus a selection bias towards higher clone acquisition was probably introduced in this approach.

Our 3 approaches to estimate FOI from molecular data resulted in different values for FOI. The question arises which approach is the most appropriate? Molecular genotyping allows distinguishing persistent from newly acquired parasite clones and therefore provides the possibility to investigate the clone acquisition in absence of antimalarial treatment. Despite

the fact that all 3 approaches showed a consistent increase of FOI by age, the measure of FOI in absence of treatment seemed to provide the best estimate of the natural FOI, because it took into account competition between parasite clones, when newly acquired parasites have to establish themselves in an already parasitized host. In contrast, measures of FOI after treatment did not account for competition between parasite clones and therefore likely represented an overestimation of FOI.

Generally, all estimates of FOI presented here, irrespective of the approach applied, were probably underestimates, since samples were only collected in 2-monthly intervals. Values of FOI were further hindered by imperfect detectability of parasite clones. However, since these shortfalls apply to all approaches to measure FOI and to all age groups the overall trend of an increasing FOI with increasing age should not have been influenced.

Our observation of numbers of new infections increasing with age suggests that risk factors are acquired with increasing age. These factors must be opposing the benefits of acquired immunity which in turn reduces the risk of acquiring new infections. A number of studies reported a positive relationship between the biting rate of mosquito vectors and the biomass of the human host, i.e. with increasing weight or body surface of the host an increased number of mosquito bites was observed [15,16]. Therefore, one would expect an increase in the number of new infections with increasing age and therefore biomass. Since the detectability was identical between all age groups in our study differential detectability cannot explain the observed difference. Therefore, we propose that a possible explanation for the observed trend in our dataset could be an increased risk of getting bitten by mosquitoes with increasing biomass (Figure 3). Other factors such as changes in behaviour as children grow older are also likely to have an impact on the observed increase in acquisition of clones.

The 2 major outcomes of this study were on the one hand the high detectability of parasite clones observed in children <5 years from PNG. This resulted in a marginal improvement of measures of MOI and FOI by short term sampling in intervals of 24 hours and led us to the conclusion not to recommend for this age group consecutive bleeds after 24 hours, because of immense additional efforts, costs, and discomforts to the study participants. The second main finding was that genotyping in longitudinal samples facilitates determination of the natural FOI since it allows measuring the number of clones acquired in absence of treatment. This avoids a bias through colonization of an empty niche which is introduced by treatment.

ACKNOWLEDGEMENTS

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Table 1: Positivity by PCR in 2 consecutive bleeds 24 hours apart

msp2 PCR result	number of pairs	frequency in relation to number of pairs with at least one positive PCR (n=311)
1st sample positive	21	6.75%
2nd sample positive	28	9.00%
Both samples positive	262	84.24%
% discordant[1]		15.76%

[1]Proportion of sample pairs carrying a malarial infection only diagnosed on one day

Table 2: Presence of *msp2* genotypes in 2 consecutive bleeds 24 hours apart

msp2 genotyping result	number of pairs	frequency in relation to number of genotype pairs with at least one genotype present on either day (n=518)
allele present only in 1st sample	93	17.95%
allele present only in 2nd sample	90	17.37%
allele present in both samples	335	64.67%
% discordant[1]		35.33%

[1]Proportion of genotype pairs for which an *msp2* allele was only diagnosed on one day

Table 3: Detectability of parasite clones in different age groups

Age group	Detectability	CI
1-2 years	0.8	[0.71 – 0.88]
2-3 years	0.81	[0.77 – 0.85]
>3 years	0.75	[0.70 – 0.81]
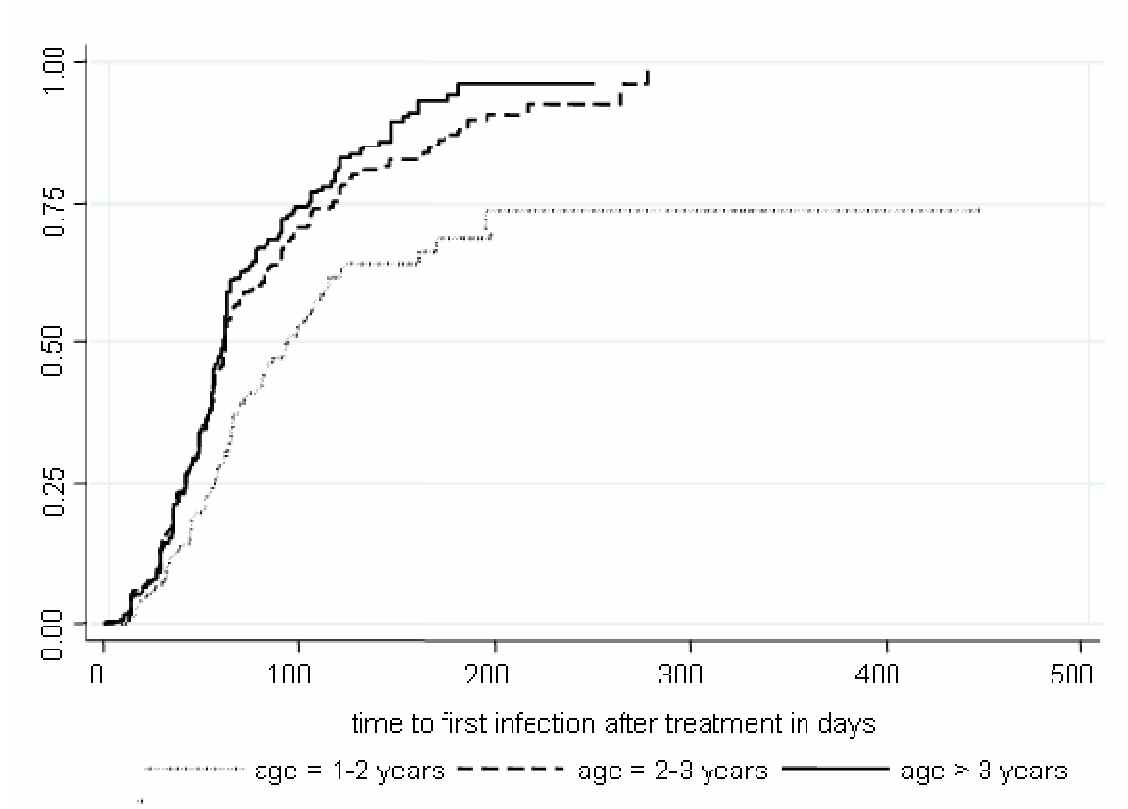
Figure1: Kaplan Meier failure estimates, showing the time to first infection after antimalarial treatment for different age groups.
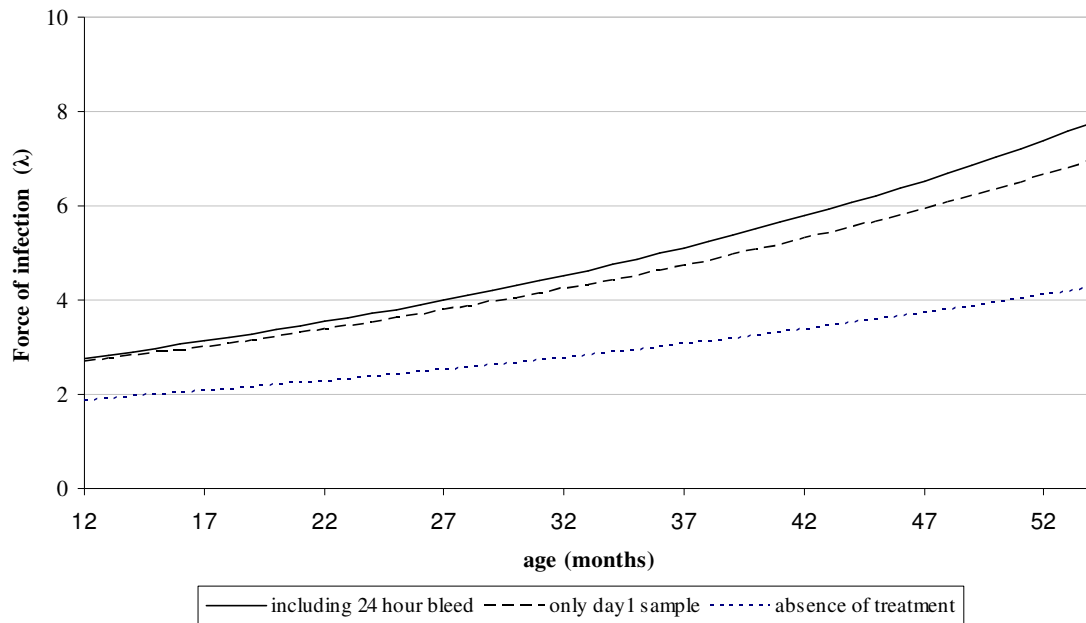
Figure 2: Force of infection in relation to age. The number of clones acquired per time interval was used to fit a Poisson regression model to describe the relationship between the force of infection (λ) and age. The FOI is depicted as the number of new infections acquired per child per year.
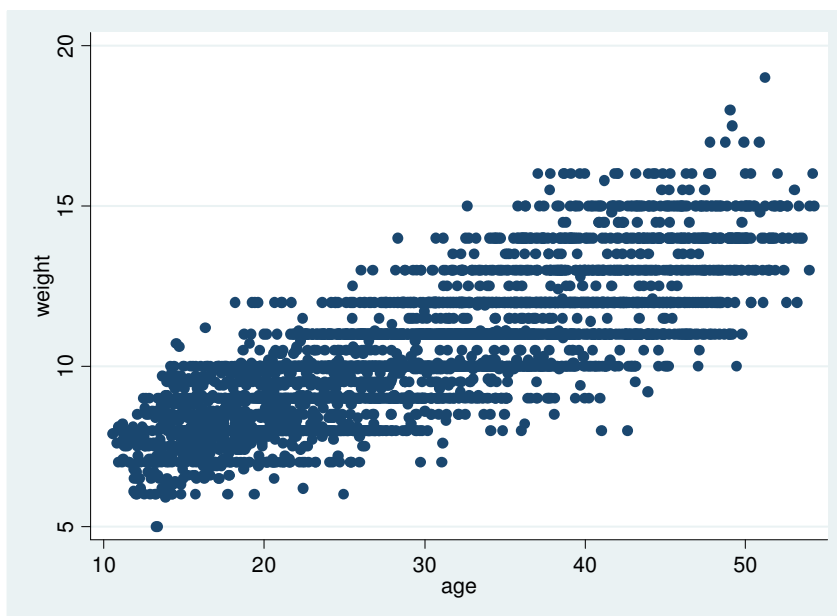
Figure 3: Relationship between age (in months) and weight (in kg) of all study participants.

CHAPTER 6

Parasitological risk factors for *P. falciparum* episodes in Papua New Guinean children

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ABSTRACT

Background: Previous studies have suggested that high multiplicity *Plasmodium falciparum* infections may protect against subsequent malaria morbidity. **Methods:** In a longitudinal study of 269 children between 1 and 4.5 years of age from Papua New Guinea (PNG) we analysed parasitological risk factors for *P. falciparum* morbidity. Repeated blood sampling was conducted in regular 2-monthly intervals and at any time point of presumptive malaria symptoms. The diversity of *P. falciparum* infections was assessed by *msp2* genotyping. **Results:** There was a very high turnover of parasites associated with high morbidity rates. A decreased proportion of episodes in patients concurrently infected with different *Plasmodium* species and a lower *P. falciparum* density in these samples were indicative for species interactions. In children >3 years, a concurrent infection with a heterologous species was associated with higher multiplicity of infection (MOI). In a prospective analysis of parasitological risk factors for episodes a higher MOI was found to protect children > 3 years old against subsequent clinical attacks, which highlighted interdependence of co-infecting parasite clones. **Discussion:** Our study adds to the evidence that ongoing asymptomatic infections appear to offer cross-protection against invading clones. The association of high *P. falciparum* MOI with the presence of other *Plasmodium* species might be due to mixed species infections being treated less often because of lower parasite densities, thus allowing accumulation of multiple parasite clones over an extended period of time. These findings might have implications for the treatment policy of asymptomatic infections and for development and introduction of malaria control interventions.

INTRODUCTION

In individuals from malaria endemic countries, protective immunity against malaria is acquired after repeated *P. falciparum* infections. Such slow and incomplete acquisition of protection is thought to depend on exposure to antigenically distinct parasites characterized by extensive antigenic diversity and antigenic variation [1]. The peak incidence of malaria morbidity in endemic areas varies with the level of endemicity; in Papua New Guinea (PNG) it is found in children under 5 years [2,3]. As a result of acquired immunity, older children and semi-immune adults in endemic countries usually have lower prevalence of infection, carry chronic, low density infections, and have developed an almost complete protection against severe illness (for example [2,4-6]). Protection against disease and protection against parasitization might be governed by quite distinct immune mechanisms [5,7].

Multiplicity of infection (MOI) is the number of parasite clones concurrently infecting one carrier. In highly endemic areas, MOI has been found to be lower in clinical episodes compared to asymptomatic carriage [8-11]. In prospective studies including mostly older children, MOI also seems to protect against subsequent malaria morbidity [12-15]. Such protective effects could be explained as result of cross-protection against super-infecting parasites (premunition). No such effect was observed in a study in infants in a highly endemic area in Tanzania, where the opposite was found: an increased risk of a febrile episode with increasing number of parasite clones [16]. Other studies confirmed MOI to be a risk factor for clinical malaria in infants or young children and in individuals from areas of low transmission intensity [17-21]. These opposing age-specific effects of MOI were delineated for areas of high malaria transmission where in children >3 years multiple concurrent infections have a protective effect, whereas in children <3 years MOI seems to be a risk factor by each additional new infection adding to the risk of developing a morbid episode [5,17,21].

Malaria is endemic in lowland areas of PNG and the malaria situation is characterized by the presence of all four *Plasmodium* species that affect humans. This makes it possible to study interactions among different *Plasmodium* species. Earlier studies found fewer mixed species infections than would be expected by chance, indicating that co-infecting parasite species interact [22]. A study in Thailand [23] showed a reduced risk of severe malaria in patients co-infected with *P. falciparum* and *P. vivax* compared to patients infected with *P. falciparum* alone. Species interactions have also been proposed in a study from Vanuatu where *P.*

falciparum and *P. vivax* co-exist [24]. In a prospective study in PNG, *P. malariae* positivity was associated with protection against subsequent morbidity, though this effect was not specific for malaria morbidity. In the same study a *P. vivax* infection was found to protect specifically against a subsequent *P. falciparum* episode [25]. In contrast, an increased risk of severe malaria was reported for Papua New Guinean children under 5 infected with *P. falciparum* plus *P. vivax* [26] and a further study in PNG also failed to find protective effects of heterologous infection at baseline against subsequent re-infection or *P. falciparum* illness [27].

None of these studies so far has taken into consideration the effect of mixed-species infections on multiple-clone infections. This shortfall is addressed by the present investigation. In a longitudinal study of 269 Papua New Guinean children participating in regular follow-up visits over a period of 16 months, we investigated the genetic profile of parasites from symptomatic and asymptomatic infections. The aim of this study was to assess the impact of mixed species infections on *P. falciparum* clone multiplicity and to define parasitological risk factors for malaria morbidity in 1-4.5 year old children from PNG.

METHODS

Field survey and patients

This study was conducted in the vicinity of Ilahita health centre, in a rural area near Maprik, East Sepik Province, Papua New Guinea. A detailed description of the study is given elsewhere (Lin et al., in preparation). Briefly, 269 study participants were enrolled at an age of one to three years starting in March 2006. Regular follow-up visits were conducted in 8-weekly intervals over a period of 16 months until July 2007. Except for the first and last round of sample collection, two consecutive blood samples were collected by finger prick at intervals of 24 hours for each study participant at each follow-up visit; i.e. each individual contributed up to seven 24 hour-sample pairs plus two rounds of simple bleeds. Active and passive case detection was performed in between the regular 8-weekly follow-up visits and a blood sample was collected from all participants with suspected malaria infection and a rapid diagnostic test (RDT) was performed. Antimalarial treatment with Coartem® (Novartis, Switzerland) was administered upon a positive RDT or if haemoglobin levels were <7.5 g/dl. Informed consent was sought from all parents or guardians prior to recruitment of each child. Scientific approval and ethical clearance for the study was obtained from the Medical Research and Advisory Committee (MRAC) of the Ministry of Health in PNG and from the Ethikkommission beider Basel in Switzerland.

Laboratory procedures

All finger prick blood samples were separated into plasma and cells. DNA was extracted from cell pellets using QIAamp® 96 DNA Blood Kit (Qiagen, Australia) according to the manufacturer's instructions. All samples were genotyped for the polymorphic marker gene merozoite surface protein 2 (*msp2*) by use of capillary electrophoresis as previously described by Falk et al. [28] with some minor changes and adaptations of PCR conditions for highly purified DNA as described by Schoepflin et al. (manuscript in preparation).

Data analysis

All samples that were positive by *msp2* PCR were classified as either an episode or asymptomatic infection. An episode was defined by a positive *msp2* PCR plus fever (i.e. axillary temperature >=37.5°C) or a history of fever in the last two days and / or a parasite

density of >5000 parasites/µl. For the analysis of this work only one sample of each 24 hour bleed was taken into account. If both consecutive samples were classified as being asymptomatic or symptomatic, respectively, the sample collected on the first day was included in the analysis. If either sample was classified as clinical episode and the other one representing an asymptomatic infection, the episode sample was considered for data analysis. Infections were classified as mixed species infection if *msp2* PCR was positive and light microscopy indicated the presence of at least one other *Plasmodium* species. A non-parametric Wilcoxon-rank sum test was used to compare parasite densities between groups. Proportions were compared between groups using chi square test. Linear regression was applied to test for correlations between numerical variables. The incidence of episodes (λ) was calculated as $\dfrac{n}{\sum t}$, where n is the total number of episodes and t is the number of days each individual was under observation during the study.

For analysing the persistence of an allele prior to the episode, those episodes were excluded that carried mixed species infections. The presence or absence of each allele was recorded for all samples collected prior to the episode. If an antimalarial treatment was given at an earlier time point, only samples collected after the last treatment were included.

The dataset used for analysing risk factors for a *P. falciparum* episode contained all asymptomatic infections, irrespective of the infecting *Plasmodium* species and all morbid episodes that were uniquely positive for *P. falciparum*. Each blood sample from an episode was matched to the sample of the same child collected at the preceding follow-up visit. Sample pairs were excluded from this analysis if antimalarial treatment was given at the time of collection of the previous sample or if there was any antimalarial treatment in between these paired samples. Logistic regression analysis was used to estimate the effect of parasitological parameters of the precursor sample on the outcome of an infection. Models were compared using AIC (Akaike's Information Criterion) as measure of goodness of fit. Lower values of AIC indicate a better fit to the data. Statistical significance was tested using likelihood ratio test.

RESULTS

Parasitological parameters in episodes and asymptomatic infections

1334 of 3216 samples genotyped were positive by *msp2* PCR. Of these 787 derived from malaria episodes and 547 from asymptomatic children. An episode was defined by a positive *msp2* PCR plus fever (i.e. axillary temperature >=37.5°C) or a history of fever in the last two days and / or a parasite density of >5000 parasites/µl. Of all PCR positive blood samples, 844 were infected by *P. falciparum* only and did not contain any other *Plasmodium* species according to the results of light microscopy. 533/844 derived from symptomatic individuals. We further detected 490 samples carrying a mixed infection with *P. falciparum* plus any other species, the majority of these (426 samples) being *P. falciparum* / *P. vivax* mixed infections. The incidence of malaria episodes (λ) was estimated approximately 2.5 episodes per child per year. When considering episodes containing only *P. falciparum* and no other *Plasmodium* species, λ was approximately 1.7 *P. falciparum* episodes per child per year.

We analyzed the representation of allelic families and parasite density in symptomatic and asymptomatic infections. A previous genotyping study had reported higher parasite densities in symptomatic episodes of FC27-type infections compared to 3D7-type infections [21]. We also observed an increased parasite density in episodes carrying a single Fc27-clone (63884.66 parasites / µl, CI [50021.6, 77747.7]) compared to 3D7-episodes (42978.9 parasites / µl, CI [32953.6, 53004.2]) (Z=-1.9, p=0.056). No difference in parasite density was observed in Fc27- and 3D7-type asymptomatic infections (p=0.53). Previous studies reported an association between the infecting allelic family and morbidity [19,29]. In our dataset, we could not confirm Fc27-type genotypes to be associated with morbidity. The proportion of *P. falciparum* episodes carrying only Fc27-type alleles was similar in symptomatic and asymptomatic infections (χ^2=0.02, p=0.89). The same was true, when only *P. falciparum* episodes were considered that contained > 5000 parasites / µl (χ^2=0.05, p=0.82).

P. falciparum episodes were further characterised by the persistence of all parasite clones prior to the episodes. For this analysis 470 samples from morbid episodes harbouring 699 *P. falciparum* clones were available. The majority of all episodes (91.8 %) contained *msp2* alleles that were not present in the samples collected prior to the episode (Figure 3). Very few genotypes (6%) persisted longer than 7 days. As our longitudinal study has scheduled sampling in 2-monthly intervals, the distance between an episode and the preceding follow up

sample of the same child ranged between 1 and 60 days. On average, the last follow-up sample was collected 30 days prior to the episode. Thus, alleles newly appearing in an episode were acquired within the preceding 30 days. The previous persistence of parasite clones in episodes was determined from all preceding samples of the corresponding child since the last antimalarial treatment. This retrospective analysis provides an estimate of the minimal persistence of a clone causing a morbid episode. The 2-monthly spacing of regular follow-up bleeds does not allow a more precise determination of the time point at which a new clone first appeared.

The prevalence of infection significantly increased with increasing age (LR χ^2=54.02, p<0.001) (Figure 1). The mean number of concurrent infections has been reported to be age dependent [5,6,11,18,30]. In contrast to these studies, mean MOI did not change when children grew older for both, asymptomatic and symptomatic infections in our study (asymptomatic infections: F= 3.31, p=0.07; episodes: F= 0.01, p=0.9) (Figure. 2). Unlike previous findings [8,9,11] mean MOI did not differ between *P. falciparum* episodes and asymptomatic infections in both age groups (< and ≥ 3 years) of our study participants (Table 1). Furthermore, we investigated the effect of a concurrent heterologous species infection on MOI. We found a higher mean MOI in infections that also harboured other *Plasmodium* species compared to those carrying only *P. falciparum* clones. This was statistically significant for children older than 3 years (Table 1).

Risk factors for *P. falciparum* episodes

The number of episodes was significantly correlated to age (LR χ^2=27.48, p<0.001). We observed an increase in the prevalence of *P. falciparum* disease with increasing age and peak prevalence of disease was reached in children between 3 and 3.5 years of age followed by a subsequent decline in older children (Figure 1). We investigated the effect of parasitological status of the preceding sample on incidence of clinical malaria. We found that the risk of *P. falciparum* episodes decreased with increasing MOI in the previous sample (Figure 4). Previous reports indicated that in children less than 3 years of age multiple infections seem to be associated with a higher prospective risk of clinical malaria [17,21], whereas in older children a higher MOI had a protective effect [12-14,21]. Therefore, we did separate logistic regression analysis for these two different age groups (Table 2). MOI at the previous visit had a significant protective effect against development of an episode in children aged 3 years or

older. This is in line with previous results from Africa [12-14,21]. No significant effect of MOI on a subsequent episode was found for younger children. We further tested, whether the presence of 3D7 or Fc27 type alleles could be defined as risk factors for a subsequent *P. falciparum* episode, but neither of them was found to have a significant effect on the outcome. For children < 3 years, the model with the best AIC value also included the presence of *P. vivax* in the preceding sample. A *P. vivax* was a risk factor for a subsequent *P. falciparum* episode in this age group. In older children the model with the best AIC included the presence of *P. malariae* in the precursor sample, which was found to have a protective effect against a *P. falciparum* episode (Table 2).

Analysis of risk factors was also performed on episodes classified according to the presence of either *msp2* allelic family. Different parameters of the precursor samples were tested as risk factors for a subsequent 3D7- or Fc27-type episode by logistic regression analysis (Table 3). No significant allele-specific effects of precursor infections were observed for the two age groups. A preceding *P. vivax* infection was found to increase the risk for subsequent Fc27-type episodes in children under 3 years. This trend was significant, irrespective of whether the precursor sample contained *P. vivax* only or in combination with other *Plasmodium* species.

DISCUSSION

Based on longitudinal sampling and genotyping of individual *P. falciparum* clones we aimed at identifying parasitological risk factors for a clinical malaria episode in children younger than 5 years in Papua New Guinea. The peak prevalence of *P. falciparum* disease was at 3 to 3.5 years, which agrees with previous studies in PNG [2,3]. A slight decline in the number of episodes in children older than 3.5 years is indicative for the development of immunity after repeated infections.

A previous study from a highly endemic area in Tanzania reported a lower mean MOI in symptomatic children between 1 and 5 years compared to asymptomatic children [8]. Similar results were reported from children living in a high transmission area in Senegal [9]. In contrast to these reports, we found mean MOI to be similar in episodes and asymptomatic infections in both, children younger and older than 3 years. This difference could be explained by the frequent treatment administered to children participating in our survey. In our tightly surveyed study group treatment was given to each child on average every 86 days. Since transmission intensity is much lower in PNG compared to the previously mentioned study sites [31], intervals between treatments were probably two short to allow for development of high multiplicity and thus we did not detect a difference in MOI between asymptomatic and symptomatic infections. As a consequence of our active case detection, the asymptomatic infections in this study do not reflect chronic asymptomatic carriage persisting in absence of treatment over extended periods of time but must be considered of quite recent origin.

Few studies have investigated the dynamics of genotypes when the status of a child changed from asymptomatic infection to a clinical episode [9,32]. Our longitudinal survey permitted to investigate in a large sample set the presence of alleles before and at morbid episodes. However, our study does not provide samples collected daily or in short intervals. We therefore estimated the minimal persistence of each allele. The measure underestimates the true duration of the *P. falciparum* clone causing an episode. This fact did not compromise our estimates substantially because the great majority of alleles detected in episodes were not present in the sample prior to the episode, collected on average 30 days ahead of the episode. These findings support previous studies [9,32], where children were followed up in closely spaced intervals and genotypes in episodes had also newly appeared. However, treatments were given in our study very frequently, on average every 86 days. Such frequent treatment

resulted in a high turnover rate of parasite clones. Therefore, very few long term persisting asymptomatic clones were observed in the entire study and we cannot conclude from our data that persisting infections did not contribute to morbidity as well.

It has been reported previously that higher MOI protects older children or semi-immune adults against subsequent morbidity [12-15,21]. The high number of antimalarial treatments in our study most likely has affected the development of acquired immunity in study participants, since frequent treatment does not allow accumulation of multiple parasite clones within a host. This might result in a lack of protective effect of persisting asymptomatic infections on subsequent malaria morbidity. However, despite this impediment through frequent treatment, we found in a prospective analysis of parasitological risk factors for *P. falciparum* episodes that high MOI protected older children (> 3 years) against a subsequent clinical attack. These results reinforce previous findings [12-15,21] and are consistent with the concept of premunition [5], i.e. ongoing asymptomatic infections offer cross-protection against invading clones. In children < 3 years no significant effect of MOI on subsequent morbidity was observed in our study. This contrasts to previous studies that found a positive correlation of MOI and subsequent episode in this younger age group [17,21] which is thought to be an indication for the lack of previous exposure and therefore development of immunity.

In previous reports Fc27-type infections were associated with symptomatic infections. This was reflected in higher Fc27-parasite densities [21,33] or a higher proportion of Fc27-type alleles in episodes [19,29]. In agreement with earlier results, we observed an increased parasite density in episodes caused by a single Fc27-type clone compared to episodes caused by a 3D7-clone. But in our study Fc27-type alleles were equally prominent in both, samples collected during morbid episodes and in asymptomatic samples. This is in agreement with results from a previous study conducted in the same area in PNG, where also no association between the presence of Fc27 alleles and clinical malaria had been found [11]. Our results indicate that both allelic families are equally contributing to morbidity, but the fact that Fc27-type infections take higher parasite densities are supporting previous suggestions that Fc27-type infections might be more successful in evading the host's immune response [21].

Our prospective analysis did not reveal any significant allele-specific effects on a subsequent *P. falciparum* episode per se (Table 2). In the separate analysis of risk factors for Fc27- or

3D7-type episodes, we did not find any allele-specific effects on the prospective risk of developing a 3D7- or Fc27-episode (Table 3). This finding suggests that within the short time interval including both paired samples, no allele-specific protection was elicited through the most recent and ongoing genotype. An asymptomatic and a symptomatic clone could establish equally well in new hosts and seem not to be prevented by a previous response against family-specific epitopes. This contrasts the assumption that protective responses to the family-specific domains of MSP2 are very short-lived and dependent on the most recent infection. Our study suggests that strain-specific immunity against MSP2 antigens is not raised within such a short period of 38 days on average and that immunity does not primarily reflect the response against the most recent infecting clone. Our data support previous findings that in an early stage of the developing immune response in younger children, defence mechanisms against parasitism are rather unspecific and act via high fever and cytokine effects [34], while the specific immune responses and possibly cross-protecting effects only develop in older children.

A *P. vivax* infection in children <3 years appeared to be a risk factor for a subsequent *P. falciparum* episode, with the risk for an Fc27-type episode being highly significantly increased. It is likely that the preceding *P. vivax* infection reflects higher exposure, as *P. vivax* and *P. falciparum* are transmitted by the same vector. If this is true, a previous *P. falciparum* infection would be expected to have a similar effect on a subsequent episode. Indeed, a similar trend was observed for a preceding *P. falciparum* infection, though this trend was not statistically significant (data not shown). We assume that the risk through a preceding *P. vivax* was only observed for Fc27-type episodes because these occur in higher densities.

A further intention of this work was to identify effects of co-infecting *Plasmodium* species. A comprehensive analysis of cross-species interactions will be presented elsewhere (Mueller et al., in preparation). An important finding was a lower proportion of episodes in mixed species infections and lower *P. falciparum* densities in sample concurrently infected with other *Plasmodium* species. This indicated interdependence among co-infecting *Plasmodium* species. We examined whether species interaction affected *P. falciparum* MOI. We observed in older children that mixed species infections harboured significantly more parasite clones than single species *P. falciparum* infections (Table 1). This is in line with the observed reduced densities in mixed species infections, as lower densities might have reduced the need for treatment and thus could have allowed accumulation of multiple clones.

In summary, our data highlights interdependence among co-infecting *Plasmodium* species and among clones of multi-clone *P. falciparum* infections. Moreover, in children >3 years multiple infections seem to confer protection against subsequent *P. falciparum* episodes. Such findings have implications for the development and introduction of malaria control interventions, e.g. for the treatment policy of asymptomatic infections in older children. Similarly, in view of protection through co-infecting species, interventions targeting only one species might have important adverse effects on the outcome of infections with other species. Future studies investigating in parallel the multiplicity of *P. vivax* infections might provide additional insights into interactions between clones of *P. falciparum* and clones of *P. vivax*.

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Table 1: Mean multiplicity of infection by morbidity status and co-infection with other *Plasmodium* species.

	age group < 3 years		age group ≥ 3 years	
	asymptomatic infections [95 % CI] n=369	symptomatic infections [95 % CI] n=551	asymptomatic infections [95 % CI] n=178	symptomatic infections [95 % CI] n=236
Mean MOI	**1.45**	**1.51**	**1.52**	**1.57**
	[1.36, 1.54]	[1.43, 1.58]	[1.4, 1.64]	[1.45, 1.68]
	p=0.1		p=0.7	

	age group < 3 years		age group ≥ 3 years	
	mixed species infections [95 % CI] n=347	*P. falciparum* infections [95 % CI] n=573	mixed species infections [95 % CI] n=143	*P. falciparum* infections [95 % CI] n=271
Mean MOI	**1.55**	**1.45**	**1.69**	**1.47**
	[1.45, 1.65]	[1.37, 1.52]	[1.52, 1.85]	[1.38, 1.57]
	p<0.07		**p<0.05**	

Table 2: Logistic regression analysis of factors affecting the morbidity outcome

CHILDREN <3 years (n=294)

Risk factors	OR	Likelihood ratio $\chi 2$	p-value
P. vivax in previous sample	1.89	6.99	**<0.01**
MOI in previous sample	0.59	1.71	0.19
Fc27 type allele in previous sample	1.63	0.77	0.38
3D7 type allele in previous sample	1.32	0.23	0.63

CHILDREN >=3 years (n=152)

Risk factors	OR	Likelihood ratio $\chi 2$	p-value
P. malariae in previous sample	0.11	5.43	**<0.05**
MOI in previous sample	0.7	3.96	**<0.05**
3D7 type allele in previous sample	1.39	0.34	0.56
Fc27 type allele in previous sample	0.91	0.03	0.86

Table 3: Logistic regression analysis of factors affecting the outcome of subsequent Fc27- or 3D7-type infections

Risk factors for an Fc27-type episode	Age group < 3 years n=193 (128 asymptomatic infections; 65 Fc27 episodes)			Age group >= 3 years n=89 (54 asymptomatic infections; 35 Fc27 episodes)		
	OR	Likelihood ratio χ2	p-value	OR	Likelihood ratio χ2	p-value
MOI in previous sample	0.63	0.46	0.5	0.94	0.01	0.93
3D7 type allele in previous sample	0.43	0.69	0.41	0.13	2.51	0.11
Fc27 type allele in previous sample	1.97	0.61	0.43	0.69	0.12	0.73
P. vivax in previous sample*	2.79	10.18	**<0.01**	0.96	0.01	0.94

Risk factors for a 3D7-type episode	Age group < 3 years n=194 (128 asymptomatic infections; 66 3D7 episodes)			Age group >= 3 years n=95 (54 asymptomatic infections; 41 3D7 episodes)		
	OR	Likelihood ratio χ2	p-value	OR	Likelihood ratio χ2	p-value
MOI in previous sample	0.5	1.66	0.2	0.89	0.03	0.86
Fc27 type allele in previous sample	1.23	0.07	0.8	0.16	2.65	0.1
3D7 type allele in previous sample	2.75	1.89	0.17	0.62	0.23	0.63
P. vivax in previous sample	1.3	0.71	0.4	0.69	0.67	0.41

* presence of *P. vivax* alone or as mixed species infection with any *Plasmodium* species
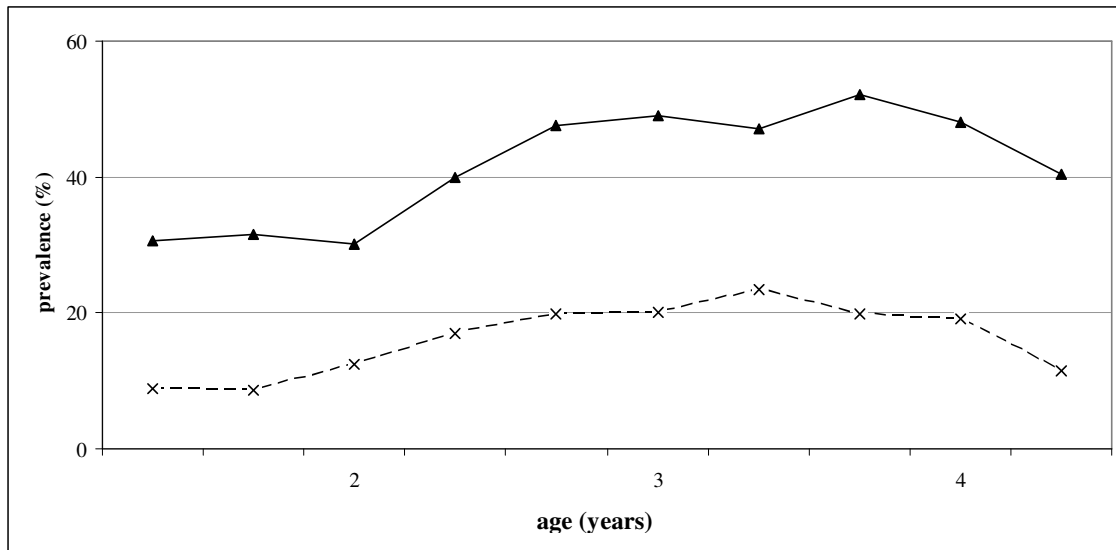
Figure 1: Prevalence of *P. falciparum* infection (solid line) and of *P. falciparum* disease[*] (dashed line) in cross sectional surveys.

[*]Disease was defined as positive *msp2* PCR plus fever (i.e. axillary temperature >=37.5°C) or a history of fever in the last two days and / or a parasite density of >5000 parasites/µl.
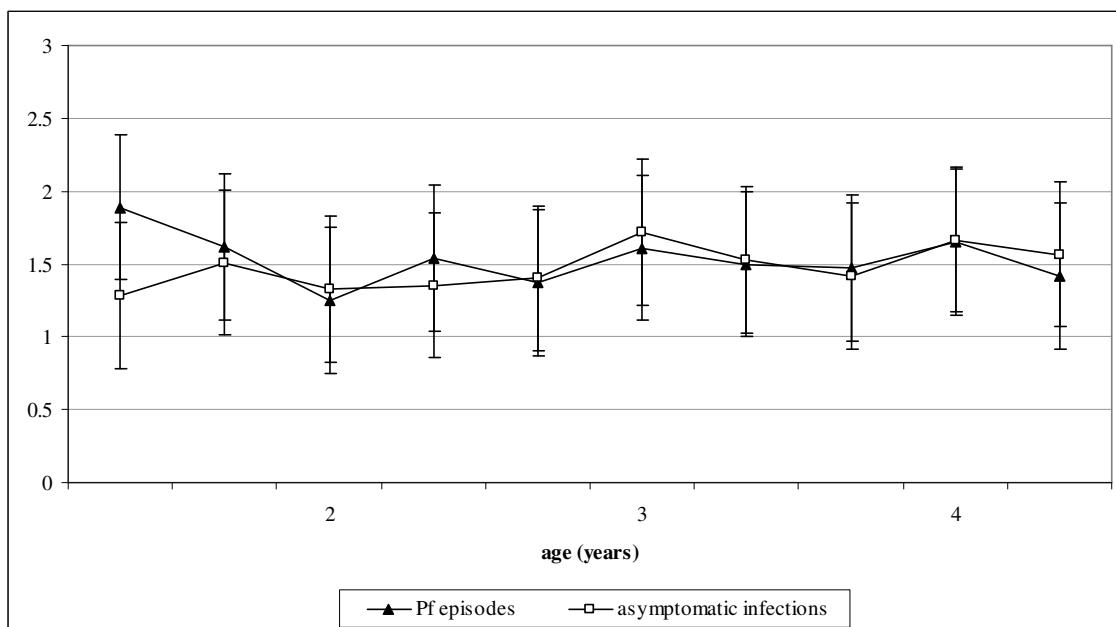
Figure 2: Mean multiplicity of infection (including only samples with MOI>0) from children aged 1 – 4.5 years. ▲ samples from symptomatic children carrying *P. falciparum* only. □ samples from asymptomatic children. Pf= *P. falciparum*
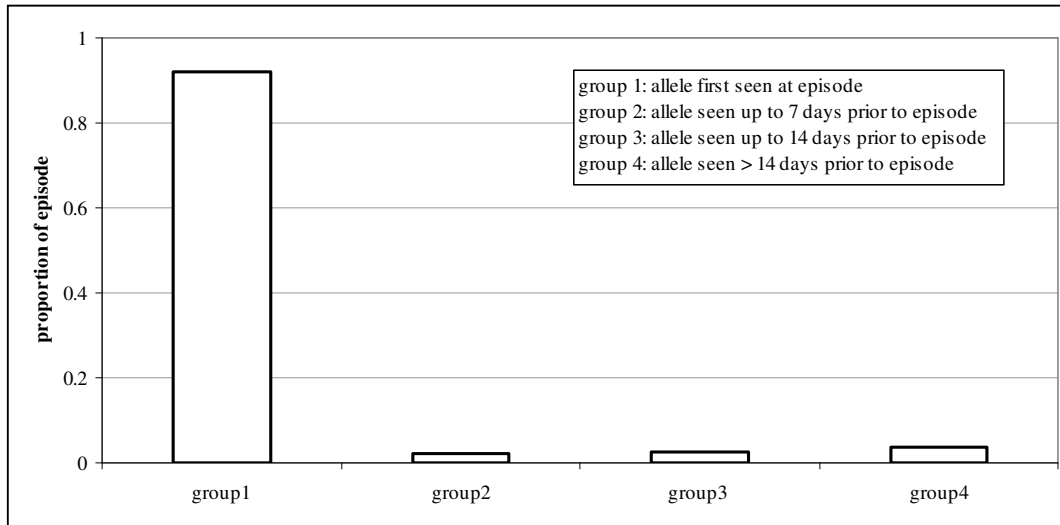
Fig. 3: Persistence of alleles prior to an episode caused by *P. falciparum*
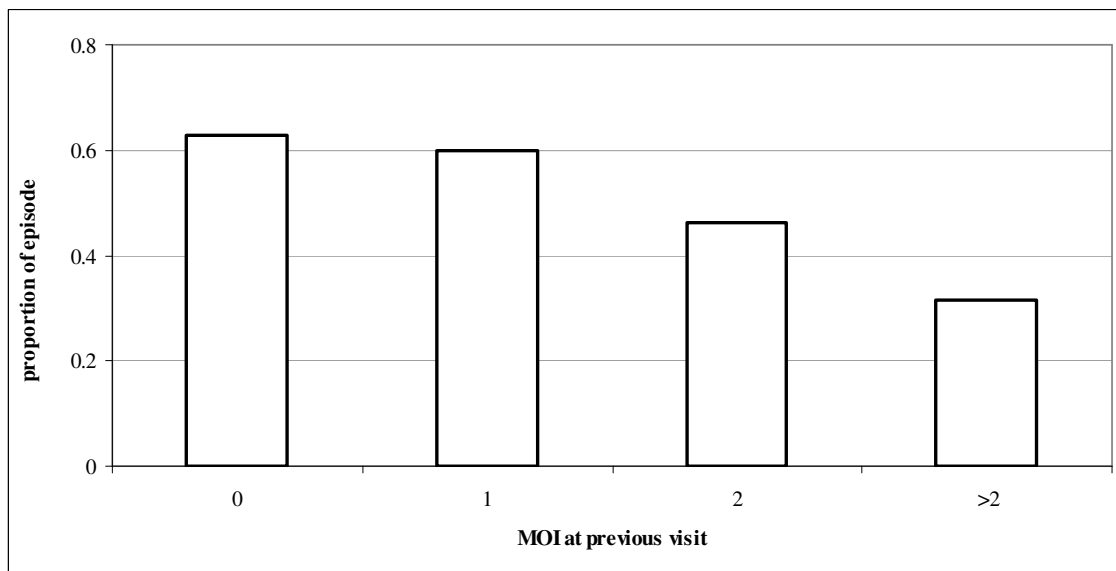
Figure 4: Risk of *P. falciparum* episodes at time t in relation to multiplicity of infection (MOI) in the preceding sample (time t-1).

CHAPTER 7: General discussion

Under the auspices of the Institute of Medical Research in Papua New Guinea, a longitudinal field survey was conducted in a cohort of 269 children between 1-4.5 years of age from a malaria endemic area. The main goal of the PNG field study was to assess the existence of cross-species protection against malarial morbidity, with a particular focus on whether asymptomatic infections protect against subsequent morbidity due to heterologous infections. The objectives of this PhD thesis were in parts nested within the field project's focus on species interactions by contributing genotyping data for *P. falciparum*. This permitted a view on species interactions on the level of individual parasite clones. Genotypes of parasites were compared between phases of asymptomatic carriage and morbid episodes in order to define parasitological risk factors for malaria morbidity and to determine the impact of co-infection with a heterologous species on the multiplicity of infection.

Other objectives of this thesis were developed as ancillary studies to the main field project making further use of the longitudinal samples collected during the 16 months follow-up period. A major focus was on estimating the loss of fitness caused by mutations associated with drug resistance. The aim was to make use of classical malariological parameters in combination with molecular parameters describing infection dynamics of *P. falciparum* to describe the survival and model the transmission success of resistant versus sensitive parasite clones. This exploratory project was carried out in 2 steps, involving samples from two different field studies: one earlier treatment to reinfection study [1] and the 16 months longitudinal study.

Fitness costs of drug resistance

Mutations associated with antimalarial drug resistance are thought to be disadvantageous for parasites once drug pressure is abolished. Experimental evidence does not only come from in vitro experiments [2] or mouse models [3,4], but also from field studies in the human host [5-7]. Because fitness in terms of transmission success of *Plasmodium* parasites cannot be determined experimentally in a simple and straight forward way, a surrogate marker for fitness would be helpful to quantify the loss of fitness associated with drug resistance mutations. We hypothesised that the survival of parasite clones within human hosts can be

used as surrogate for transmission probability. If this assumption was true, one would expect to find differences in drug resistance mutations in persisting versus short term infections. If drug resistance mutations incur a fitness cost by impairing the parasites' survival, long term, chronic infections should harbour less SNPs compared to rapidly cleared infections. This difference could be observed only in absence of drug pressure. Our approach considers all known SNPs from several genes associated with drug resistance summarized as drug resistance haplotype.

Determination of the haplotype of a clonal infection is straight forward only in single clone infections. In areas of high MOI, identification of all haplotypes of multiple co-infecting clones is impossible. In PNG, mean MOI for *P. falciparum* is low and most individuals harbour single clone infections ([8,9], and own data) making it possible to clearly assign drug resistance haplotypes. Thus, low MOI in PNG offers optimal study conditions to investigate effects of haplotypes of drug resistance mutations on parasite fitness.

In a first attempt to measure fitness costs incurred by drug resistance we tested whether reduced parasite fitness could be measured as effect on parasite survival. We compared the drug resistance haplotypes of single clone infections between new infections acquired after radical cure and chronic infections in adults from an endemic area in PNG. A reduced frequency of a 7-fold mutated haplotype and increased frequency of a 5-fold mutated haplotype in long term persistent infections indicated an impaired fitness of highly mutated parasites to develop into chronic infections. These results suggested that it's a suitable approach to use the duration of an infection as marker for parasite fitness. However, the disadvantage of this pilot study was that samples from adults derived from a cross-sectional study and consequently, the duration of persistence of the detected parasite clones in these samples was not known. Instead it was assumed that adults carry long term infections, based on previous results from an endemic area in Africa [10].

Better estimates of the reduction in parasite survival due to the presence of mutations would be achieved by correlating the actual persistence determined by longitudinal tracking of individual clones to the drug resistance haplotype of each clone. Such an analysis requires a sufficient number of infections that have not been cleared by antimalarial treatment. We intended to retrieve these samples from untreated periods of several months from the longitudinal study in children from PNG. However, a great proportion of all newly acquired

infections caused a clinical episode which had to be treated with antimalarials. This prevented the establishment of long-term persisting infections and led to a high turn-over of parasites in our study children. Only very few long term infections were observed in our cohort of young children. Therefore, the samples collected in our longitudinal field study could be used to estimate the clone acquisition rate, but were not suitable for estimating natural clearance rates and clone persistence.

There are several reasons for the high number of treatments administered to the study participants. On one hand, the age group enrolled in our study accounts for precisely the fraction of the population with the greatest burden of disease. On the other hand it is possible that the study design including active and passive case detection might have resulted in an increased frequency of treatment. This might have led to an impaired development of immunity, and as a consequence each newly acquired infection caused another symptomatic episode that had to be treated. Thus, the establishment of asymptomatic long-term infections was affected. Furthermore, according to the standard treatment in PNG all *Plasmodium* infections that were associated with anaemia (Hb<7.5g/dl) had to be treated with antimalarials, irrespective of the presence of malaria symptoms. This further increased the amount of Coartem® dispensed during the course of our study.

Deprived of the possibility to estimate duration of infections, we were not able to determine the association between molecular markers of drug resistance and the persistence of parasite clones. This part of the initial work plan had to be postponed until a new set of samples from older and untreated individuals becomes available. We expect that in an age group older than the one studied here, infections less frequently cause morbid episodes requiring treatment. While adults seem to be optimal, these age groups are not easily available for longitudinal surveillance because of occupational constraints or migration. Thus, the group of oldest school children seems to be the most suitable study group for obtaining infections of potentially long duration.

Outlook

After completion of this PhD thesis, it is foreseen to subject the limited number of persisting single clone infections detected in our field study to characterization for drug resistance SNPs and to compare results to haplotypes of the abundant new infections. This approach is similar to the one of our pilot study except that our first approach [11] only assumed a persisting

infection, while now we can delineate the duration of haplotyped clones. We expect to collect further data supporting our previous results of differential distribution of drug resistance haplotypes in new versus persisting infections. A more pronounced difference in the haplotype frequency between new and persistent infections may be detected, because the data from our longitudinal study are less prone to assumption errors. However, as the available samples from persisting infections will not exceed 50 parasite clones, and because durations are limited to >2 months, further mathematical modelling might not be possible due to scarce data.

Relevance of this project

Detailed investigations of fitness costs of drug resistance might have important implications for the guidance of treatment policies and for the ongoing efforts to best manage the implementation of new antimalarial drugs in a country. If fitness costs of drug resistance are high enough to lead to a resurgence of wild type alleles once the specific drug usage is discontinued, this might results in the possibility of re-using this drug at a later time point. This is a particular advantage for the use of CQ, as this drug has been a widely available, effective, safe and affordable antimalarial for a long time. However, it should be decided with great care about the re-introduction of drugs, and in any case this should be done in a combination with other effective partner drugs. A rotative usage of drugs over intervals of years in well matched combination therapies has recently been proposed [12]. Though this might be a conceivable solution to control the spread of drug resistance, it might practically be very difficult to achieve in routine clinical practice as it seems to be important to achieve a complete replacement of drugs. This emerged from of a recent first approach to estimate the fitness costs of drug resistance based on the reduction of the mutant *pfcrt*76 allele in Kenya and Malawi [5,6] after the cessation of CQ as first line treatment. A much lower estimate of fitness costs was estimated for Kenya compared to Malawi (5% vs. 12%). Ineffective replacement of CQ and sustained use of AQ, which is a close analogue of CQ and known to select for *pfcrt*K76T were proposed to have contributed to the lower fitness costs in Kenya. These findings further indicate that the type of drug that is used to replace the ineffective treatment regimen might play a crucial role whether and to what extent a reduction of the resistant parasite population can be achieved. Since Coartem® was found to select for the wild type *pfmdr1* N86 and *pfcrt* K76 alleles [13-18] which are associated with CQ sensitivity this seems to be an ideal drug for replacement of CQ.

Infection dynamics of *P. falciparum*

Advances in the development of molecular genotyping techniques have greatly increased our knowledge of the population structure and genetic profile of *Plasmodium* infections and has given insights into the dynamics and complexity of infections. Optimal discrimination of parasites within a host can be achieved by choosing a marker gene that is highly polymorphic and shows a homogeneous distribution of allele frequencies. The quality of genotyping can further be improved by the technique applied to determine individual clones. In the course of this thesis we have developed an improved protocol for genotyping the polymorphic marker gene merozoite surface protein 1 (*msp1*) based on capillary electrophoresis. It has previously been shown for *msp2* that capillary electrophoresis has a greater discrimination power than previously applied techniques [10]. We have compared the allele frequency distribution of *msp1* and *msp2* for two countries of different endemicity (PNG vs. Tanzania (TZ)) and showed that in both countries *msp2* was most polymorphic. Our results confirmed previous reports of an increased diversity of *msp2* with increasing endemicity [19-22]. In TZ the probability of two parasites sharing the same *msp2* allele was low and indicated that *msp2* could qualify to be used as a single marker for genotyping. However, the high MOI observed in Tanzanian samples likely has a negative impact on discrimination power because each additional co-infecting clone increases the chance to become superinfected with a clone already present. To quantify this effect of MOI we made an attempt to estimate the probability of observing the true number of alleles present in a host. This probability was found to rapidly decrease with increasing MOI. The fact that high MOI reduced discrimination power highlights the importance of genotyping more than one marker gene. Our results are of particular importance for genotyping samples from drug efficacy trials where recrudescent and new infections have to be reliably distinguished and the probability of newly infecting parasites having the same allele as an initial infection should be minimized.

Consecutive blood sampling from the same individual has previously shown that parasite populations undergo rapid fluctuations [23,24] and that a single sample underestimates the true MOI. In order to estimate the detectability of parasite clones in our study and to assess the benefit of drawing repeated blood samples 24 hours apart, we genotyped two consecutive blood samples from each participant collected at all regular follow-up bleeds. In contrast to previous estimates [10,25], which had included participants of all age groups, we estimated an overall very high detectability in our group of young children. In endemic areas parasite densities are usually higher in young children compared to adolescent or adults [26-29]. This

suggests that the high detectability determined by us is likely a result of high parasite densities in our age group.

We have investigated the effect of collecting 2 samples 24 hours apart on the outcome of basic epidemiological parameters. As expected, we obtained more accurate estimates of the parasite population within a host which was reflected in a slight increase of the mean MOI when genotyping results from both days were combined. We further showed that in our study cohort the risk of acquiring new infections increased as children grew older and that collecting 24 hour bleeds resulted in a small increase of the measured FOI. Since the observed age-trend remained similar and the increase in MOI was only marginal, it does in our opinion neither justify the immense additional costs and logistical efforts associated with this study design nor the additional discomfort caused to the study participants. Therefore, for this age group we consider it unnecessary to collect repeated samples in short intervals, i.e. after 24 hours, for studies investigating basic epidemiological parameters. It has been shown that in endemic areas, detectability greatly depends on the age of the host with a drastic decrease in detectability in adults [25]. Therefore, the above conclusion on repeated sampling might not be appropriate for semi-immune individuals in endemic areas who better control parasites and consequently carry low-parasite density infections that fluctuate around the detection limit of PCR techniques.

Imperfect detectability likely has important implications for the accuracy of results from drug efficacy trials. Suboptimal clone detection during molecular genotyping of baseline and follow-up samples leads to an underestimation of the number of recrudescent infections. A recent drug efficacy trial conducted in Tanzania indeed reported a lower efficacy of the tested drug therapies when an enhanced protocol was applied which included consecutive blood sampling following enrolment and follow-up visits compared to the standard protocol generally applied [30]. Despite the reported benefit of repeated sampling reported from this study, WHO and Medicines for Malaria Venture (MMV) currently do not recommend conducting 24 hour sample collection after baseline and follow-up samples. Reasons for this include very fast acting new drugs leading to negative samples on day 2, additional costs, involvement of complex statistical analysis, lack of convincing evidence in favour of 24 hour repeated sampling, and the fact that patients would have been given rescue treatment on the follow-up visit in case of presence of parasites, irrespective of whether these are recrudescent or new infections [31]. Our finding of high detectability of parasite clones in our study

children supports these current recommendations, however for reasons stated above, this might not apply for drug trials conducted in older age groups.

Coartem® treatment failures

During the analysis of *msp2* genotyping in our longitudinal samples, a high number of Coartem® treatment failures in treated children became evident. To confirm these treatment failures, we have genotyped a second marker, *msp1*. This genotyping system is also based on capillary electrophoresis and has high discrimination power. Genotyping of a third marker (microsatellite TA81) was performed by our collaborators from Case Western Reserve University (USA). We have shown in chapter 3 that genotyping of *msp1* and *msp2* resulted in a very low probability of observing a new infection with the same genotype than the one present in the treatment sample. Genotyping 3 molecular markers to distinguish recrudescent from new infections therefore provided a very high discriminatory power and confident identification of treatment failures.

The high rate of parasitological and clinical treatment failures observed in our study differs remarkable from the report of a recent drug efficacy trial in PNG where Coartem® was found to be highly effective against *P. falciparum* [32]. Our results indicate poor adherence to the complex dosing regimen in combination with insufficient fat supplementation as potential explanation for the observed difference. Drug trials are likely to represent artificial conditions where compliance to drug regimens and fat supplementation are optimal. Our study better reflects the situation in routine practice, because treatments were mostly administered unsupervised and we did not provide any fat supplementation. Therefore, our results highlight a potential problem of Coartem® treatment in routine clinical practice and emphasize that the forthcoming introduction of Coartem® as first line treatment in PNG should be paralleled with provision of training and education of health workers in order to guarantee optimal compliance to the recommended treatment guidelines. It is essential to make great efforts to convince patients to complete the full regimen even after relief of symptoms.

Risk factors for clinical episodes

The high frequency of clinical episodes in participants of our longitudinal field survey made it possible to assess parasitological risk factors for malaria morbidity in children under 5 years from PNG. Our prospective analysis indicated that interactions between different parasite clones do occur. In children >3 years, multiple clone infections were found to protect against a subsequent *P. falciparum* episode which was consistent with previous findings [33-36]. The occurrence of clone-interactions was further supported by our findings from estimating FOI. Using molecular typing techniques to distinguish individual parasite clones allowed us to estimate FOI in absence of antimalarial treatment. Comparison of estimates between absence and presence of treatment showed a lower FOI in absence of treatment. One explanation for this observed difference might be that asymptomatic infections cross-protect against newly invading parasites which might have resulted in a lower number of new clones acquired per survey interval. Substantial competitive interactions were also found in artificial infections in mice [37], where the outcome of competition was determined by several factors. The extent to which parasite clones suffered from competition was affected by the genotype of the infecting parasite, by the order in which parasites were inoculated, and by the duration of prior residency. All clones suffered from competition when they were simultaneously inoculated or after their competitor. Moreover, the longer the time between two inoculations, the greater was the competitive suppression of the first over the second clone. These results support our and previous ([29] and references therein) findings of asymptomatic infections protecting against superinfections. If asymptomatic multi-clonal infections offer cross-protection against superinfections, interventions targeting asymptomatic infections might have significant adverse effects on malaria morbidity, because treatment of asymptomatic infections might lead to clearance of the protective effect of already persisting infections.

The presence of all four species infecting humans in PNG provides suitable conditions to study interspecific interactions between different *Plasmodium* species. If hosts harbour several related parasite species, these species are likely to interact by competing for resources, e.g. host cells. Such interactions could be antagonistic or asymmetrical, i.e. one species could have a negative effect on density or reproduction of the other. In contrast, sharing a host could also provide benefits for one of the species: the presence of one species could interfere with host defense mechanisms and could thus promote the multiplication rates of the other species. To elucidate this aspect of parasite ecology might have implications for antimalarial intervention strategies.

A number of previous studies indicated that interactions between species exist in natural populations, though results about cross-species protection have been inconsistent [1,38-40]. A comprehensive analysis of species interactions in our longitudinal study will be published elsewhere. In the course of this thesis, interactions were analysed with respect to multi-clonal *P. falciparum* infections. We observed a significantly higher MOI in samples that were concurrently infected with other *Plasmodium* species, supporting evidence for interactions among co-infecting species. This is in line with lower *P. falciparum* densities in infections simultaneously harbouring other *Plasmodium* species. Lower *P. falciparum* densities might have led to the decreased proportion of episodes observed in mixed species infections and consequently to fewer treatments which in turn might have allowed accumulation of multiple parasite clones. These findings are consistent with the idea that cross-species protection mainly acts via species transcending density regulation. In a study conducted in PNG, comprising sampling in short-term intervals over 61 days, Bruce and co-workers (reviewed in [41]) found that the total parasite density of all *Plasmodium* species fluctuated around a threshold and that peaks of infection with different species did not coincide. If more than one *Plasmodium* species occupy the same host, these must divide the available resources and will be affected by cross-reactive immune responses. Increasing densities of one species can have a negative effect on another by stimulating a host response that acts against both species. This might lead to lower parasite densities and, as morbidity is associated with high densities, to fewer episodes.

Our results bear relevance for antimalarial intervention programmes. Should heterologous species interact within a host, interventions targeting only one species, such as vaccines, could potentially increase the burden of morbidity attributable to the other species.

Outlook

Important insights into species and clone interactions could be gained by genotyping *P. vivax* and *P. falciparum* co-infecting the same individual or by genotyping both species in samples collected longitudinally. A capillary electrophoresis based genotyping technique to distinguish individual *P. vivax* clones was recently developed at STI [42]. Using this technique, all *P. vivax* samples collected in our study cohort will be genotyped for the two most polymorphic markers. Complementing our data with genotypes of *P. vivax* will permit to study in great detail interactions between multi-clone infections of both *Plasmodium* species and their prospective effects on morbidity.

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Effect of the malaria vaccine Combination B on merozoite surface antigen 2 diversity

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Abstract

Extensive genetic polymorphism is generally found in *Plasmodium falciparum* surface antigens. This poses a considerable obstacle to the development of a malaria vaccine. In order to assess possible effects of a polymorphic vaccine, we have analyzed the genetic diversity of parasites collected in the course of a phase 2b field trial of the blood stage vaccine Combination B in Papua New Guinea. The full-length 3D7 allele of the merozoite surface protein 2 (MSP2) was included in Combination B as one of three subunits. Vaccinees had a lower prevalence of parasites carrying a 3D7-type allele (corresponding to that in the vaccine) and selection appeared to favour the alternative FC27-type alleles resulting in a higher incidence of morbid episodes associated with FC27-type parasites. We sequenced MSP2 alleles detected in study participants after vaccination to identify breakthrough genotypes. Extensive genetic diversity of MSP2 was observed in both the repetitive and family-specific domains, but alleles occurring in vaccine recipients were no different from those found in placebo recipients. A phylogenetic analysis showed no clustering of 3D7-type breakthrough infections from vaccine recipients. The repeat unit present in the vaccine molecule occurred in a number of alleles from the trial area and was also observed in vaccinated individuals. Thus the anti-repeat immune response did not lead to elimination of parasites carrying the same repeat unit. We conclude that the conserved epitopes in the family-specific domain were the most important determinants of the vaccine effect against new 3D7-type infections and that the hypervariable domains were not subject to selective effects of the vaccine.
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1. Introduction

Plasmodium falciparum surface proteins, and in particular those coating the invasive merozoite stage, are considered prime candidates for vaccine development. Merozoite surface proteins are accessible to the immune system between the rupture of a schizont-infected erythrocyte and reinvasion. Antigens located on the merozoite surface are generally well recognized by the immune system, but unfortunately these antigens also exhibit extensive polymorphism. Antigenic diversity is thought to help the parasite in escaping human immune defences (Anders, 1986).

Extensive genetic polymorphism poses considerable obstacles to vaccine design. If only a single allele of a polymorphic antigen is used as a malaria vaccine, the protection achieved might be directed only against the same or similar variants. The merozoite surface protein 2 (MSP2) of *P. falciparum*, which constitutes a major component of the surface coat of the merozoite, is an example of a highly polymorphic antigen that has been used for vaccination. The recombinant, full length MSP2 molecule was one component of the Combination B subunit vaccine along with MSP1 (190LCS.T3), and the Ring-infected Erythrocyte Surface Antigen (RESA). In 1998 Combination B was tested in a randomised, 4-armed placebo-controlled, double-blind Phase I/IIb trial (natural challenge) in 120 Papua New Guinean children aged 5–9 years. The subgroup of vaccinated children that were not treated with the anti-malarial sulfadoxine-pyrimethamine prior to vaccination, had on average 62% lower parasite densities than controls (Genton et al., 2002).

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A single allele of the highly polymorphic MSP2, deriving from the 3D7 strain, was included in the Combination B vaccine. The major portion of MSP2 is polymorphic, only the N- and C-terminal domains are conserved. A dimorphic region flanking a repetitive domain identifies the two allelic families of MSP2, the 3D7-type and FC27-type alleles. The units of tandem repeats vary considerably in length and sequence between different *msp2* alleles. Difference in repeat copy number causes extensive length polymorphism, which forms the basis of various *msp2* genotyping schemes.

Genotyping all blood samples collected during the trial at fortnightly intervals over 18 weeks revealed that the vaccine exhibited specificity for infections belonging to the 3D7 allelic family. This efficacy against 3D7 parasites was not evident in blood samples collected from morbid episodes during an extended 1-year follow-up. However, during the follow-up there was a higher incidence of clinical episodes with FC27-type parasites in vaccinated children than in placebo recipients. These were the first reports of a selective effect exerted by vaccination with a polymorphic malaria vaccine (Genton et al., 2002; Felger et al., 2003).

Combination B, as most other malaria vaccines currently in development, was not expected to provide sterilizing immunity. Such imperfect vaccines may select for specific breakthrough parasites, comprising variants not cleared by vaccine-induced immunity. While genotyping of all samples from the vaccine trial had shown that the 3D7 vaccine differentially affected the alleles of the FC27 and 3D7 family (Genton et al., 2002), the vaccine effect on individual 3D7-type MSP2 alleles from subsequent infections has not yet been examined. To monitor the impact of the imperfect MSP2 vaccine subunit of Combination B on the subsequent genetic diversity of MSP2, we have now sequenced *msp2* alleles from both breakthrough and placebo group infections. Phylogenetic sequence analysis was applied to identify characteristics of genotypes resistant to vaccine effects. Such analysis has been postulated to be of great relevance, since partially effective vaccines could potentially select for more virulent pathogens (Gandon et al., 2001).

2. Materials and methods

2.1. Study population

One hundred and twenty children aged 5–9 years from the Wosera area of Papua New Guinea were recruited for a phase I/IIb field trial of Combination B, starting in February 1998. The study was designed as a four-armed placebo controlled trial with one half of the children being pre-treated with SP at baseline. Venous or finger prick blood samples were collected at baseline and during eight consecutive cross-sectional surveys as described by Genton et al. (2002). During a 1-year morbidity follow-up finger prick blood samples were taken from all children reporting with a history of fever in the last 3 days.

2.2. Sample collection

From the 120 children enrolled in the trial, 1079 blood samples were collected at baseline and during the 18 weeks

follow-up period. Isocode stix dip sticks (Schleicher and Schuell) were used for transport and storage of blood pellets after removal of serum. All samples were analyzed by PCR, and 257 of these were found to be positive for *P. falciparum*. In addition, 449 blood samples were collected from those 120 children during the 1-year morbidity follow-up (296 samples from self-reported case detection at the health center and 153 samples from community-based case detection through weekly visits by village reporters). From these 449 morbidity follow-up samples, 202 were positive for *P. falciparum* by PCR.

2.3. Genotyping

Isolation of *P. falciparum* DNA and *msp2* genotyping was performed as previously described (Felger et al., 1994; Felger and Beck, 2002). The variable central part of the *P. falciparum msp2* gene was amplified by PCR. Subsequent restriction digests produced a genotype-specific RFLP pattern for each different parasite clone in a blood sample. PCR-RFLP genotyping distinguished 39 *msp2* alleles in 257 parasite-positive blood samples of the trial. During the following year of morbidity follow-up, three additional *msp2* alleles were detected in morbid episodes. This amounts to 42 *msp2* alleles (eight of the FC27 family, 34 of the 3D7 family) present in the study area.

2.4. Sequencing

During the entire study we sequenced 32/34 different 3D7-type *msp2* alleles identified by PCR-RFLP. Alleles from Single infections were chosen for analysis. Two alleles only occurred in multiple infections also harbouring other alleles of the same allelic family. Thus direct sequencing was not possible for 2/34 alleles. The nested *msp2* PCR product was directly sequenced using both PCR primers and two internal sequencing primers (5′-CAGTTTGTTCGGCTGTTGGA-3′ and 5′-CTGAA-GAGGTACTGGTAGA-3′). The sequencing reaction was performed with Big Dye sequencing reagents (Applied Biosystems) according to the supplier's instructions and loaded to an ABI PRISM 310 genetic analyzer. The ABI Sequence Navigator program was used for sequence analysis. Sequences were submitted to Genbank under the accession numbers: U07001, AY534507, U07009, U16840, U16842, DQ162622, DQ168572, DQ166534, DQ185319, AJ318755, AJ318753, AJ318752, AJ318754, DQ166535, DQ174442, DQ166536, DQ158904, DQ185320, DQ168571, DQ166545, DQ166537, DQ166538, DQ166546, DQ166539, DQ166540, DQ166541, DQ166542, DQ166543, DQ166544, DQ171731, DQ171732, M73810.

2.5. Phylogenetic analysis

For sequence alignment the ClustalX program (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) was used. Phylogenetic analysis was performed with PHYLIP (Phylogeny Inference Package) version 3.6.a3 (Felsenstein, 2002).

APPENDIX

Distance and parsimony methods were chosen to calculate the fractions of sites that differ between MSP2 alleles. Trees were constructed by the Neighbor Joining as well as by the parsimony algorithm using 1000 bootstrap replicates. Phylogenetic trees were viewed by the Tree View program version 1.5.2 (Page, 1996).

2.6. Definition

A breakthrough infection was defined as a new infection which occurred from week 8 onwards in children immunized with Combination B, and which has not been present in the blood sample of the same individual at baseline and at weeks 4 and 6. Immunizations were carried out at baseline and at week 4.

3. Results

3.1. Genetic diversity of 3D7-type msp2 alleles

Thirty-four distinct genotypes of the 3D7 family were identified by PCR-RFLP in the course of the entire study. Two genotypes could not be directly sequenced due to high multiplicity of infection in the respective blood samples. Seven genotypes were detected in baseline samples only, or persisted from baseline onwards but did not occur in new infections.

We analyzed 37 breakthrough parasite clones and 41 control clones from placebo recipients. Twelve RFLP genotypes occurred in both, vaccine and placebo, 7 only in vaccine and 6 only in placebo. There is no evidence that a subset of genotypes was selectively eliminated by vaccination. This might reflect chance effects in transmission.

3.2. MSP2 gene trees

Phylogenetic analysis of genes with intragenic repeats is difficult. Sequence alignment of 3D7-type *msp2* alleles was problematic because repeat units of the various alleles differed in sequence and lengths and were often scrambled. The repeat region includes tandem and scrambled repeats and is defined on nucleotide sequence level by regular spacing of thymidines, giving rise to solely (XXT) codons (Felger et al., 1997). This previous observation was confirmed by all new *msp2* alleles sequenced in this study. Fig. 1 shows an alignment of the polymorphic domains of 32 3D7-type MSP2 alleles detected in the trial area. For comparison, the 3D7 vaccine molecule is added to the alignment, despite the fact that this variant was not present in the study area. The repeats are followed by a non-repetitive but polymorphic region of variable length, which reveals several clusters of similar sequences. Downstream of this variable region all 3D7-type *msp2* alleles contain a poly-threonine (T) stretch, also varying in lengths, which represents on the nucleotide sequence level in fact another tandem repeat. Further downstream of the poly-threonine stretch follows the so-called family-specific domain of 90 residues that in parts is well conserved with the exception of a region of microheter-

ogeneity (starting at position 14 after poly-T). This region of microheterogeneity revealed 10 SNPs within a stretch of 25 residues, and synthetic peptides representing a part of this region bound to human red blood cells and inhibited parasite invasion of erythrocytes (Ocampo et al., 2000). Just upstream of the repeats, a second small region of microheterogeneity within the family-specific domain is located, spanning six residues of which only the serine at position 3 is conserved.

In order to quantify the sequence similarity between each allele and the 3D7 vaccine molecule, we performed a distance analysis and applied the Neighbour Joining algorithm. First the analysis was performed with the entire polymorphic region between the N- and C-terminal constant domains. The resulting MSP2 gene tree is shown in Fig. 2a. The vaccine molecule 3D7 (accession number M28891) was chosen as outgroup when drawing the tree. We found that breakthrough infections were not clustered in any branch of the tree, and that alleles not found in breakthrough infections were distributed over all clusters. The same analysis was performed with alignments from which increasing portions of the variable regions had been removed. Fig. 2b shows the MSP2 gene tree obtained after the (XXT)n repeat region had been removed. Both trees were consistent in their key finding, that breakthrough infections found in vaccinated individuals showed no greater distance to the vaccine molecule than alleles from the placebo group. In addition to the distance method for constructing a gene tree, we also applied the maximum parsimony algorithm. Trees obtained were similar with the major clusters maintained (data not shown). Breakthrough alleles were again evenly spread over all clusters of the tree.

Because the region of microheterogeneity located downstream of the poly-T Stretch and spanning 25 residues was implied in competitive invasion inhibition (Ocampo et al., 2000), we also analyzed this region in greater detail. For Fig. 2c this region of 25 residues was aligned together with a stretch of six residues preceding the tandem repeats and also displaying sequence heterogeneity. The gene tree showed that breakthrough infections were not clustered and did not reveal less similarity to the 3D7 vaccine molecule than sequences found in the placebo group. Thus, vaccination obviously did not select for alternative variants. However, the considerable number of variants detected in our samples suggests that this region is nevertheless under selection.

3.3. Repeats of breakthrough infections

The intragenic repeats of 3D7-type alleles are mainly responsible for length polymorphism. Some alleles share the same repeat units, but vary in copy number of repeats (Felger et al., 1997). We were interested in how far the 3D7 repeats, which had been found to be immunogenic in the Combination B vaccine trial (Flück et al., 2004), might have cross-protected vaccinated children against becoming infected with an allele carrying the same repeat unit. We found that, while the 3D7 allele (accession number M28891) representing the vaccine molecule, was not present in the study area, its 4-mer repeat glycine-glycine-serine-alanine (GGSA) was present in three

APPENDIX

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DQ166534  SIRRSMAESNPSTGAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGS----------------------------------------------------
DQ166540  SIRRSMAESNPSTGGSGSGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGS-------------------------
DQ166535  SIRRSMAESNPSTGAGGSGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGS---------------------------------------------
DQ166539  SIRRSMAESNPSTGAGGSGSAGGSGSAGGSAGGSGSAGGSAGGSAGGSAGGSGSAGGSGSAGGSAGGSAGS------------------------------------
DQ166543  SIRRSMAESNPSTGAGGSGSAGGSGSAGGSGSAGGSGSAGGSAGGSAGGSAGGSAGGSGSAGGSGSAGGSAGGSAGS--------------------------------
DQ174442  SIRRSMAESNPSTGAGGSGSAGGSGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSGSAGGSGSAGGSGSAGGSAGGSAGS------------------------------
DQ171732  SIRRSMAESNPSTGAGASGSAGGSGSAGGSGSAGGSGSAGGSAGGSAGGSAGGSAGGSAGGSGSAGGSGSAGGSAGGSAGGSAGS-------------------------
3D7       SIRRSMAESKPSTGAGTAGGSAGGSAGGSAGGSAGGSAGS----------------------------------------------------------------
DQ162622  SIRRSMEESKPPTGASGSAGGSAVASAGSGAVASAGSGAVASAGSGAVASAGSGAVASAGSGAVASAGNGAVASA------------------------------
DQ168572  SIRRSMEESKPPTGASGSAGGSAVASAGSGAVASAGSGAVASAGSGAVASAGSGAVASAGSGAVASAGNGAVASA------------------------------
M73810    SIRRSMAESKPPTGTGASGSAGSGAGASGSAGSGDGAVASA-----------------------------------------------------------------
U16840    SIRRSMAESKSPTGTGASGSAGSGDGASGSAGSGDGASGSAGSGDGAVASA------------------------------------------------------
U07001    SIRRSMEESNPSTGAGGSGSAGSRDGAVASAGSRDGAVASAGSRDGAVASAGSRDGAVASAGSRDGAVASA----------------------------------
DQ158904  SIRRSMEESNPSTGAGGSGSAGSRDGAVASAGSRDGAVASAGSRDGAVASAGSRDGAVASAGSRDGAVASAGSRDGAVASA-------------------------
DQ166536  SIRRSMAESKPPTGAGGSGSAGSRDGAVASAGSRDGAVASAGSRDGAVASAGSRDGAVASAGSRDGAVASAGSRDGAVASAGSRDGAVASARN------------
DQ166544  SIRRSMEESNPSTGAGGSGSAGSRDGAVASAGSRDGAVASAGSRDGAVASAGSRDGAVASARN---------------------------------------
DQ166545  SIRRSMAVSNPPTGAGASGRAGAGAGAGASGRAGAGGASGRAGSGDGAVASAGSGDGAVASAGSGDGAVASARN--------------------------------
U07009    SIRRSMTESNPPTPTGAGAGAGDGAVASAGSGDGAVASA----------------------------------------------------------------
DQ166537  SIRRSMAESKPPTGAGAGASGSAGS----------------------------------------------------------------------------------
U16842    SIRRSMEESNPPTGAGAVAGSGAGAVAGSGAGAVAGSGAGAGAGAVAGSGAGAGAVAGSGAGAGAVAGSGAGAVAGSGAGASA---------------------
DQ185319  SIRRSMEESNPPTGAGAVAGSGAGAVAGSGAGAVAGSGAGAVAGSGAGAGAGAVAGSGAGAGAVAGSGAGAGAVAGSGAGASA--------------------
DQ185320  SIRRSMEESNPPTGAGAVAGSGAGAVAGSGAGAGAGAVAGSGAGAGAGSGAGASA---------------------------------------------------
DQ171731  SIRRSMEESNPPTGAGAVAGSGAGAVAGSGAGAGAVAGSGAGAGAVAGSGAGAVAGSGAGAGAVAGSGAGAVAGSGAGASA----------------------
AY534507  SIRRSMTESNPPTGASGSAGAGAGASGSAGAGASGSAGAGASGSAGAGASGSASGSAGASGSAGAGASGSAGS-------------------------------
DQ166541  SIRRSMAESNPPTGASGSAGAGAGASGSAGAGASGSAGAGASGSAGAGAGASGSASGSAGASGSAGAGASGSAGS---------------------------
DQ166538  SIRRSMAESKPPTGASGSAGAGAGASGSAGAGASGSAGAGASGSAGAGASGSASGSAGASGSAGAGASGSAGS------------------------------
DQ168571  SIRRSMAESKPPTPTGAGAGASGSAGSGDGASGSASGSAGASGSASGSAGASGSASGSAGASGSAGASGSASGSA----------------------------
AJ318755  SIRRSMAVSKAPTSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAAGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSA
AJ318752  SIRRSMAVSKAPTSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSA------
AJ318754  SIRRSMAVSKAPTSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSA-----------
AJ318753  SIRRSMAVSKAPTSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSA-----------
DQ166542  SIRRSMAVSKAPTSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSA---------------------------
DQ166546  SIRRSMAESNPPAGAGASGNPPAGAGASGNPPAGAGASGNPPAGAGASGNPPAGAGASGNPPAGAGASGSA--------------------------------


DQ166534  GDGNGANPGADAEGSSSTPATTTTTTTTTTTTTNDAEASTSTSSENPNHNNAKTNPKGNGGVQEPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166540  GDGNGANPGADAEGSSSTPATTTTTTTTTTTTTNDAEASTSTSSENPNHNNAKTNPKGNGGVQKPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166535  GDGNGANPGADAEGSSSTPATTTTTTTTTTTTTNDAEASTSTSSENPNHNNAKTNPKGNGGVQKPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166539  GDGNGANPGADAEGSSSTPATTTTTTTTTTTTTNDAEASTSTSSENPNHNNAKTNPKGNGGVQKPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166543  GDGNGANPGADAEGSSSTPATTTTTTTTTTTTTNDAEASTSTSSENPNHNNAKTNPKGNGGVQKPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ174442  GDGNGANPGADAEGSSSTPATTTTTTTTTTTTTNDAEASTSTSSENPNHNNAKTNPKGNGGVQKPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ171732  GDGNGANPGADAEGSSSTPATTTTTTTTTTTTTNDAEASTSTSSENPNHNNAKTNPKGNGGVQKPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
3D7       GDGN----GADAEGSSSTPATTTTTKTTTTTTTNDAEASTSTSSENPHKNAETNPKGKEVQEPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ162622  --GNGANPGADAAGSSSTRATTTTTTTTTTT---NDAEASTSTSSENPNHKNAETNPKGKGEVQKPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ168572  --GNGANPGADAAGSSSTRATTTTTTTTTTT---NDAEASTSTSSENPNHKNAETNPKGKGEVQKPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ158904  --RNGANPGADAEGSSSTRATTTTTTTTT-----NDAEASTSTSSENPNHKNAETNPKGKGEVQTNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
M73810    --RNGANPGADAEGSSSTPATTTTTTTTTTTTTNDAEASTSTSSENPNHNNAETNPKGNGEVQKSNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
U16840    --RNGANPGADAEGSSSTPATTTTTTTTTTTTTNDAEASTSTSSENPNHNNAETNPKGNGEVQEPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
U07001    --RNGANPGADAEGSSSTRATTTTTTTT------NDAEASTSTSSENPNHKNAETNPKGKGEVQKTNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166536  ----GANPGADAEGSSSTRATTTTTTTTT-----NDAEASTSTSSENPNHKNAETNPKGKGEVQKTNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166544  ----GANPGADAEGSSSTRATTTTTTTTT-----NDAEASTSTSSENPNHKNAETNPKGKGEVQKTNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166545  ----GANPGADAKRSPSTPATTTTTTTTT-----NDAEASTSTSSENSNHNNAETNPKGKGEVQEPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
U07009    --GNGANPGADAERSPSTPATTTTTTTTT-----NDAEASTSTSSENTNHNNAETNPKGNGEVQEPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166537  GDGNGANPGADAERSPSTPATTTTTTTTT-----NDAEASTSTSSENPNHNNAETNPKGNGEVQEPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
U16842    --GN----GADAKRSPSTPATTTTTTTTT-----NDAEASTSTSSENPNHNNAETNPKGKGQVQEPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ185319  --GN----GADAKRSPSTPATTTTTTTTT-----NDAEASTSTSSENPNHNNAETNPKGKGQVQEPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ185320  --GN----GADAKRSPSTPATTTTTTTTT-----NDAEASTSTSSENPNHNNAETNPKGKGQVQEPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ171731  --GN----GADAKRSPSTPATTTTTTTTT-----NDAEASTSTSSENPNHNNAETNPKGKGQVQEPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
AY534507  --------GADAERSPSTPATTTTTTTTT-----NDAEASTSTSSENPNHNNAKTNPKGKGEVQKPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166541  --------GADAERSPSTPATTTTTTTTT-----NDAEASTSTSSENPNHNNAKTNPKGKGEVQKPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166538  --------GADAERSPSTPATTTTTTTTT-----NDAEASTSTSSENPNHNNAKTNPKGKGEVQKPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ168571  --------GA--ERSPSTPATTTTTTTTT-----NDAEASTSTSSENPNHNNAKTNPKGK-EVQEPNKANTETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
AJ318755  --------GA--EGSSSTPATTTTTTTTT-----NDAKASTSTSSENPNHNNAETNPKGNGEVQEPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
AJ318752  --------GA--EGSSSTPATTTTTTTTT-----NDAKASTSTSSENPNHNNAETNPKGNGEVQEPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
AJ318754  --------GA--EGSSSTPATTTTTTTTT-----NDAKASTSTSSENPNHNNAETNPKGNGEVQEPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
AJ318753  --------GA--EGSSSTPATTTTTTTTT-----NDAKASTSTSSENPNHNNAETNPKGNGEVQEPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166542  --------GA--EGSSSTPATTTTTTTTT-----NDAEASTSTSSENPNHNNAETNPKGNGEVQEPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166546  --------GA--EGSSSTPATTTTT---------NDAEASTSTSSENPNHNNAKTNPKGNGGVQEPNQANKETQNNSNVQQDSQTKSNVPPTQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
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Fig. 1. Sequence alignment of the 3D7-MSP2 vaccine molecule and 32 3D7-type MSP2 alleles that were detected in children participating in the Combination B vaccine trial in PNG. Names of sequences correspond to Genbank accession numbers. Stretches of sequence microheterogeneity are bold.

alleles from the trial area. One allele harbouring a GGSA repeat (accession number DQ166535) was detected in two vaccinated children. The same 4-mer motif was also represented in another, larger repeat unit present in four additional alleles (DQ174442, DQ171732, DQ166539, DQ166543), three of which were found in vaccinees.

3.4. Sequence fidelity and persistence in time of msp2 alleles in the study area

At the site of the vaccine trial, in the Wosera area in PNG, *msp2* diversity has been studied 6 years prior to the trial by using the same genotyping technique. In this previous cross-sectional survey in 1992, 38 different *msp2* alleles of both families were detected in two villages (Felger et al., 1994). When we compared the nucleotide sequence and frequencies of

RFLP-genotypes, we found that the most frequent genotypes in 1992 were still frequent 6 years later, and that some alleles had been maintained without a single point mutation. Alleles of low allelic frequency seem to fluctuate and were mostly new.

Because PCR-RFLP detects length polymorphism and mutations at restriction sites, it does not reveal all sequence diversity present. In order to establish sequence fidelity within a RFLP genotype, we chose the most frequent allele, KF1916, for a detailed sequencing analysis. Seven nested *msp2* PCR products, all classified as KF1916 genotype by PCR-RFLP, were directly sequenced. These KF1916 sequences were aligned together with the original KF1916 sequence from Genbank (accession number M73810) that derived from a PNG isolate adapted to culture in the 1980s (Marshall et al., 1992). Three KF1916 sequences from the 1992 survey were also added to the alignment. Fig. 3 shows that KF1916 is well conserved,

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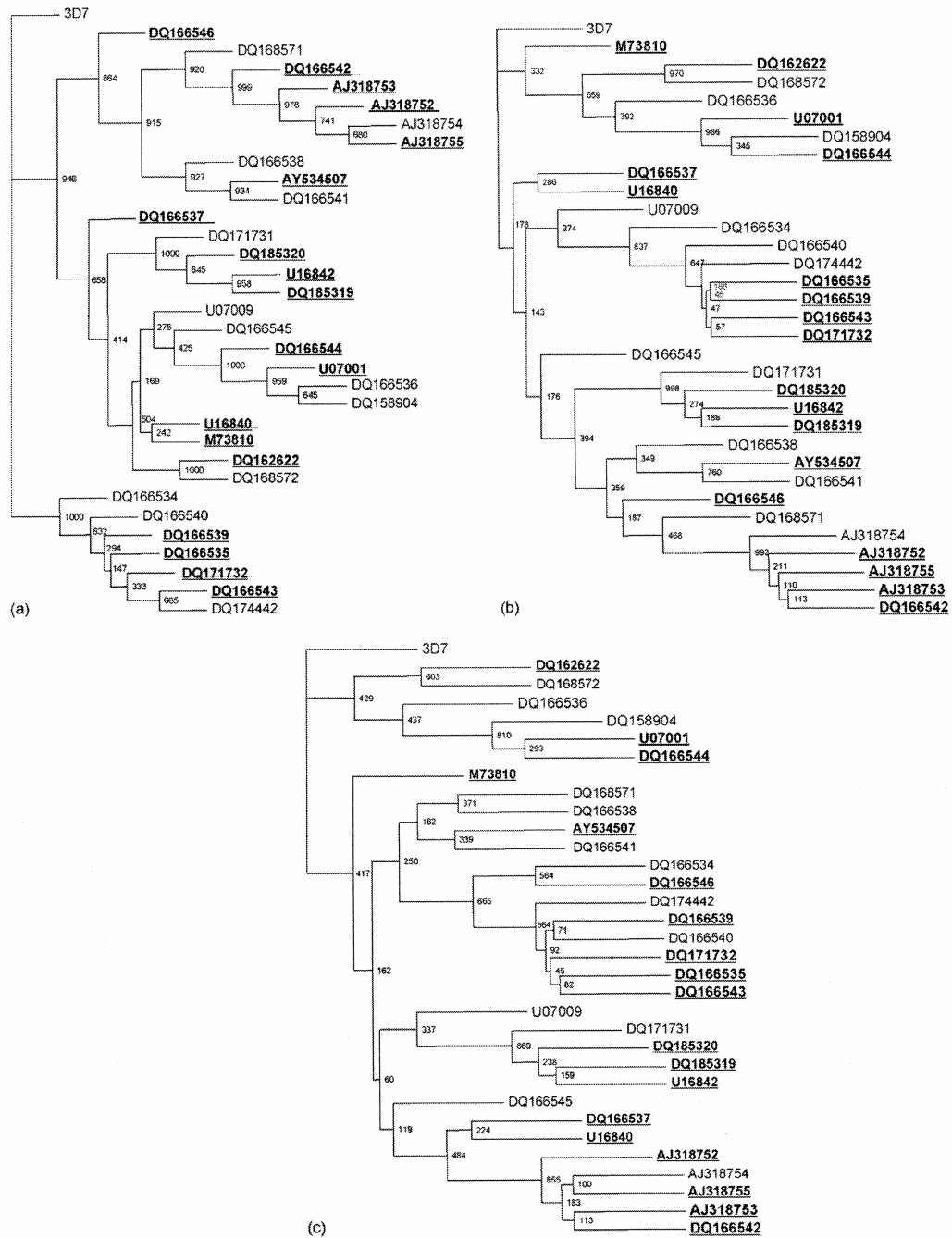

Fig. 2. Phylogenetic analysis of 3D7-type *msp2* alleles detected in children from the Combination B trial. Sequences occurring in breakthrough infections (new infections after week 8 post-vaccination) of vaccinated children are underlined. The phylogenetic tree was built using the Neighbor Joining method with 1000 bootstrap replicates. The tree was drawn with the vaccine molecule 3D7 as outgroup. The scale bar indicates sequence distance. (a) *msp2* gene tree obtained from an alignment of the entire variable region including repeats. (b) *msp2* gene tree based on 3D7-type *msp2* sequences from which the repeats had been deleted. (c) *msp2* gene tree based on regions of microheterogeneity flanking the repetitive domain.

```
KF1916 M73810 S I R R S M A E S K P P T G T G A S G S A G S G
A G A S G
1992 (n=3)    - - - - - - S(2/3)- - - - - - - - - - - - - - -
- - - - -
1998 (n=7)    - - - - - - E(1/6)- - S(3/6)- - - - - - - - - -
- - - - -


KF1916_M73810 S A G S G D G A V A S A R N G A N P G A D A E G
S S S T P
1992 (n=3)    - - - - - - - - - - - - - - - - - - - - - - - -
- - - - -
1998 (n=7)    - - - - - - - - - - - - - - - - - - - - - - - -
- - - - -


KF1916_M73810 A T T T T T T T T T T T T T T T N D A E A S T S T
S S E N P
1992 (n=3)    - - - - - - - - - - - - - - - - - - - - - - - - -
- - - - -
1998 (n=7)    - - - - - - - - - - - - - - - - - - - - - - - - -
- - - - -


KF1916_M73810 N H N N A E T N P K G K G E V Q K S N Q A N K E
T Q N N S
1992 (n=3)    - - - - - - - - - - - - - - - - - - - - - - - -
- - - - -
1998 (n=7)    - - - - - - - - - - - - N(1/6)- - - E(1/6)- - - -
- - - - -


KF1916_M73810 N V Q Q D S Q T K S N V P P T Q D A D T K S P T
A Q P E Q
1992 (n=3)    - - - - - - - - - - - - - - - - - - - - - - - -
- - - - -
1998 (n=7)    - - - - - - - - - - - - - - - - - - - - - - - -
- - - - -


KF1916_M73810 A E N S A P T A E Q T E S P E L Q S A P E N
1992 (n=3)    - - - - - - - - - - - - - - - - - - - - - - -
1998 (n=7)    - - - - - - - - - - - - - - - - - - - - - - -
```

Fig. 3. Sequence fidelity of the PCR-RFLP genotype KF1916 of MSP2 over a period of 20 years. Isolates were collected in Papua New Guinea in 1992 and 1998 and compared to the KF1916 sequence submitted to Genbank that derived from an isolate collected in the 1980s. The polymorphic central part of *msp2* corresponding to the nested PCR product is shown. Underlined residues correspond to the two regions of microheterogeneity where SNPs are frequent.

mutations did not accumulate over time, and SNPs were found at an average frequency of one per clone sequenced. The repetitive domain and most part of the family-specific domain were totally conserved whereas SNPs were only detected in the two regions of microheterogeneity. From these data we concluded that PCR-RFLP genotypes are stable over time.

In summary, our results showed a high amount of sequence heterogeneity at the trial site. Allelic diversity in *msp2* was not restricted to the actual repetitive domain alone. High diversity was also found in a considerable part of the dimorphic or family-specific domain. Strictly conserved within all 3D7-type MSP2 alleles was a stretch of 53 residues upstream of the C-terminal constant domain. Specifying the boundaries of strictly conserved dimorphic domains has implications for design of other MSP2 vaccine molecules. We showed that in the Combination B vaccine trial, the diverse domains seem to have not contributed to vaccine efficacy, because breakthrough infections in vaccinated individuals were independent of similarity with the 3D7 vaccine molecule. This suggests that the well-conserved epitopes in the family-specific domain must have been the important determinants of the vaccine effect against 3D7-type infections.

4. Discussion

Antigenic diversity in *P. falciparum* represents a significant challenge for the development of a malaria vaccine. As polymorphism is prevalent in most *P. falciparum* antigens, it is unrealistic to expect complete parasite clearance in vaccinated individuals. Not even natural immunity prevents infection entirely, and sterilizing immunity is never achieved in individuals from endemic areas. Most current efforts in malaria vaccine development consider partially effective vaccines and combination of multiple subunits consisting of several candidate antigens or of several variants of a polymorphic vaccine molecule is the currently favoured strategy (Mahanty et al., 2003).

Little evidence from field data is available on selective effects in malaria vaccine trials. Despite the extensive polymorphism of MSP2, the MSP2 allele of the 3D7 strain was included as a subunit in the Combination B malaria vaccine. Thus, it is likely that escape mutants emerge in the population by filling ecological niches emptied by variants eliminated by vaccine-induced immunity. Our previously published genotyping results from the Combination B trial

had shown selection acting on the level of allelic families (Genton et al., 2002). Now we have investigated whether a vaccine effect is evident also on the level of individual alleles. The impact of the imperfect 3D7-MSP2 vaccine on breakthrough infections occurring in vaccinated children was assessed by sequence analysis of all *msp2* alleles detected during the trial.

One objective of our analysis was to verify that genotypes detected by PCR-RFPL were stable in time, and thus matched sequences submitted to GenBank. To date, little is known on the temporal and geographic genetic differentiation among parasite populations. The concept of microepidemics is still under debate. The 3D7-type allele (KF1916) was the most frequent in the Wosera area, both in 1992 and 1998, and showed little variation in frequency. We have so far not been able to analyse the sequence conservation over time in the less frequent RFLP-defined genotypes. More longitudinal analyses similar to those of KF1916 are needed to understand the dynamics of *P. falciparum* population structure.

4.1. Msp2 repeat units as smoke screen epitopes

The function of intragenic tandem repeats in plasmodial surface antigens remains obscure. It has been speculated that the arrays of repeats represent "smoke screen" epitopes, which divert the immune system from protective responses by directing the response to irrelevant repetitive epitopes (Anders, 1986; Kemp et al., 1987). It has been proposed that repeats are immunodominant, but even so induce only non-neutralizing antibodies by crosslinking hapten-specific surface immunoglobulin on B cells thus providing a thymus-independent activation with no memory elicited (Schofield, 1991). It is generally assumed that levels of anti-repeat antibodies are not correlated with protection, but this has not yet been shown conclusively for MSP2. Our data cannot provide much evidence to either support or reject this hypothesis, mainly because the $(Gly-Gly-Ser-Ala)_5$ repeat of the vaccine molecule was found only rarely in alleles in the study area. Nevertheless, we inspected the anti-$(Gly-Gly-Ser-Ala)_5$ response at week 12 post-vaccination in the few vaccinated individuals infected with a parasite displaying the (Gly-Gly-Ser-Ala) motif, either as tandem repeat or as part of a larger repeat unit. From those individuals antibody titres were available in the serological database of Flück et al. (2004). Antibody titres were either not raised at week 12 compared to baseline values, or only a minor increase was observed (data not shown). Thus, it remains unclear whether infection by these genotypes could have been prevented if an anti-$(Gly-Gly-Ser-Ala)_5$ response had been induced in these vaccinated children.

4.2. Variable non-repetitive domain

After deleting the repeat regions from all sequences of the alignment of 3D7-type MSP2 alleles, a region of about 50 residues of the dimorphic domain proved totally conserved. This region is located adjacent to the C-terminal conserved region. Further upstream, flanking the repeats, highly poly-morphic non-repetitive blocks are found characterized by an accumulation of point mutations. We have tested particularly whether these regions of microheterogeneity were subject to selection in vaccinated individuals. We found that sequence similarity of this SNP-rich region to the vaccine molecule did not affect a genotype's presence or absence in the immunized group. Therefore, it has to be assumed that responses elicited against these regions were not protective. It follows that the 50-residues-long totally conserved dimorphic region might have been responsible for the selective effect acting on the level of the allelic family.

We assume that the regions of microheterogeneity were immunogenic, because several studies have mapped immunogenicity to these regions. Immunization with a short peptide including a part of the C-terminal region of microheterogeneity (peptide 40 in Jones et al., 1992) elicited IFA-positive antibodies. Lawrence et al. (2000) have mapped linear antibody epitopes within MSP2 after vaccinating a human volunteer with the 3D7 variant of MSP2, corresponding to the 3D7 component of Combination B. The same analysis was performed after immunizing mice with the same molecule. Both experiments showed in parallel that the regions of local microheterogeneity were found to be the major targets of antibody response in the family-specific domain of MSP2. This hypervariable region downstream of the poly-threonine stretch revealed 10 sites of non-synonymous mutations clustered in a stretch of 25 amino acids (underlined in Fig. 3). It is exactly this block that seems to play an important role in merozoite invasion of human red blood cells. Ocampo et al. (2000) have identified an MSP2 peptide with high specific binding to human erythrocytes, which is identical with this block. The peptide also inhibited in vitro parasite invasion by up to 95%. It remains unclear how a possible function in invasion can be reconciled with the hypervariability we have documented in our small sample size.

4.3. Conserved regions within the family-specific domain

The Combination B vaccine was effective in reducing parasite densities, yet the effect was incomplete. Despite vaccination, some 3D7-type infections could establish themselves in immunized children but the vaccine effect may have lead to faster elimination and thus to the reduced prevalence of 3D7-type parasites observed in the trial (Genton et al., 2002). In search of new improved vaccine formulations the question arises which domain of MSP2 could have caused the selective effect? The 3D7 family-specific domain contains 50 residues of invariant sequence. This is a likely candidate to account for selection observed on the level of the allelic family. We have previously shown that antibody levels against the recombinant 3D7 family-specific domain and against the 3D7 repeats were significantly higher in vaccinated children (Flück et al., 2004). However, our phylogenetic analysis does not allow us to pinpoint the active component of the 3D7 subunit vaccine; it can only indicate whether a polymorphic region is selected and thus subject to protective antibodies. As with naturally induced immune responses, responses elicited by vaccination are directed against many different epitopes, only a fraction of

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which might lead to protection. We assume that an antibody response against both the repeats and regions of microheterogeneity was elicited, but did not protect the vaccinated children against new infections. This does, however, not exclude activity of these antibodies against high parasite densities.

This analysis does not take into account the pre-existing acquired immunity to 3D7 genotypes nor the possibility that an ongoing 3D7-type infection inhibits a newly occurring infection of the same allelic family via within-host competition (e.g., for resources). The extent of competitive interactions in multiple clone infections is only recently being studied (de Roode et al., 2004). It is unclear whether such conditions can be ignored in the analysis of selectivity.

5. Conclusion

The vaccine trial has shown that the 3D7-MSP2 component had some efficacy, though this was imperfect and numerous breakthrough infections occurred. When analyzing the breakthrough genotypes, we found no evidence that responses against the Gly-Gly-Ser-Ala repeats and hypervariable stretches might have protected against new infection by similar variants. We conclude that a response against the conserved Stretches within the family-specific dimorphic domain is more likely to account for the MSP2 family-specific selective effect seen in the Combination B trial.

The occurrence of replacement by FC27-type infections in vaccinees in the Combination B trial, leading to increased morbidity, confirms that selective effects of imperfect polymorphic malaria vaccines are of real concern. As more results from other and bigger trials of polymorphic vaccines become available, it will become clear whether such selective effects are a general side effect of vaccine interventions. There is a clear need to include several variants of a polymorphic vaccine candidate in a single vaccine formulation.

Acknowledgement

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THE RISK OF MALARIAL INFECTIONS AND DISEASE IN PAPUA NEW GUINEAN CHILDREN

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Abstract. In a treatment re-infection study of 206 Papua New Guinean school children, we examined risk of re-infection and symptomatic malaria caused by different *Plasmodium* species. Although children acquired a similar number of polymerase chain reaction–detectable *Plasmodium falciparum* and *P. vivax* infections in six months of active follow-up (*P. falciparum* = 5.00, *P. vivax* = 5.28), they were 21 times more likely to develop symptomatic *P. falciparum* malaria (1.17/year) than *P. vivax* malaria (0.06/year). Children greater than nine years of age had a reduced risk of acquiring *P. vivax* infections of low-to-moderate (>150/μL) density (adjusted hazard rate [AHR] = 0.65 and 0.42), whereas similar reductions in risk with age of *P. falciparum* infection was only seen for parasitemias > 5,000/μL (AHR = 0.49) and symptomatic episodes (AHR = 0.51). Infection and symptomatic episodes with *P. malariae* and *P. ovale* were rare. By nine years of age, children have thus acquired almost complete clinical immunity to *P. vivax* characterized by a very tight control of parasite density, whereas the acquisition of immunity to symptomatic *P. falciparum* malaria remained incomplete. These observations suggest that different mechanisms of immunity may be important for protection from these malaria species.

INTRODUCTION

The epidemiology of *Plasmodium falciparum* malaria suggests children first acquire immunity against severe disease after relatively few infections.[1,2] However, uncomplicated *P. falciparum* malaria remains common throughout most of childhood, and a significant decrease in risk of infection is only seen in adolescence and early adulthood.[1] Similar patterns have also been described in area of areas of Papua New Guinea highly endemic for malaria.[3,4] It has therefore been argued that the mechanisms responsible for protection against severe disease may be distinct from those that protect against infections *per se* and mild episodes of disease,[1,5] and that immunity might be acquired in stages. Although many potential targets and mechanisms of protective immunity have been identified,[1,5–8] we still know little about mechanisms involved in the acquisition of protective immunity against *P. falciparum*.

Even less is known about the acquisition of immunity to non-*P. falciparum* malarias. In highly endemic areas such as Papua New Guinea where the different species co-occur, prevalence of infection with *P. vivax* peaks at younger ages[3,9,10] and contributes proportionally less to the burden of febrile illness[11] than *P. falciparum*. Conversely, *P. malariae* reaches maximum prevalence only in adolescents.[9,10] These data indicate that immunity to *P. vivax* may be acquired more quickly than immunity to *P. falciparum* despite lower transmission rates.[12] Although a number of potential targets and mechanisms for immunity have been identified for *P. vivax*,[13–15] little is known about acquisition of immunity to *P. malariae* and *P. ovale*.

A better understanding of the incidence of infection and disease caused by different *Plasmodium* species in areas co-endemic for all species is needed to properly assess differences in the acquisition of clinical immunity to different species. Because mixed infections are common in malaria-endemic areas, but often remain undetected by light

microscopy,[10,16] polymerase chain reaction (PCR)–based diagnostic methods are needed for quantifying risk of infection and morbidity reliably.

To determine epidemiologic patterns of infections and disease with *P. falciparum* and *P. vivax* and investigate possible mechanisms of immune protection, we conducted a longitudinal treatment re-infection study of 206 Papua New Guinean elementary school children that combines repeated blood sampling and molecular detection of parasitemia with a large array of classic and functional immune assays. We describe the general study design and report patterns of incidence of infection and disease with all four human malaria parasite species. Detailed investigations of immunity to *P. falciparum* and *P. vivax* malaria will be the topic of future reports.

MATERIALS AND METHODS

Field study. This study was conducted between June and December 2004 at the Mugil and Megiar elementary schools situated on the northern coast of Papua New Guinea, 50 km north of Madang. The catchment area of both schools is serviced by a single health center at Mugil (Figure 1) run by the Catholic Health Services. Although the Mugil school is within easy walking distance of the health center, the Megiar schools are 4 km away along a sealed road but with frequent transport available. Bed net use in the study area is limited, with re-treatment of bed nets virtually absent. This study was reviewed and approved by institutional review boards of the Papua New Guinea Medical Research Advisory Council, the Walter and Eliza Hall Institute, and the Veteran's Affairs Medical Center (Cleveland, OH).

After obtaining community support and written parental consent, children from all three grades in Mugil and grades 1 and 2 in Megiar were enrolled. Demographic information was collected from all participating children; the location of each child's home was recorded using a hand-held global positioning system (GPS) receiver (GPS 315; Magellan, Santa Clara, CA).

Before starting treatment, each child was clinically examined: axillary temperature was measured using digital thermometers, the spleen was palpated, and a standard question-

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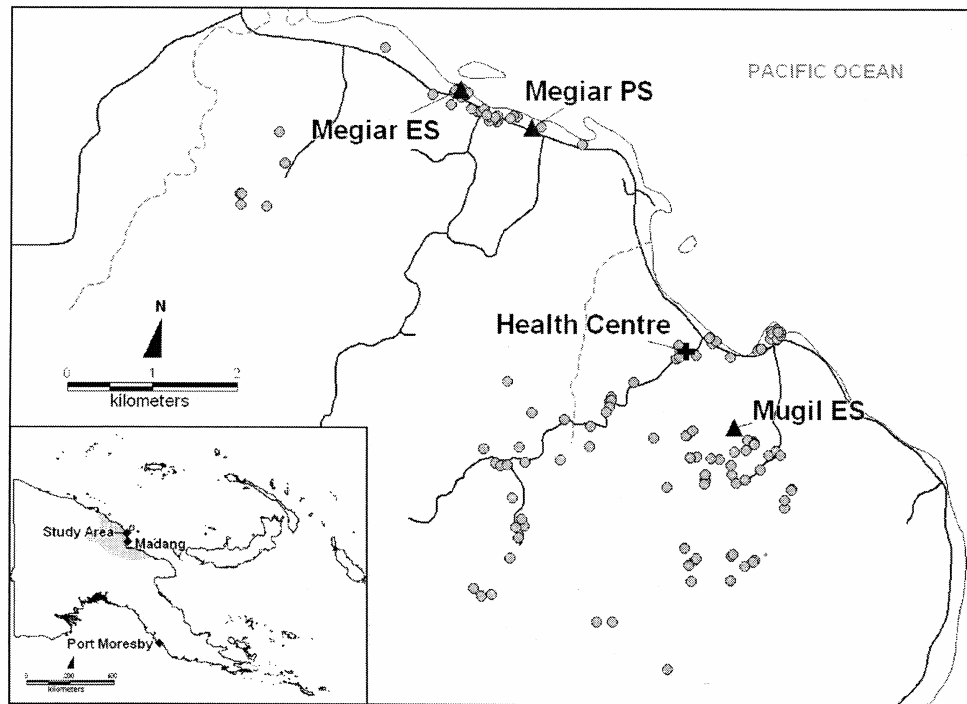
FIGURE 1.   Location of study site, schools, and participants' houses in Papua New Guinea.

naire of common signs and symptoms of malarial illness was administered. Hemoglobin (Hb) levels were measured using a portable device (HemoCue, Ångholm, Sweden). A 10-mL venous blood sample was collected using EDTA-Vacutainer® tubes (Becton Dickinson, Franklin Lakes, NJ) and two blood slides (thick and thin films) were made for determination of malarial infection. All children were subsequently treated with a seven-day course of artesunate monotherapy according to Papua New Guinea National treatment guidelines (i.e., 4 mg/kg on day 1 and 2 mg on days 2–7). All children received all seven doses with at least five of them directly observed.

After treatment, children were actively followed-up at the schools every two weeks for new infections and febrile illness with the first visit taking place two weeks after receiving the first artesunate treatment. A total of 12 follow-up visits were conducted. Because of national holidays that prevented field work, the eighth follow-up period had to be extended to three weeks, which resulted in a total length of active follow-up of 25 week. Follow-up visits were conducted on a class-by-class basis with one class checked every day. Children that did not attend school on the day of scheduled follow-up were checked the next day or at their homes at the earliest possible time within the next week. During school holidays (follow-up time points weeks 4 and 14), special clinics that included the screening of children's videos were set up at the schools or in the villages that regrouped most students. Children who did not come to these clinics were individually followed-up at their homes, when possible.

At each active follow-up, all children were clinically examined, their axillary temperatures were taken, their health

books were checked for recent antimalarial treatments, and they were questioned for recent bed net use. Hemoglobin levels were measured every four weeks and spleens were palpated every eight weeks. At the same time, two malarial blood films were made, a rapid diagnostic test (ICT Diagnostics, Brookvale, New South Wales, Australia) was conducted and a 250-μL blood sample was collected from each child into an K+-EDTA Microtainer® tube (Becton Dickinson) by finger prick using a retractable lancet (Genie® Lancet; Becton Dickinson). Children with symptoms of clinical malaria were transported to the Mugil health center after a third blood slide was made for parasitologic assessment, and treatment was given at the health center according to blood slide results. The scheduled active follow-ups, together with collection of additional venous blood samples, resulted in the study team visiting the Mugil school five times and the Megiar schools three times over each two-week period.

For six months after treatment, a passive case-detection system was maintained at the Mugil health center. In addition, children, their parents, and teachers were encouraged to report any illness to the study team at any time the team was visiting the school. All study children attending the outpatient clinic at Mugil health center with any illness, as well as those diagnosed by the study teams during their school visit, were referred to specific study staff based at the Mugil health center for clinical and parasitologic assessment. After a detailed clinical assessment, a 250-μL finger prick sample was collected, 3 blood slides were made, and Hb levels were measured. One of the slides was stained using rapid Giemsa staining (10% Giemsa for 10 minutes) and read immediately for

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diagnostic purposes. The other two slides were stained for research readings (5% Giemsa for 30 minutes). If children had signs of a febrile illness and a *P. falciparum* parasitemia > 500/μL or a *P. vivax* parasitemia > 250/μL, an additional 5-mL venous blood sample was collected within 24 hours. All children with clinical signs of malaria and a positive blood slide (irrespective of parasite density) were treated by the study team according to Papua New Guinea national treatment guidelines with chloroquine (three days) and sulfadoxine-pyrimethamine (single dose). A concurrent *in vivo* efficacy study at Mugil health center showed an overall 28-day failure rate (corrected by polymerase chain reaction [PCR]) of 12% for the treatment of *P. falciparum* infections (4% late clinical failures and 8% parasitologic failures) (Marfurt J, Mueller I, Genton B, unpublished data) Any other medical conditions were referred to the health center for appropriate treatment.

During the study period, monthly mosquito sampling was conducted in two villages using the all-night landing catch method[17] and specimens were identified morphologically as described by Belkin.[18] A subset of morphologically identified mosquitoes was processed by PCR to assess species identity.[19] Mosquitoes were tested by enzyme-linked immunosorbent assay (ELISA)[20] to detect circumsporozoite antigens using monoclonal antibodies specific for *P. falciparum*, *P. vivax* (PK210), and its variant (PK240).

Laboratory methods. All venous blood samples were separated into plasma, peripheral mononuclear cells, and remaining blood cells, centrifuged, and aliquoted accordingly. Finger prick blood samples were separated into plasma and cell pellets. DNA was extracted using the QIAamp 96 DNA Blood kit (Qiagen, Valencia, CA) from the cell pellet fraction of all samples.

All research blood films were read by two expert microscopists independently. Slides with discrepant results were re-read by a third microscopist. Thick blood films were examined by light microscopy (LM) for 100 thick-film fields (under a 100× oil-immersion lens) before being declared infection negative. Parasite species in positive films were identified and densities were recorded as the number of parasites per 200 white blood cells (WBCs). Densities were converted to the number of parasites per microliter of blood assuming 8,000 WBCs/μL (population average WBC count[3]). Slides were scored as LM positive for an individual *Plasmodium* species if the species was detected independently by at least two microscopists and subsequent PCR-based analysis confirmed the presence of the species. Densities were calculated as the geometric mean densities of all positive results.

Infection by each of the four human malaria species was assessed in all blood samples collected using a semiquantitative post-PCR, ligase detection reaction–fluorescent microsphere assay (LDR-FMA).[21] This assay combines PCR amplification of the small subunit (SSU) ribosomal RNA gene (491–500-basepair fragments) using genus specific primers, followed by a multiplex species-specific LDR. The LDR products are hybridized to FlexMAP™ classification bead sets (5′) (Luminex, Austin, TX) and receive reporter labeling after incubation with streptavidin-R-phycoerythrin that binds to biotin (3′). Double-labeled species-specific LDR complexes are detected using a Bio-Plex array reader (Bio-Rad Laboratories, Hercules, CA). Species-specific fluorescence

data were collected with Bio-Plex Manager version 3.0 software (Bio-Rad Laboratories). To ensure maximum sensitivity for the detection of *Plasmodium* infections, the PCR cycle number was set at 35. Differentiation of negative from positive fluorescent signals was performed by comparing median fluorescent intensity from study participants with values obtained from uninfected North American controls. Cut-off values for positivity were set at the 99% quantile of signals in controls as reported by Kasehagen and others.[10] The design and sensitivity of this assay has been previously described.[10,21,22] In these studies, the assay demonstrated high sensitivity compared with LM and real-time PCR. Nevertheless, we are not able to exclude the possibility that sequence polymorphisms in the *Plasmodium* species SSU ribosomal RNA gene sequences may contribute to false-negative results. However, to date, we have observed no direct evidence identifying specific variants of the *P. falciparum*, *P. vivax*, *P. malariae*, or *P. ovale* target sequences in our malaria prevalence studies in Papua New Guinea (Zimmerman PA, unpublished data).

To differentiate between treatment failures and newly established blood stage infections, all infections with *P. falciparum* or *P. vivax* (by PCR or LM) that occurred between the pre-treatment time point and six weeks post-treatment were genotyped to identify individual *P. falciparum* merozoite surface protein 2 alleles (*P. falciparum* infections[23,24]) or the highly polymorphic *P. vivax* Duffy binding protein alleles (*P. vivax* infections[25]).

Statistical analysis. Children were monitored for acquiring new infections until they withdrew from the study, did not provide two consecutive bi-weekly blood samples, or were re-treated with antimalarial drugs. Time-to-first infection with each species was calculated as the time between the date of first treatment and an infection-positive result by PCR or LM by either active or passive case detection. In addition, we calculated time-to-first infections with > 500 and > 5,000 parasites/μL for *P. falciparum* and > 150 parasites/μL for *P. vivax*. Children that remained negative for a particular species and/ or density cut-off until the end of active follow-up were censored after the last active follow-up time point (after 169–181 days of follow-up). Prevalence of infection during follow-up was calculated for only those children who had not been re-treated.

The risk of symptomatic malarial illness was assessed as time-to-first clinical episode and incidence of clinical episodes during follow-up. Clinical malaria was defined as a measured fever (axillary temperature ≥ 37.5°C) or history of febrile illness during the 48 hours preceding examination in conjunction with any malaria infection. The cut-off value for clinical disease was set at 5,000/μL for *P. falciparum*[26] and 1,000/μL for all other species (Mueller I, unpublished data). Children were monitored for clinical disease until they either withdrew from the study or were re-treated with antimalarial drugs. Children without a clinical episode were excluded after the last bi-weekly follow-up time point.

For the calculation of incidence rates, all disease episodes observed during active follow-up and passive morbidity surveillance were considered. In contrast to analyses for risk of infections, a child was considered at risk until he or she withdrew or reached the end of the study with the exception of the four weeks after further antimalarial treatments for the analy-

ses of difference in incidence rates. If a child was recorded as ill with the same species twice within a two-week period, this was considered a single episode.

Time-to-event (infection or clinical episode) data were analyzed using standard survival analysis techniques. A log-rank test was used to evaluate the difference in non-parametric survival curves. Because hazards for the different explanatory variables were generally proportional over the follow-up study, Cox regression was used to test for univariate and multivariate risk factors. Poisson regression was used for univariate and multivariate analyses of factors associated with the incidence of clinical P. falciparum disease. In all multivariate analyses, backwards selection and likelihood ratio tests were used to identify the best fitting models. All statistical analyses were performed using STATA 8 statistical analysis software (Stata Corporation, College Station, TX).

RESULTS

Enrollment and baseline characteristics. After obtaining informed consent and baseline health assessment, 206 children 5–14 years of age (inter-quartile range = 8.1–9.3 years) were enrolled into the study. Of these, 152 attended Mugil Elementary School, 44 attended Megiar Elementary School, and 10 attended Megiar Primary School. Of these children, 51.5% were girls and 35.9% reported having slept under a bet net the previous night.

At the time of enrollment, 92 children (44.7%) were positive for *Plasmodium* trophozoites by LM with *P. falciparum* the most common infection followed by *P. vivax* and *P. malariae* (Table 1). An additional 12 children were positive for *P. falciparum* gametocytes only. Geometric mean parasite densities for all species were generally low (Table 1). Densities of infections with *P. vivax* were significantly lower than those with *P. falciparum* ($P < 0.001$) or mixed infections ($P < 0.001$). The prevalence of infection by all species increased significantly when infections were diagnosed by LDR-FMA. Overall, 166 children (80.6%) were positive for any malarial infection with 54 (26.2%) having mixed species infections (Table 1). There was no significant association with prevalence between any species (by LDR-FMA) and bed net use.

Ninety-eight children (47.6%) had an enlarged spleen that

TABLE 1
Parasitologic and clinical assessment prior to treatment*

Infection species†	PCR-LDR-FMA		Light microscopy		
	No. pos	%	No. pos	%	Geometric mean density‡
Pf	85	41.3	68	33.0	361 (240, 544)
Pv	25	12.1	17	8.3	102 (74, 142)
Pm	2	1.0	4	1.9	421 (38, 4,646)
Pf and Pv	40	19.4	3	1.5	315 (183, 540)
Pf and Pm	8	3.9	0	0.0	–
Pf and Po	1	0.5	0	0.0	–
Pf, Pv, and Pm	4	1.9	0	0.0	–
Pf, Pv, and Po	1	0.5	0	0.0	–
Pf gametocyte only	–		12	5.8	68 (51, 90)
all positive	166	80.6%	104	50.5%	

* PCR = polymerase chain reaction; LDR = ligation detection reaction; FMA = fluorescent microsphere assay; Pf = *Plasmodium falciparum*; Pv = *P. vivax*; Pm = *P. malariae*; Po = *P. ovale*.
† Only species combinations that were observed are shown.
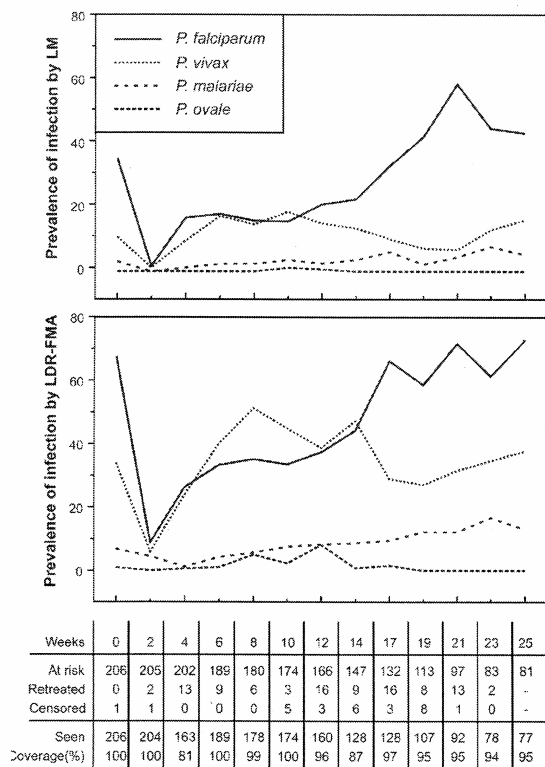‡ Mean number of parasites per microliter (95% confidence interval).

was associated with a concurrent malarial infection by either LM (odds ratio [OR] = 2.49, $P = 0.001$) or LDR-FMA (OR = 2.51, $P = 0.01$). Six children (2.9%) had moderate-to-severe anemia (Hb level = 5–8 g/dL) and 74 (35.9%) had mild anemia (Hb level = 8–11 g/dL) with no significant difference between boys and girls. All five children who had an axillary temperature ≥ 37.5°C were positive for *P. falciparum* by both LM and LDR-FMA.

Initial antimalarial treatment. Of the 139 children with an LDR-FMA-positive *P. falciparum* infection at baseline, only 12 (8.6%) showed evidence of infection with the same merozoite surface protein 2 genotype in the six weeks after the seven-day treatment with artesunate. In one child, the genotype of the first re-infection could not be ascertained and the infection was thus considered as a suspected treatment failure. Genotyping for *P. vivax* showed that only one PCR-positive infection observed within the first six weeks after treatment had the same DBP genotype as the infection at baseline. Among *P. malariae* infections, there was one infection that remained PCR-positive at day 14. Because of the lack of a genotyping assay for *P. malariae*, this case was therefore also treated as a suspected treatment failure. No treatment failure was observed for *P. ovale*. Overall, the artesunate treatment had an efficacy of 91.4% for treating infections with *P. falciparum*, 92.9% for infections with *P. malariae*, and 98.6% for infections with *P. vivax*. All treatment failure children were excluded from further analysis of re-infection with the homologous *Plasmodium* species. However, they were included in analyses for heterologous *Plasmodium* infections.

Risk of re-infections during active follow-up. When treatment failure cases were excluded, only one child was positive for *P. falciparum* and none for any other species by LM two weeks after treatment. However, when we used LDR-FMA, 17 (8.9%) of 192 children were positive for *P. falciparum*, 12 (5.9%) of 204 were positive for *P. vivax*, and 9 (4.4%) of 203 were positive for *P. malariae* two weeks after treatment. Prevalence of infection increased steadily over the active follow-up period (Figure 2), with prevalence rates for *P. vivax* and *P. malariae* reaching pre-treatment levels 6 and 10 weeks after treatment, respectively. The prevalence of *P. falciparum* reached pre-treatment levels after 16 weeks. The comparatively slower increase in *P. falciparum* prevalence may be partly due to the large number of *P. falciparum* infections that became symptomatic (see below), which needed to be treated and were thus excluded from the analyses of prevalence rates after re-treatment time points (Figure 2).

The risk of acquiring blood stage *Plasmodium* infection was thus better estimated by the time to re-infection (Table 2 and Figure 3). When diagnosed by LDR-FMA, re-infection rates for *P. falciparum* and *P. vivax* were identical (incidence of 5.00 new infections with *P. falciparum* and 5.28 with *P. vivax*/child/year; $P = 0.29$, by log-rank test). However, the relative incidence of infections with either species changed over the course of follow-up. A significantly higher incidence of new *P. vivax* infections compared with *P. falciparum* was observed during the first 90 days of follow-up (Figure 3) (0–90 days: 4.33 *P. falciparum* and 5.28 *P. vivax* infections/child/year; $P = 0.04$), whereas the incidence of *P. falciparum* was significantly higher in the second 90 days (90–180 days: 9.04 *P. falciparum* and 5.23 *P. vivax* infections/child/year; $P = 0.03$). Infections with *P. malariae* (0.98/child/year) and *P. ovale* (0.42/child/

Weeks	0	2	4	6	8	10	12	14	17	19	21	23	25
At risk	206	205	202	189	180	174	166	147	132	113	97	83	81
Retreated	0	2	13	9	6	3	16	9	16	8	13	2	-
Censored	1	1	0	0	0	5	3	6	3	8	1	0	-
Seen	206	204	163	189	178	174	160	128	128	107	92	78	77
Coverage(%)	100	100	81	100	99	100	96	87	97	95	95	94	95

FIGURE 2. Prevalence of malarial infections at baseline and at active bi-weekly follow-up obtaining of blood samples. **Top**, Diagnosis by light microscopy (LM). **Bottom**, Diagnosis using post-polymerase chain reaction and ligation detection reaction-fluorescent microsphere assay (LDR-FMA). The number of children at risk, treated, and withdrawn during each two-week period and number of children seen during each follow-up are shown below the bottom panel.

year) were significantly less frequent than those with the two other species ($P < 0.001$).

Re-infection rates decreased significantly in all *Plasmodium* species when infections were diagnosed by LM ($P < 0.001$), with children acquiring 3.25 LM-detectable *P. falciparum* infections, 2.03 *P. vivax* infections, 0.24 *P. malariae* infections, and 0.04 *P. ovale* infections/child/year. Although children had significantly less new *P. vivax* infections than *P. falciparum* infections ($P < 0.001$), this effect was almost exclusively observed in the second half of follow-up (Figure 3: 0–90 days: 2.23 *P. falciparum* and 1.98 *P. vivax* infections/child/year; $P = 0.46$; after 90 days, 6.63 *P. falciparum* and 2.19 *P. vivax* infections/child/year; $P < 0.001$). The difference in incidence between these two species increased when only new moderate (*P. falciparum* > 500/μL and *P. vivax* > 150/μL) and high-density (*P. falciparum* > 5,000/μL and *P. vivax* > 1,000/ μL) infections were considered (Figure 4). Although 78.9% and 47.8% of the children were re-infected with *P. falciparum* infections > 500/μL and > 5,000/μL, respectively, only 25.1% and 5.6% had *P. vivax* infections > 150/μL and > 1000/μL, respectively ($P < 0.001$, by log-rank test).

For all *Plasmodium* species except *P. vivax*, some geo-

graphic variation in infection rates was observed, with children in more remote areas of the study area (those further away from health center and schools) having a significantly higher risk of acquiring new infections (Table 2). No significant associations were found between frequency of bed net use (average use = 35.9%) and risk of re-infections with any *Plasmodium* species. However, effects were small and not consistent between LDR-FMA- and LM-positive infections. Although some significant associations were observed between infection risk during the active follow-up period and infection status prior to artesunate treatment, no consistent relationships were detected. Time-to-first LM-positive *P. falciparum* infection was decreased in children who were PCR positive for *P. falciparum* at baseline but not in those who were only LM positive (Table 2). Infection status at baseline was also not significantly associated with time-to-first PCR-positive infections. Similarly, no clear association of infection status at baseline with risk of infection during follow-up was found for *P. vivax*. Although the risk of acquiring a new PCR-positive *P. vivax* infection was associated with an increased risk of having a *P. vivax*-positive blood slide at baseline, this association was not found with PCR positivity at baseline or with a risk of acquiring new LM-positive infections (Table 2). Although having a PCR-positive *P. malariae* infection was associated with a decreased risk for later infections with *P. ovale*, no association in infection status was observed for risk of infection with *P. malariae*.

A decrease in risk of acquiring infections with increasing age was only observed in LM-detectable *P. vivax* (adjusted hazard ratio [AHR] = 0.65, $P = 0.028$) and PCR-diagnosed *P. ovale* (AHR = 0.41, $P = 0.038$) infections. However, progressively larger decreases in risk with age were observed not only for increasing density cut-offs of *P. vivax* infections (> 150/μL, AHR = 0.42, $P = 0.01$) but also for *P. falciparum* infections (Figure 5). However, this effect only reached statistical significance for moderate and high-density infections (i.e. *P. falciparum* > 500/μL, AHR = 0.71, $P = 0.053$ and *P. falciparum* > 5,000/μL, AHR = 0.48, $P = 0.002$).

Incidence and risk factors for symptomatic malarial infections. Overall, 162 febrile episodes with concurrent parasitemia were observed in 109 children (52.9%, Table 3). Of these, 111 (65.7%) exceeded species-specific pyrogenic thresholds of 5,000/μL for *P. falciparum* and > 1,000/μL for non-*P. falciparum*. Most of these malaria-attributable illness episodes (92.8%) were caused by *P. falciparum*, with only five episodes caused by *P. vivax* (4.5%), two each by *P. malariae* and *P. ovale* (1.8%), and one by a *P. falciparum*/*P. vivax* mixed infection (0.9%). The distribution of febrile illness episodes with any concurrent parasitemia and malaria-attributable episodes was consistent with a Poisson distribution both overall and for *P. falciparum* episodes ($P > 0.5$, by Kolmogorow-Smirnov test). Although 20 children (9.7%) had more than one *P. falciparum*-attributable malaria episode, none had more than one non-*P. falciparum* malaria episode. With an incidence rate of 1.17 episodes/child/year, *P. falciparum*-attributable illness episodes were 21 times more frequent than those caused by *P. vivax* (0.06/child/year) and 52 times more frequent than *P. malariae* or *P. ovale* (0.02/child/year) ($P < 0.001$).

The incidence of febrile illness with any *P. falciparum* density was significantly lower in children greater than nine years of age (adjusted incidence rate ratio [IRR] = 0.67, 95% CI =

TABLE 2
Median time to first infections, incidence of new infections, and multivariate risk factors for acquiring new infections with different *Plasmodium* species diagnosed by LM and LDR-FMA*

	P. falciparum		*P. vivax*		*P. malariae*		*P. ovale*	
	PCR	LM	PCR	LM	PCR	LM	PCR	LM
% Re-infected†	95.3	87.6	82.0	49.5	29.3	8.3	13.6	1.5
Median time to re-infections (days)	55	99	54	118	–‡	–‡	–‡	–‡
Incidence rate (years)	5.00 (4.30, 5.80)	3.23 (2.76, 3.78)	5.28 (4.54, 6.14)	2.03 (1.68, 2.46)	0.98 (0.76, 1.26)	0.24 (0.15, 0.39)	0.42 (0.29, 0.61)	0.04 (0.01, 0.13)
Multivariate predictors§	AHR	AHR	AHR	AHR	AHR	AHR	AHR	–¶
Age > 9 years				0.67 (0.44, 0.96)			0.41 (0.17, 0.95)	
Distance from Mugil HC#	1.14 (1.02, 1.27)					1.44 (1.03, 2.01)		
Distance from school#		0.76 (0.64, 0.91)				1.68 (1.16, 2.45)	1.54 (1.08, 2.19)	
Enrolled at Megiar Elementary School							5.68 (2.45, 13.2)	
Pf LDR-FMA + at baseline		1.67 (1.18, 2.35)						
Pf LM + at baseline							3.79 (1.75, 8.18)	
Pv LM + at baseline				1.70 (1.03, 2.83)				

* LM = light microscopy; AHR = adjusted hazard ratio. For definitions other abbreviations, see Table 1. Values in parentheses are 95% confidence intervals.
† Proporation of children with at least one positive sample while at risk. Total children at risk: Pf: n = 194, Pv: n = 206, Pm: n = 205; Po: n = 206.
‡ Not estimated because less than 50% of children re-infected.
§ Only significant factors (p < 0.05) from multivariate Cox regression analyses are given. Other factors included sex, distance from seacoast, presence of enlarged spleen and/or mild anemia (hemoglobin < 11 g/dL), reported bed net use (slept > 50% of nights under bed net), and infection status with any infection by PCR and LM at baseline.
¶ Not estimated due to very low re-infection rate (n = 3).
# Distances calculated as straight line distance (km) from child's residence to Mugil Health Center (HC) or school.

0.48–0.93, *P* = 0.019) and in children with an LM-positive *P. falciparum* infection at baseline (IRR = 0.66, 95% CI = 0.45, 0.97, *P* = 0.032). In addition, children living within one kilometer of the coastline more frequently had a fever and concurrent parasitemia (IRR = 1.57, 95% CI = 1.11–2.23, *P* = 0.011).

The decrease in incidence of *P. falciparum* illness with increasing age was more pronounced when only febrile episodes with a *P. falciparum* parasitemia greater than 5,000/μL were considered (IRR = 0.55, 95% CI = 0.37–0.83, *P* = 0.005). However, the incidence of high *P. falciparum* density episodes was no longer significantly associated with infection status at baseline (*P* > 0.1). As for fevers with any *P. falciparum* density, spatial differences in incidence of high *P. falciparum* density episodes were observed with those children living near the coastline (IRR = 1.61, 95% CI = 1.05, 2.46, *P* = 0.030) and/or with those attending Mugil Elementary School (IRR = 2.02, 95% CI = 1.20, 3.40, *P* = 0.008) who had an increased risk. A tendency for lower incidence of high-density clinical *P. falciparum* infections was observed in mildly anemic children (Hb level < 11 g/dL, IRR = 0.69, 955 CI = 0.45–1.05, *P* = 0.084).

Of the 103 children with ≥ 1 febrile illness episodes and concurrent *P. falciparum* parasitemia of any density, five (4.9%) were re-treated with antimalarials prior to the episode and were thus excluded from time-to-event analyses. Because all febrile episodes with any concurrent parastaemia were treated, 12 (15.0%) of 80 children who had ≥ 1 episode with a *P. falciparum* parasitemia > 5,000/μL were excluded because of earlier re-treatment. Similar to our preceding analysis, the hazard of having a *P. falciparum* episode, both overall and for high-density infections, was higher in children attending the Mugil school and/or coastal hamlets, but lower in

children greater than nine years of age (Table 4 and Figure 6). In addition, female children tended to present more quickly with *P. falciparum* illness than males. Children with mild anemia (Hb level < 11 g/dL) had a significantly a lower risk of having a febrile illness (all episodes: AHR = 0.59, *P* = 0.020 and *P. falciparum* > 5,000/μL: AHR = 0.52, *P* = 0.016).

Mosquito transmission dynamics. The biting rates of *Anopheles* sp. in the coastal and inland (> 2.5 km) villages were 1.2 bites/person/night and 5.81 bites/person/night, respectively. A sampling effort of 188 person-nights over a 12-month period, identified 659 mosquitoes belonging to the *An. punctulatus* group. These included *An. punctulatus* (34.1%), *An. koliensis* (51.6%), and *An. farauti* (14.3%). *Anopheles farauti* (41.6%) and *An. koliensis* (41.6%) were equally abundant in coastal villages where *An. punctulatus* accounted for only 16% of mosquitoes caught. In inland villages, *An. punctulatus* (37.3%) and *An. koliensis* (51.9%) were notably more prevalent than *An. farauti* (8.6%).

Only 113 *Anopheles* mosquitoes were caught in the coastal village and none of the 53 processed by ELISA was positive for malaria parasites. In inland villages, the *P. falciparum* and *P. vivax* sporozoite rates for *An. punctulatus* s.l were 1.89% and 1.26%, respectively. The corresponding entomologic inoculation rates were 36.56 and 24.38 infective bites/person/year for *P. falciparum* and *P. vivax*, respectively.

DISCUSSION

The present study shows that Papua New Guinean children 5–13 years of age have acquired significantly different levels of immunity against *P. falciparum* and *P. vivax*. Despite comparable number of PCR-detectable new blood infections, children were 21 times more likely to have illness from *P.*
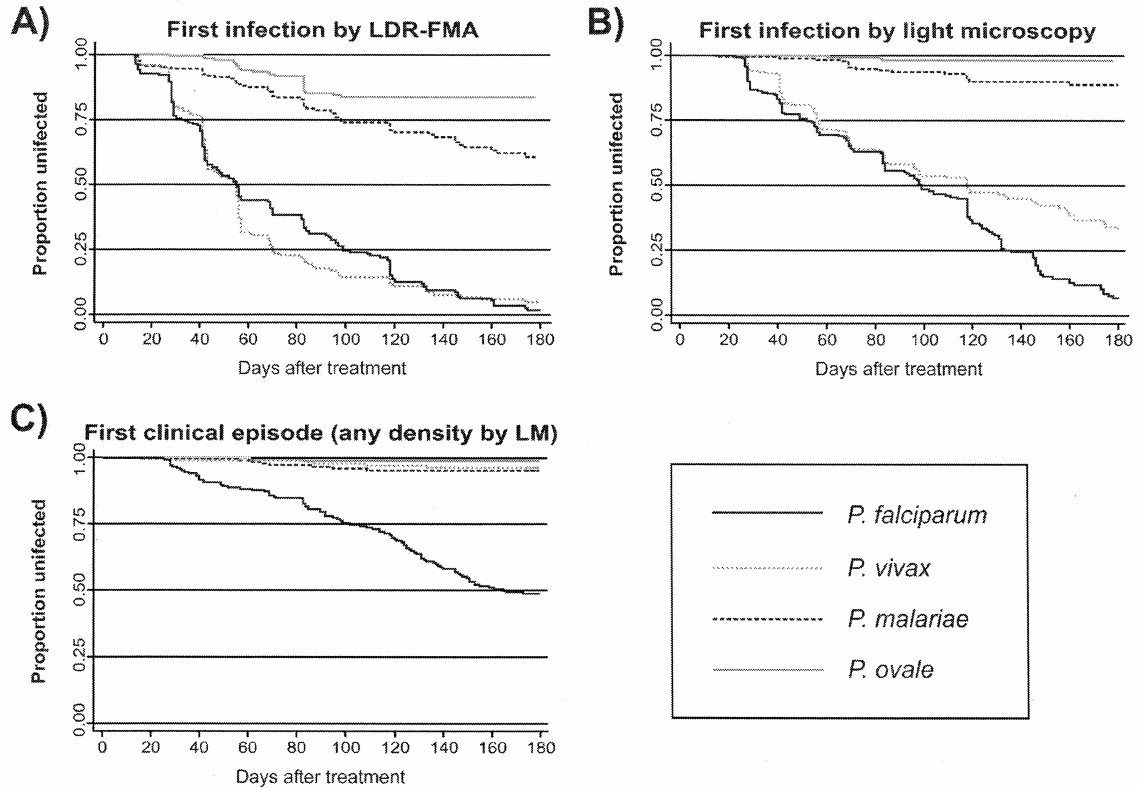
FIGURE 3. Risk of acquiring new infections after treatment of blood-stage infections. Kaplan-Meier curves for **A**, Asymptomatic infections diagnosed by post–polymerase chain reaction and ligation detection reaction–fluorescent microsphere assay (LDR-FMA), **B**, Asymptomatic infections diagnosed by light microscopy (LM), and **C**, Episodes of febrile illness with concurrent malarial infection of any density (by LM).

falciparum than from *P. vivax* infections. By the time they reach elementary school age, children in the study appeared to have acquired almost complete clinical immunity to *P. vivax*, whereas the acquisition of clinical immunity against *P falciparum* was ongoing, with older children showing reduced risk.

After treatment with a very short half-life drug, we saw rapid re-infection of children. In particular, prevalence of *P. vivax* increased quickly and parasite prevalence (by LDR-FMA and LM) exceeded those observed prior to treatment. Although by the end of follow-up virtually all children had been infected at least once with both parasites, it took significantly longer for *P. falciparum* prevalence rates to reach the levels seen at baseline. The fact that we did not use a drug with activity against liver stages may have influenced the rapid recurrence of *P. vivax* infections. However, in an area with year-round, albeit moderately seasonal, transmission such as Madang,[27] the rates of acquiring new liver stage infections through mosquito bites and establishing new blood stage infections from liver stages are likely to be comparable. The clearance of liver stages by drug treatment may lead to an underestimation of the incidence of *P. vivax* blood stage infections, particularly early in the follow-up period when new liver stage infections are acquired, but no new blood stage infections are established from existing liver stages. The high

number of blood stage infections observed is thus likely to be reflective of the true burden of infection with *P. vivax* in the study community.

The varying delay between time of initial infection by mosquito bite and appearance of blood stages in *P. vivax* may mean that seasonality of blood stage infections is partially uncoupled from seasonality of transmission. Some evidence for this is seen in this study cohort. The study was started and treatment was given in June, at the end of the high transmission season with the first half of the follow-up coinciding with the period of lowest transmission (July–September).[11,27] Although the incidence of new *P. falciparum* infections (by LDR-FMA and LM) was lower during the first half of follow-up, the incidence of *P. vivax* was relatively constant over time. Relapses from long-lasting liver stages may be an important contributor to the more limited seasonality of *P. vivax* that was also observed in earlier population surveys in the Madang area.[11,27]

It was evident that the children in the cohort were significantly better at controlling moderate and high-density *P. vivax* infections than *P. falciparum* infections (Figure 4). A considerable number of LDR-FMA–detectable *P. vivax* infections never became detectable by LM. Of those that did, few reached the pyrogenic threshold of 1,000 parasite/µL and only five children had a symptomatic *P. vivax* episode. This
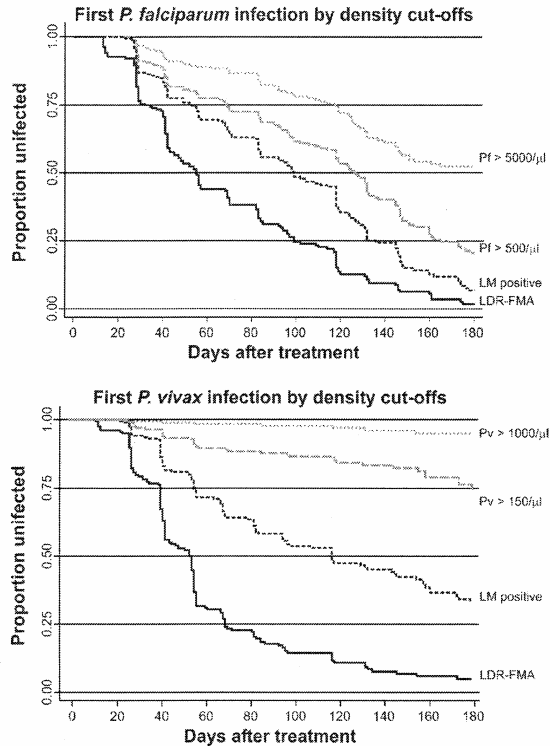
FIGURE 4. Kaplan-Meier curves of time to first *Plasmodium falciparum* (Pf) and *P. vivax* (Pv) infections exceeding increasing density thresholds. LM = positive by light microscopy; LDR-FMA = positive by post–ligation polymerase chain reaction ligation detection reaction–fluorescent microsphere assay. Shown are species-specific density cut-offs: higher cut-offs are set at species-specific pyrogenic thresholds and lower cut-offs are set at approximately 1.5 times the geometric mean density observed at baseline.
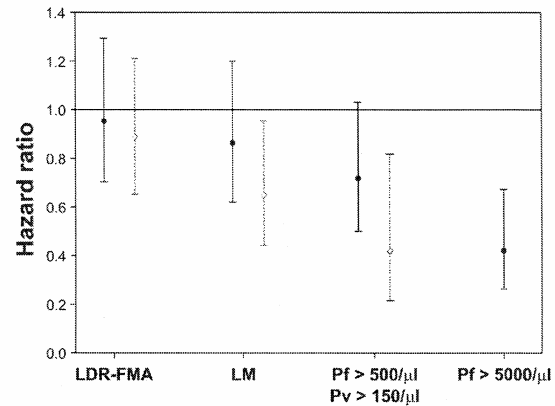

FIGURE 5. Risk of acquiring new infections in relations to age and increasing density cut-offs. Values are adjusted hazard ratios and 95% confidence intervals. ● = *Plasmodium falciparum* (Pf); ○ = *P. vivax* (Pv). Because of low number of infections, no age effect for Pv parasitemias > 1,000/mL was estimated. LDR-FMA = positive by post–polymerase chain reaction ligation detection reaction–fluorescent microsphere assay; LM = positive by light microscopy. Shown are species-specific density cut-offs: higher cut-offs for Pf are set at species-specific pyrogenic thresholds and lower cut-offs set at approximately 1.5 times the geometric mean density observed at baseline.

low incidence of clinical *P. vivax* disease in children 5–13 years of age is consistent with data from a passive morbidity surveillance system in another community in Papua New Guinea,[4] which showed that the incidence of *P. vivax* illness peaks in children 1–2 years of age and becomes rare in children greater than five years of age (Mueller I, Genton B, unpublished data). Conversely, almost half of all children had at least one *P. falciparum* infection > 5,000/μL and 80 (39%) children were diagnosed with 1–3 episodes of *P. falciparum* illness during the six months of follow-up. In a similar treatment re-infection study conducted in northern Ghana,[28] the incidence of *P. falciparum* disease after radical treatment for blood stage infections was higher than that usually observed for the same age group. On the basis of evidence that in older children asymptomatic infections of high multiplicities offered protection against subsequent morbidity,[29] Smith and others reported that the initial treatment of asymptomatic infections increased the children's risk of clinical illness. Although the incidence of *P. falciparum* illness in our study was high, it was similar to that observed in an earlier study in a neighboring population that measured malarial associated fever rates in children 2–15 years of age using a weekly morbidity surveillance program.[11] Because this earlier study did not treat children prior to follow-up and overall *P. falciparum* prevalence in asymptomatic individuals (39%) was comparable to what we observed at baseline, we believe it is unlikely that the initial treatment led to an increase in incidence of *P. falciparum* disease.

The relatively limited heterogeneity in the cohort in re-infection risk in relation to sex and place of residence indicates that age is likely to be closely correlated with lifetime exposure and thus is a good proxy for overall immune status. Comparing differences in risk of infections with different parasite densities not only shows that high-density *P. vivax* infections are rare, but that despite a similar number of new blood stage infections (as detected by LDR-FMA), the risk of acquiring even moderate and low-density *P. vivax* infections is significantly lower in older children (i.e. > 9 years of age). This indicates that by nine years of age children have acquired an ability to control *P. vivax* densities at levels well below pyrogenic thresholds with many infections not becoming patent by LM. In contrast, control of *P. falciparum* densities is less developed. Most blood stage *P. falciparum* infections do eventually become LM positive, and only at moderate-to-high densities is a reduction in risk apparent in older children. Nevertheless, some older children still have frequent high-density *P. falciparum* infections and clinical illness. These differences in ability to control parasite densities may explain why in most studies in areas with moderate-to-high endemicity for malaria, *P. vivax* prevalence[3,10,11,27] and incidence rates[30] peak in younger age groups than *P. falciparum* prevalence and incidence rates.

The observed difference in rates of immune acquisition for *P. falciparum* and *P. vivax* is remarkable, given that entomologic inoculation rates, as well as re-infection rates (by PCR), for both species are comparable. By the time children reach elementary school age, i.e. 6–7 years, they are likely to have

APPENDIX

TABLE 3
Incidence of symptomatic malaria episodes by species and case definition*

	Any episode		P. falciparum		P. vivax		P. malariae		P. ovale	
	Any	Threshold	Any	> 5,000/μL	Any	< 1,000/μL	Any	> 1,000/μL	Any	> 1,000/μL
No. episodes	162	111	148	103	10	5	8	2	3	2
No. mixed	7	1	5	1	4	1	5	0	0	0
No. of children with										
0 episodes	97	123	103	126	196	201	198	204	203	204
1 episode	62	57	64	60	10	5	8	2	3	2
2 episodes	36	23	33	17						
3 episodes	9	3	6	3						
4 episodes	2	0	0	0						
% children with ≥ 1 episode	52.9	40.3	50.0	38.8	4.9	2.4	3.9	1.0	1.5	1.0
Incidence rate† (/child/year)	1.92 (1.65, 2.23)	1.27 (1.06, 1.53)	1.68 (1.43, 1.98)	1.17 (0.97, 1.42)	0.11 (0.06, 0.21)	0.06 (0.02, 0.14)	0.09 (0.05, 0.18)	0.02 (0.01, 0.09)	0.03 (0.01, 0.11)	0.02 (0.01, 0.09)

* Values in parentheses are 95% confidence intervals.
† Estimated from Poisson regression model. Total time at risk = 32,125 person days.

TABLE 4
Multivariate predictors for time to first *Plasmodium falciparum* (Pf) episode: infections with any density versus infections > 5,000/μL*

	Any Pf density		Pf > 5,000/μL	
	AHR	(95% CI)	AHR	(95% CI)
Multivariate predictors†				
Enrolled at Mugil Elementary School	**1.62**	**(0.98, 2.68)**	3.35	(1.58, 7.10)
Coastal residence‡	2.08	(1.34, 3.21)	1.98	(1.19, 3.30)
Female	**1.41**	**(0.94, 2.12)**	**1.52**	**(0.93, 2.40)**
Age > 9 years	0.56	(0.37, 0.85)	0.51	(0.31, 0.84)
Hb < 11 g/dL	0.59	(0.38, 0.92)	0.52	(0.30, 0.88)

* AHR = adjusted hazard ratio; CI = confidence interval; Hb = hemoglobin.
† Only factors with a *P* value < 0.05 and 0.05–0.10 (**bold**) from multivariate Cox regression analyses are given. Other factors included distance from school, distance from health center, presence of enlarged spleen, reported bed net use (slept > 50% of nights under bed net) and infection status with any *Plasmodium* species diagnosed by PCR and LM at baseline.
‡ Residence of child situated less than one kilometer from coastline.

been exposed to 150–200 infected bites and had 30 successful blood stage infections with each species. Why a similar amount of exposure can lead to these apparent differences in immunity to the two species is not clear. However, similar differences in rates of immune acquisition have been described in Vanuatu[30] and other areas of the world where the species co-existed.[31,32] These results are consistent with data from malaria therapy patients in whom immunity to *P. vivax* was more rapidly acquired than that to *P. falciparum*.[33] Whereas a single infection with *P. vivax* resulted in a strongly reduced incidence of a febrile episode upon homologous and to a lesser extent heterologous re-infection,[34] most secondary *P. falciparum* infections were associated with fever, and in some cases high-density parasitemia, even if re-infected with the same strain.[35] Similar patterns were observed under natural conditions in Sri Lankan patients.[36]

Contrary to the relative homogeneity in re-infection risk in relation to sex and place of residence, there were significant, spatially structured differences in risk of *P. falciparum* disease between children attending the Mugil Elementary School and those living near the coast who more often had a febrile episode of *P. falciparum* parasitemia; girls also tended to be at higher risk of illness. However, these differences are more likely to be related to differences in accessibility and health-seeking behavior rather than reflect true difference in risk of
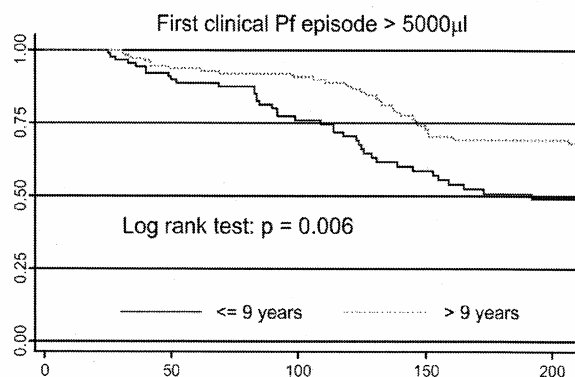

FIGURE 6. Kaplan-Meier curves of risk of experiencing an episode of symptomatic *Plasmodium falciparum* (Pf) malaria with a parasite density greater than 5,000 parasites/μL in relation to age.

disease. The higher frequency of study team visits to the Mugil school indicated that children who were sick while attending school were more likely to be reported to the study team on non-surveillance days than at the other schools. Similarly, the vicinity to the schools and the sealed road leading to the health center indicated that it was substantially simpler for children living in costal hamlets to seek treatment from either study team or health center. Such a decrease in attendance at health centers with increasing distance has been previously observed in Papua New Guinea.[37]

The observed association of mild anemia (Hb level < 11 g/dL) with protection against clinical *P. falciparum* disease is intriguing because such a mild degree of anemia is by itself unlikely to be an impediment to parasite growth. Because malaria is an important contributor to anemia,[38–40] Hb levels at baseline may be a marker of recent malarial exposure rather than directly affecting risk of *P. falciparum* illness.

Although almost 40% of children did have at least one LDR-FMA–detectable *P. malariae* infection, most of these infections were not apparent by LM, and as in areas of Africa highly endemic for malaria,[41,42] episodes of *P. malariae* illness were rare. The low number of episodes precludes drawing firm conclusion with regards to acquisition on immunity to *P. malariae* in this population. However, because seven of eight clinical episodes occurred in children greater than nine years of age, older children are still at least as susceptible as younger children to *P. malariae* and the acquisition of immunity to this species may consequently be rather slow. Similar slow acquisition of immunity to *P. malariae* is also supported by observations by Smith and others[9] and Kasehagen and others[10] that in another population in Papua New Guinea the prevalence of *P. malariae* infections peaked later than those for *P. vivax* and *P. falciparum*. As observed in earlier cross-sectional surveys[10,16] infections and illness with *P. ovale* were rare and, as observed in Africa,[43] most *P. ovale* infections were of very short duration. In contrast to infections with other species, *P. ovale* infections were clustered in time and space with significantly higher risk of infection between weeks 6 and 12 of follow-up and in children attending Megiar Elementary School.

Since the suggestion by Williams and others that an increased incidence of *P. vivax* infection in early life may protect children with α-thalassemia from later severe *P. falciparum* malaria,[44] a number of studies have investigated potential cross-species protection. Although clinical studies from Thailand,[45,46] as well as an epidemiologic study from Papua New Guinea,[47] provide some support to the idea of cross-species protection by *P. vivax* against *P. falciparum* morbidity, it has been remarkably difficult to find consistent evidence that infection with one species protects against (concurrent or subsequent) heterologous infections.[16,47–50] With the exception of a possible positive interaction between prior infection with *P. falciparum* and subsequent risk of infection with *P. ovale*, the present study did not find any evidence that the presence of a heterologous infection at baseline provided protection against subsequent re-infections or *P. falciparum* illness. However, persistent, concurrent parasitemia may be required for cross-species protection. Thus, it is possible that the lack of associations between heterologous species is caused by clearance of blood stage infections by the initial treatment rather than truly indicating a lack of cross-species protection. The existence of cross-species protection is thus better studied in conventional cohort rather than a treatment re-infection design.

The current study clearly demonstrates differences in the acquisition of clinical immunity to *P. falciparum* and *P. vivax* among Papua New Guinean children. Despite similar incidence of infections, *P. vivax* is no longer a consistent source of morbidity by the time children reach school age. Although it continues to decrease with age, the burden of *P. falciparum* illness remains high. In the age group studied (i.e., 5–14 years), clinical immunity seems to be linked closely to the ability to control parasite densities, with children able to control *P. vivax* at much lower densities than *P. falciparum*. Potential reasons for the differential rates in the acquisition of immunity may include key biologic differences between the two species, particularly in relation to red blood cell invasion, adhesion or sequestration in vascular beds, and the nature of variant surface antigens. Understanding these differences may provide key insights into immunity and pathogenesis with *P. falciparum* and *P. vivax* and is the focus of on-going studies. Comparative studies on immune acquisition to both *P. falciparum* and *P. vivax* species may thus provide valuable insight into the important different immune targets and mechanisms underlying acquisition of clinical protection.

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Immunoglobulin G Subclass-Specific Responses against *Plasmodium falciparum* Merozoite Antigens Are Associated with Control of Parasitemia and Protection from Symptomatic Illness[▽][†]

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Substantial evidence indicates that antibodies to *Plasmodium falciparum* merozoite antigens play a role in protection from malaria, although the precise targets and mechanisms mediating immunity remain unclear. Different malaria antigens induce distinct immunoglobulin G (IgG) subclass responses, but the importance of different responses in protective immunity from malaria is not known and the factors determining subclass responses in vivo are poorly understood. We examined IgG and IgG subclass responses to the merozoite antigens MSP1-19 (the 19-kDa C-terminal region of merozoite surface protein 1), MSP2 (merozoite surface protein 2), and AMA-1 (apical membrane antigen 1), including different polymorphic variants of these antigens, in a longitudinal cohort of children in Papua New Guinea. IgG1 and IgG3 were the predominant subclasses of antibodies to each antigen, and all antibody responses increased in association with age and exposure without evidence of increasing polarization toward one subclass. The profiles of IgG subclasses differed somewhat for different alleles of MSP2 but not for different variants of AMA-1. Individuals did not appear to have a propensity to make a specific subclass response irrespective of the antigen. Instead, data suggest that subclass responses to each antigen are generated independently among individuals and that antigen properties, rather than host factors, are the major determinants of IgG subclass responses. High levels of AMA-1-specific IgG3 and MSP1-19-specific IgG1 were strongly predictive of a reduced risk of symptomatic malaria and high-density *P. falciparum* infections. However, no antibody response was significantly associated with protection from parasitization per se. Our findings have major implications for understanding human immunity and for malaria vaccine development and evaluation.

Effective immunity against *Plasmodium falciparum* malaria in humans develops slowly over time after repeated exposure and protects against the development of symptomatic and severe illness. Although the targets of protective immunity in humans remain ill-defined, substantial evidence suggests that antibodies against merozoite antigens play an important role, and several merozoite antigens are leading vaccine candidates (5, 15, 29, 35, 37, 45). Antibodies to merozoite antigens are thought to function in vivo by inhibition of merozoite invasion of erythrocytes, opsonization of merozoites for phagocytosis, and antibody-dependent cellular inhibition (3, 9, 13, 21, 24).

The subclass of antibodies produced against antigens is likely to be important for protective activity, as immunoglobulin G (IgG) subclasses differ in their structures and mediate different immune effector functions (32). Knowledge of subclass responses associated with protection against malaria is important for understanding immunity and guiding vaccine development. IgG1 and IgG3 are the predominant subclasses produced in response to merozoite antigens (31, 37, 40, 43, 46, 48). IgG1 and IgG3 are cytophilic and T cell dependent, have high affinity for Fc receptors, and mediate phagocyte activation and complement fixation (7). It has been suggested that IgG3 is more efficient at mediating these processes (7). For reasons that are not well understood, different merozoite antigens induce different relative levels of IgG1 and IgG3 (14, 29, 31, 37, 40, 46, 48). It is unclear whether individuals have a bias toward producing a specific subclass regardless of the antigen or if instead the IgG subclass response is generated independently for each antigen and how this relates to protective immunity. While factors determining subclass responses to antigens are not clearly defined, antigen properties, host age, cumulative exposure, and genetic determinants have been linked with the nature of subclass responses (2, 4, 17, 33, 34, 41, 42, 47, 48). Some studies have suggested that increasing age (and therefore malaria exposure) leads to an increasing polarization of IgG subclass responses to merozoite antigens (41, 48).

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Antibodies to merozoite antigens have been linked with protection from malaria in humans in some longitudinal studies (6, 11, 15, 23, 25, 29, 31, 35, 37–39, 45). Results from these studies have been conflicting, which results partly from the use of different endpoints for evaluating the protective role of antibodies (i.e., different parasitemia thresholds versus symptomatic illness). It is thought that acquired immunity largely targets blood-stage antigens and acts by limiting parasite replication, thereby preventing the development of high-density parasitemia, but is less effective at protecting from parasitization per se (26). However, there are limited data that directly address this question and few studies have evaluated antibody associations with protection from symptomatic malaria, high-density parasitemia, and parasitization per se in the same cohort because of challenges in performing these studies in community-based settings. Additionally, the detection of parasitization has generally been performed using light microscopy, which is not sufficiently sensitive to detect parasitemias of very low density. The development of high-throughput molecular methods to detect parasitemia in cohort studies has provided new opportunities to better define these associations between immune responses and parasitization and symptomatic malaria. Furthermore, most studies of immunity have been conducted in sub-Saharan Africa, and there are little data from populations in Asia, where a large portion of the global malaria burden occurs (44).

We addressed these important issues in a treatment-reinfection study of 206 children resident in an area of malaria endemicity in Papua New Guinea. We prospectively examined associations between subclass-specific responses to *P. falciparum* merozoite antigens (the 19-kDa C-terminal region of merozoite surface protein 1 [MSP1-19], apical membrane antigen 1 [AMA-1], and merozoite surface protein 2 [MSP2]) and the risks of high-density parasitemia, symptomatic malaria, and reinfection, as detected by sensitive molecular-based methods. Furthermore, we evaluated the influences of host age, exposure, and concurrent *P. falciparum* infection on the nature of responses and assessed whether individuals demonstrated a bias toward specific subclass responses and whether polymorphisms in antigens influenced the nature of subclass responses.

MATERIALS AND METHODS

Study population. A prospective treatment-reinfection study was undertaken in the Mugil and Megiar area 50 km north of Madang, Papua New Guinea. Details of the study are described elsewhere (30). Briefly, 206 children aged 5 to 14 years (median, 9 years; interquartile range, 8.1 to 10.3 years) were enrolled in the study, and venous blood samples were collected. All children were treated with 7 days of artesunate taken orally and were monitored for 6 months by active (twice-weekly) and passive case detection for reinfection and symptomatic illness. New infections were distinguished from treatment failures by MSP2-based genotyping. A symptomatic episode of *P. falciparum* malaria was defined as the presence of fever and parasitemia of >5,000 parasites/µl. Parasitemia was determined by a semiquantitative post-PCR ligase detection reaction-fluorescent microsphere assay (LDR-FMA) (28), and light microscopy. All analyses were performed using parasitemia determined by LDR-FMA, unless otherwise indicated. *P. falciparum* was detected in 67.5% of subjects at enrollment by LDR-FMA and in 40.3% by light microscopy. Samples collected at baseline and from the first symptomatic infection were genotyped to identify MSP2 alleles (FC27 or 3D7), according to methods published previously (16, 22).

Samples were also collected from children and adults (0 to 3 years old [*n* = 50; median age of 2 years], 4 to 6 years old [*n* = 48; median age of 5 years], 7 to 9 years old [*n* = 50; median age of 7.9 years], and ≥10 years old [*n* = 59; range from 10 to 56 years; median age of 17.7 years]) in the Madang area of Papua New

Guinea to further evaluate the associations between age and IgG subclass response to merozoite antigens. Plasma samples were obtained from anonymous Melbourne, Australia, residents with no known previous exposure to malaria to act as negative controls in all assays.

Informed consent was obtained from all participants in the studies, and ethics approval was obtained from the Medical Research Advisory Council, PNG, and the Human Research Ethics Committee, The Walter and Eliza Hall Institute.

ELISA. Samples collected from the enrollment bleed were used in an enzyme-linked immunosorbent assay (ELISA). All available samples were tested for total levels of IgG, IgG1, and IgG3 to each antigen. A subset of samples was tested for IgG2 and IgG4 (*n* = 129 for MSP1-19 and MSP2 IgG2 and IgG4; *n* = 121 for AMA-1 IgG2; *n* = 120 for AMA-1 IgG4). AMA-1 was expressed as a His-tagged recombinant protein in *Escherichia coli*, using the full ectodomains of 3D7 and W2mef, and was purified and refolded as described previously (21). Recombinant MSP1-19 (3D7 sequence) was expressed as a His-tagged protein in *E. coli*, purified over nickel-nitrilotriacetic acid resin (Qiagen, Victoria, Australia), and refolded, as described previously (12). Full-length MSP2 (corresponding to the 3D7 or FC27 gene sequence) was expressed in *E. coli* as a C-terminally His-tagged protein which was purified by nickel chelate, anion-exchange, and reversed-phase chromatography. Schizont parasite protein extract was prepared from *P. falciparum* (3D7). Schizonts were lysed with saponin (0.09% in RPMI-HEPES) on ice for 10 min and then centrifuged at 4°C, and the pellet was washed in cold phosphate-buffered saline (PBS). Schizonts were resuspended in cold PBS, vortexed and freeze-thawed on dry ice twice, and sonicated for 30 s to solubilize proteins. This mixture was then centrifuged, and the supernatant was collected and used in the assays.

ELISAs were performed using established methods (36). Ninety-six-well plates (Immulon 4 plates [ThermoLabsystems, MA] or Maxisorp plates [Nunc, Roskilde, Denmark]) were coated with 0.5 µg/ml of recombinant antigen or 4.6 µg/ml schizont lysate in PBS and incubated overnight at 4°C. Skim milk-PBS-0.05% Tween was used for blocking and diluting plasma and antibodies. Plasma was added in duplicate at previously determined dilutions. For measurement of total IgG, horseradish peroxidase-conjugated sheep anti-human IgG (Chemicon, Melbourne, Australia) was used at a 1:2,500 concentration. For measurement of IgG subclasses, secondary antibodies were added at a dilution of 1:1,000 using mouse anti-human IgG subclass antibodies (IgG1 clone HP6069 [Invitrogen Corporation, CA], IgG3 clone HP6047 [Invitrogen Corporation, CA], IgG4 clone HP6023 [Calbiochem-Novabiochem Corp., CA], and IgG2 clone HP6002 [Caltag Laboratories, CA]). The tertiary antibody for the subclass assays was a sheep anti-mouse antibody (Chemicon International, CA), added at 1:2,500. Finally, ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] substrate (Sigma, Castle Hill, Australia) was added to the plates and the reaction stopped with 1% sodium dodecyl sulfate. The optical density (OD) was determined at 405 nm. All samples were tested in duplicate, and samples were retested if there was a discrepancy of greater than 25% between duplicates. Standardization of the plates was achieved using positive-control plasma pools on each plate. Background (determined from the wells with no plasma) was deducted from the mean of each sample and a cutoff threshold for positivity determined as the mean plus 3 standard deviations from the nine negative-control plasma samples (Melbourne residents) included in each assay.

Analysis. As antibody levels were not normally distributed, nonparametric tests were used for analyses. Correlations between ODs of different subclasses and/or to different antigens were determined using Spearman's rank correlation, and differences in the median ODs with age and infection status were compared using a two-sample Wilcoxon rank sum test. Differences in the proportions of children positive for different subclasses and associations between age and infection status and antibody prevalence were assessed using the chi-square or Fisher's exact test. To determine how exposure influences the subclass profile, children were grouped into four equal groups (quartiles) according to their IgG responses to *P. falciparum* schizont extract and the subclass responses within each group examined.

For determining the association between antibody levels and *P. falciparum* infection and symptomatic malaria, children were stratified into three equal groups (tertiles), reflecting low, medium, and high responders according to OD values for each antigen (tertile cutoffs are given in Table S1 in the supplemental material). A Poisson regression was used to test for associations between antibody levels and incidence of disease, while associations with time to first infection were assessed by Cox regression, adjusting for known confounders, as previously described (30). In all multivariate analyses, backward selection and likelihood ratio tests were used to identify the best-fitting models. All statistical analyses were performed using STATA 8 statistical analysis software (Stata Corporation, College Station, TX). *P* values of ≤0.05 were considered statistically significant, and *P* values of >0.1 were classified as not significant (NS).
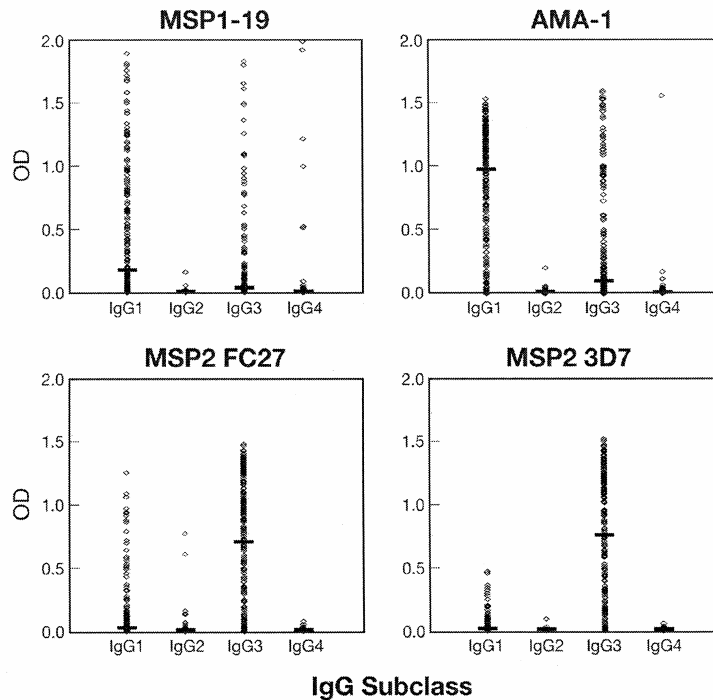
FIG. 1. IgG subclass profiles for the levels of reactivity to merozoite antigens of *P. falciparum* among the study cohort, as measured by ELISA. Horizontal bars indicate the median antibody levels. The 3D7 allele of MSP1-19 and AMA-1 was used.

RESULTS

IgG subclass responses to merozoite antigens and effect of polymorphisms on these responses. In agreement with previous studies, antibody responses were predominantly IgG1 and IgG3 and there was little IgG2 and IgG4 reactivity (Fig. 1). Therefore, analyses were restricted to IgG1 and IgG3. The levels of predominance of IgG1 versus IgG3 varied among antigens (Fig. 1; also see Table S1 in the supplemental material). For MSP1-19 and AMA-1, IgG1 levels were significantly higher than IgG3 levels (median IgG1 OD of 0.17 versus median IgG3 OD of 0.03 [$P < 0.001$] for MSP1-19; median IgG1 OD of 0.97 versus median IgG3 OD of 0.09 [$P < 0.001$] for AMA-1). In contrast, IgG3 was the dominant subclass for both allelic forms of MSP2 (median IgG1 OD of 0.01 versus median IgG3 OD of 0.74 for the 3D7 allele; median IgG1 OD of 0.02 versus median IgG3 OD of 0.70 for the FC27 allele [$P < 0.001$]).

We next evaluated the effect of antigen polymorphisms on subclass responses. Polymorphisms in AMA-1 appeared to have little effect on the profile of IgG subclass responses. In a subset of children ($n = 73$), IgG1 and IgG3 were measured against both the 3D7 and the W2mef AMA-1 variants, which have been shown to be antigenically different (20). For both AMA-1 variants, IgG1 was predominant, and similar numbers of individuals were positive for each allele (93.2% IgG1 versus 82.2% IgG3 for 3D7; 93.2% IgG1 versus 76.7% IgG3 for W2mef). For MSP2, responses were less strongly biased toward IgG3 for the FC27 allele than for the 3D7 allele. The

prevalence of IgG1 was significantly higher for the FC27 than for the 3D7 allele (41.3% and 51.5% IgG1 [$P = 0.04$] for MSP2 3D7 and FC27, respectively), whereas the prevalences of IgG3 were similar (84.2% and 80.7% IgG3 for MSP2 3D7 and FC27, respectively [$P = 0.35$]).

IgG subclass responses are associated with age, exposure, and concurrent parasitemia. Antibody responses were significantly associated with age in the cohort; both IgG1 and IgG3 levels were significantly higher among older children than among younger children for all four antigens (Fig. 2). Antibody prevalence was also significantly higher for older children for all responses except that of IgG1 to MSP2 (see Table S2 in the supplemental material). We did not find any evidence that increasing age or malaria exposure leads to an increasing polarization of responses from a mixed IgG1/IgG3 profile to a particular IgG subclass response, as reported from studies of African populations (45, 48). Higher levels of IgG to schizont protein extract (reflecting greater exposure to malaria) were significantly correlated with higher levels and prevalence of IgG1 and IgG3 for all antigens (Fig. 3 and Table 1; also see Fig. S1 in the supplemental material). Because the age range in the cohort was narrow, we tested an additional set of samples from 207 randomly selected individuals aged 0 to 56 years resident in the same geographical area to further examine this relationship. For all four antigens, both IgG1 and IgG3 increased significantly in association with age (Fig. 4; also see Fig. S2 in the supplemental material).

In the main cohort, levels of IgG1 and IgG3 to AMA-1,

AMA-1

IgG1   p = 0.01

p < 0.001   p = 0.05

IgG3   p = 0.02

p < 0.001   p = 0.05

Pf Neg / Pf Pos (<= 9 yrs)   Pf Neg / Pf Pos (> 9 yrs)

MSP1-19

IgG1   p < 0.001

p = 0.006   p = 0.65

IgG3   p < 0.001

p < 0.001   p = 0.20

Pf Neg / Pf Pos (<= 9 yrs)   Pf Neg / Pf Pos (> 9 yrs)

MSP2 3D7

IgG1   p = 0.004

p = 0.01   p = 0.04

IgG3   p = 0.02

p = 0.004   p = 0.0

Pf Neg / Pf Pos (<= 9 yrs)   Pf Neg / Pf Pos (> 9 yrs)

MSP2 FC27

IgG1   p = 0.004

p = 0.01   p = 0.04

IgG3   p < 0.001

p = 0.05   p = 0.0

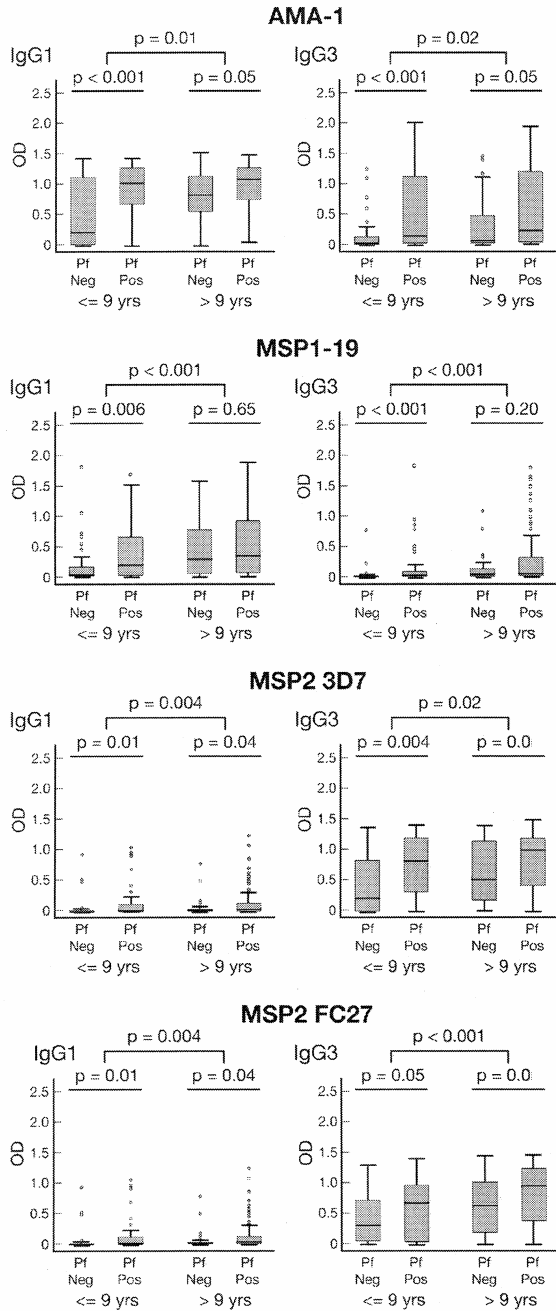Pf Neg / Pf Pos (<= 9 yrs)   Pf Neg / Pf Pos (> 9 yrs)

FIG. 2. Associations between age and presence of parasitemia and IgG subclass responses to merozoite antigens of *P. falciparum*. Children were divided into two age groups, ≤ 9 years ($n = 91$) and >9 years ($n = 115$), to examine associations with age. The presence (Pf Pos [$n = 139$]) or absence (Pf Neg [$n = 67$]) of *P. falciparum* infection was determined by PCR. Data are plotted as box-and-whisker plots (boxes show medians and interquartile ranges; error bars show 95% confidence intervals).

MSP1-19, and MSP2 were higher among children with concurrent *P. falciparum* infection at enrollment than among uninfected children; for MSP1-19, this association was observed only in children of <9 years (Fig. 2). For AMA-1 and MSP1-19, the prevalences of IgG1 and IgG3 were also higher in infected children than in uninfected children (see Table S2 in the supplemental material). For MSP2, only the prevalence of the less dominant IgG1 was higher among infected children (see Table S2 in the supplemental material). When the MSP2 allelic type was considered, a higher prevalence of allele-specific IgG3 was associated with infection only by the corresponding genotype (i.e., the prevalence of MSP2 3D7 IgG3 was higher among children with 3D7-type infections than among children with FC27-type infections) (Fig. 5). A similar, although less pronounced, strain-specific boosting was observed for IgG1 (data not shown).

Relatedness of subclass responses to different antigens within individuals. For all antigens, the total IgG levels were strongly correlated with the IgG1 and IgG3 responses ($P < 0.001$) (Table 1). Correlations with the total IgG levels were highest for the predominant subclass for each antigen (higher for IgG1 than IgG3 for AMA-1 and MSP1-19, but higher for IgG3 than IgG1 for MSP2). Antibody responses were also significantly correlated between different antigens, although the strengths of the correlations varied substantially (median rho = 0.42; interquartile range, 0.36 to 0.48) (Table 1).

As subclass responses varied between different individuals, we examined whether individuals might have a propensity to make a particular subclass response irrespective of the antigen. When comparing individual responses to different merozoite antigens, we found no evidence that this was the case. Individuals who were high responders (defined as >50th percentile; $n = 103$) for IgG3 to AMA-1 (3D7 variant) were no more likely to be high responders for IgG3 to MSP1-19 than for IgG1 to MSP1-19 (67% were high responders for IgG3, versus 64% for IgG1; P value was NS). Similarly, high responders for IgG1 to AMA-1 (3D7) were no more likely to be high responders for IgG1 to MSP1-19 than for IgG3 to MSP1-19 (70% were high responders for IgG1, versus 62% for IgG3; P value was NS). Similar results were obtained when comparing AMA-1 3D7 subclass responses of high responders for IgG1 and IgG3 to MSP2 (data not shown).

We then examined the relatedness of subclass responses between different forms of the same antigen. High responders for IgG3 to AMA-1 3D7 were significantly more likely to be high responders for IgG3 to AMA-1 W2mef than high responders for IgG1 to AMA-1 W2mef (92% were high responders for IgG3, versus 64% for IgG1; $P = 0.005$). A similar association was observed when comparing high responders for IgG1 to AMA-1 3D7 with high responders for IgG1 to AMA-1 W2mef (86% were high responders for IgG1, versus 67% for IgG3; $P = 0.052$). The IgG subclass responses to the two AMA-1 variants were also strongly correlated ($n = 73$; IgG1 rho = 0.92; IgG3 rho = 0.92; $P < 0.001$). Conversely, there was little evidence of relatedness between IgG subclass responses to the two MSP2 alleles. High responders for IgG1 to MSP2 3D7 were no more likely to be high responders for IgG1 to MSP2 FC27 than for IgG3 to MSP2 FC27 (67% were high responders for IgG1, versus 61% for IgG3; P value was NS). Similar observations were made with MSP2 IgG3 responses
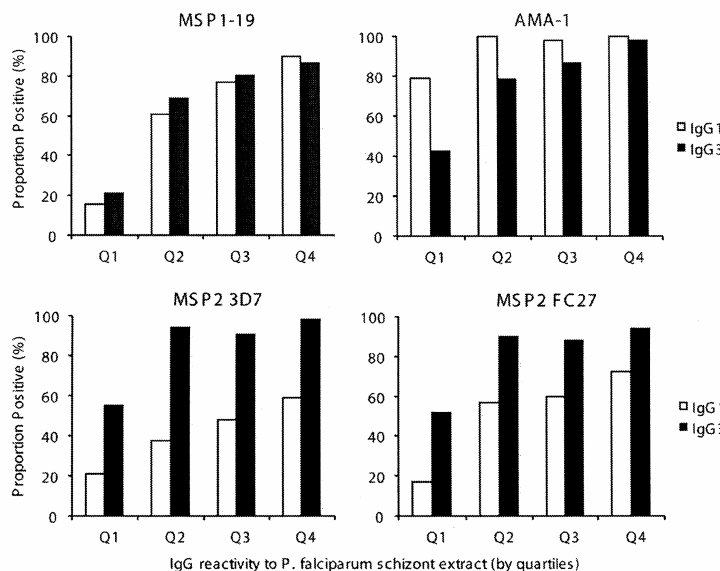
FIG. 3. Effect of exposure on prevalence of IgG subclass responses to different merozoite antigens. Exposure was determined by IgG reactivity to *P. falciparum* schizont extract. Children were grouped into quartiles (Q1 to Q4, where Q1 represents the group with the lowest IgG response to *P. falciparum* schizont extract), and the proportions of individuals who were positive for IgG1 and IgG3 for each quartile are shown. *P* < 0.0001 for differences in prevalence of IgG1 or IgG3 between groups for all antigens, except *P* = 0.001 for MSP2 3D7 IgG1. The 3D7 allele was used for MSP1-19 and AMA-1.

(data not shown). IgG subclass reactivities against the 3D7 and FC27 alleles were significantly correlated (*n* = 199; IgG1 rho = 0.36; IgG3 rho = 0.37; *P* < 0.001) but not as strongly as those observed for AMA-1.

Association between subclass response and risk of reinfection, high-density parasitemia, and symptomatic malaria. We examined associations between antibodies and symptomatic malaria, reinfection, or parasitemias of different densities (Fig.

TABLE 1. Correlations between antibody responses to merozoite antigens of *P. falciparum*

Antigen and antibody	Correlation coefficient[a]											
	AMA-1			MSP1-19			MSP2 3D7			MSP2 FC27		
	IgG	IgG1	IgG3	IgG	IgG1	IgG3	IgG	IgG1	IgG3	IgG	IgG1	IgG3
AMA-1												
IgG		0.87	0.46	0.48	0.52	0.43	0.49	0.20**	0.48	0.48	0.37	0.41
IgG1			0.47	0.46	0.50	0.40	0.48	0.24	0.49	0.48	0.39	0.41
IgG3				0.40	0.44	0.55	0.42	0.22**	0.42	0.42	0.16*	0.47
MSP1-19												
IgG					0.86	0.32	0.41	0.14*	0.42	0.47	0.31	0.38
IgG1						0.34	0.48	0.21**	0.48	0.43	0.37	0.37
IgG3							0.43	0.26	0.44	0.54	0.31	0.56
MSP2 3D7												
IgG								0.48	0.89	0.46	0.36	0.39
IgG1									0.39	0.32	0.36	0.25
IgG3										0.40	0.29	0.37
MSP2 FC27												
IgG											0.72	0.91
IgG1												0.6
IgG3												
Schizont protein extract IgG	0.52	0.58		0.68	0.52		0.36	0.58		0.44	0.58	

[a] Correlation coefficients were determined by Spearman's method, using samples that had complete data only (*n* = 198). All correlations are significant at a *P* value of <0.001 unless otherwise indicated (*, *P* < 0.05; **, *P* < 0.01).
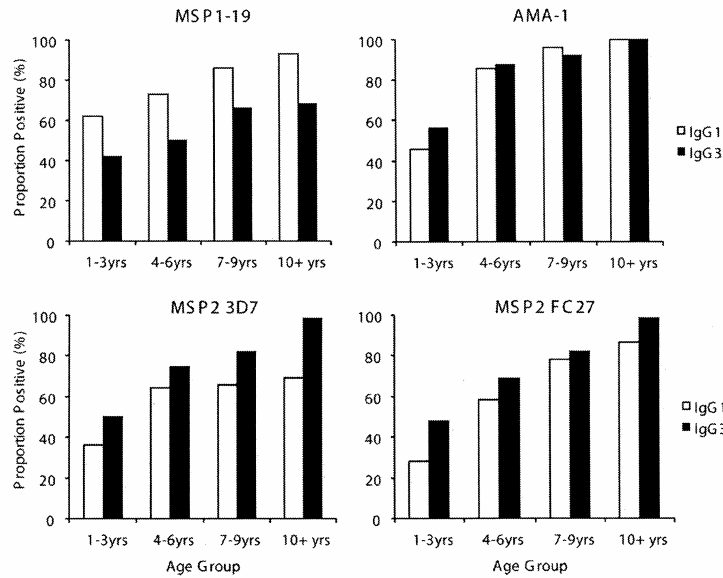
FIG. 4. Association between age and IgG subclass reactivity to different merozoite antigens. The proportions of individuals who were positive for IgG1 and IgG3 for each age group are shown (0 to 3 years [$n = 50$], 4 to 6 years [$n = 48$], 7 to 9 years [$n = 50$], and >10 years [$n = 59$]). Associations between IgG subclass responses and age were significant for all antigens (for MSP1-19, $P < 0.001$ and $P = 0.018$ for IgG1 and IgG3, respectively; for AMA-1, $P < 0.0001$ for IgG1 and IgG3; for MSP2 3D7, $P = 0.002$ and $P < 0.0001$ for IgG1 and IgG3, respectively; and for MSP2 FC27, $P < 0.0001$ for IgG1 and IgG3). The 3D7 allele of MSP1-19 and AMA-1 was used.
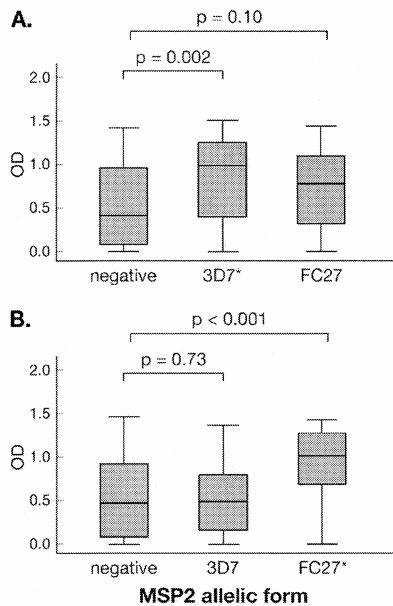

MSP2 allelic form

FIG. 5. Association between MSP2 3D7 (A) and MSP2 FC27 (B) IgG3 responses and MSP2 genotype of concurrent *P. falciparum* infection. *P. falciparum* infection status was determined by PCR. *, results include mixed 3D7/FC27 infections. Data are plotted as box-and-whisker plots (boxes show medians and interquartile ranges; error bars show 95% confidence intervals).

6). Univariate analyses established that the risk of *P. falciparum* malaria was associated with older age and location of residence but not with other demographic parameters (30); therefore, analyses of associations between antibodies and malaria risk were adjusted for location and age. Erythrocyte genetic polymorphisms (SAO, Gerbich, α^+-thalassemia, and CR1 polymorphisms) were not associated with risk of *P. falciparum* malaria (E. Lin, P. Michon, and I. Mueller, unpublished data).

Antibody levels were grouped as high, medium, or low, based on tertiles (which divide the data into three equally sized groups), and related to the risk of symptomatic malaria. The strongest association between IgG subclass reactivity to merozoite antigens and protection from symptomatic malaria was observed for IgG3 to AMA-1. After adjustment for location, a strong reduction in risk (i.e., evidence of protection) was found for high (compared to low) levels of AMA-1 IgG3 (adjusted hazard ratio [AHR] of 0.23, $P < 0.001$). There was also a smaller reduction in risk among children with high IgG1 responses (AHR of 0.51, $P = 0.028$). However, in a model combining IgG1 and IgG3 responses, only high IgG3 responses were predictive of protection. Total IgG to AMA-1 was associated only weakly with protection against symptomatic malaria (AHR of 0.56 for low versus moderate levels, $P = 0.045$; AHR of 0.63 for high versus low levels, $P = 0.14$). Individuals with high levels of IgG1 and IgG to MSP1-19 had a significantly reduced risk of malaria compared to those with low levels, after adjusting for location (AHR of 0.43 for IgG1, $P = 0.007$; AHR of 0.39 for total IgG, $P = 0.004$). Individuals with medium IgG1 and IgG levels also had a reduced risk of malaria compared to those with low levels (AHR of 0.48 for IgG1, $P = $
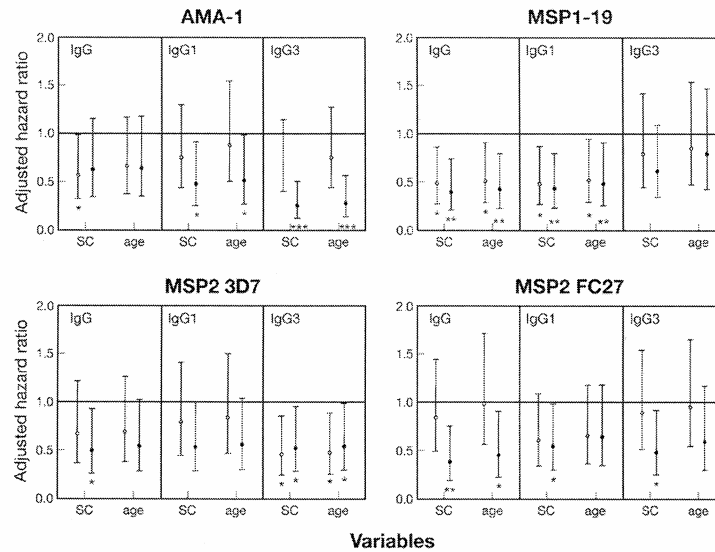
FIG. 6. Association between IgG antibody responses to merozoite antigens and protection against symptomatic *P. falciparum* malaria. Children were stratified into groups of low, moderate, and high responders (based on tertiles) in order to test for associations between antibody responses and risk of symptomatic malaria (defined as parasitemia of >5,000 parasites/μl and fever). Values represent AHRs ± 95% confidence intervals, adjusted for spatial confounders (SC) (attendance at Mugil school and living 1 km from seaboard) or age and SC. Open circles, AHRs for medium versus low responders; filled circles, AHRs for high versus low responders. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The 3D7 allele of MSP1-19 and AMA-1 was used.

0.015; AHR of 0.48 for total IgG, $P = 0.014$). In contrast, IgG3 responses to MSP1-19 were not associated with a reduced risk of malaria.

Medium (AHR of 0.47, $P = 0.015$) and high (AHR of 0.53, $P = 0.037$) IgG3 responses to MSP2 3D7 were associated with protection, after adjusting for location (AHR of 0.53, $P = 0.037$), whereas IgG1 was not. In contrast, high IgG1 (AHR of 0.55, $P = 0.046$) and IgG3 (AHR of 0.49, $P = 0.027$) responses to MSP2 FC27 were associated with a reduced risk of symptomatic malaria. Performing the analysis using the combined IgG3 responses to both MSP2 alleles did not substantially change the strength of the association with malaria risk. High IgG responses to MSP2 FC27 were significantly associated with protection against symptomatic malaria, but the association with IgG to MSP2 3D7 was of borderline significance (3D7 AHR of 0.50, $P = 0.031$; FC27 AHR of 0.39, $P = 0.006$).

We further investigated the association between antibodies to MSP2 and protection from malaria by examining the MSP2 genotype of the first symptomatic episode in relation to MSP2 allele-specific antibodies at baseline. Of 96 clinical episodes with available DNA, 56 (58.3%) were caused by a 3D7-type infection, 24 (25%) were caused by an FC27-type infection, and 16 (16.7%) by mixed 3D7/FC27 infections. If only episodes containing 3D7-type infections were considered, high levels of IgG3 to MSP2 3D7 were associated with a reduced risk of symptomatic malaria (AHR of 0.43, $P = 0.026$). However, high levels of IgG1 (AHR of 0.42, $P = 0.03$) and IgG3 (AHR of 0.35, $P = 0.009$) to MSP2 FC27 were also associated with a reduced risk of symptomatic malaria containing 3D7-type infections. Due to the low numbers of FC27-type infections, it

was not possible to examine the associations between MSP2 FC27 antibodies and FC27-type malaria episodes.

After further adjusting analyses for age in addition to location, the associations between the reduced risk of symptomatic malaria (comparing high and low responders) and IgG3 to AMA-1 (AHR of 0.28, $P < 0.021$), IgG1 to AMA-1 (AHR of 0.49, $P = 0.025$), and IgG1 to MSP1-19 (AHR of 0.48, $P = 0.024$) remained significant (Fig. 6). Age adjustment had a more substantial effect on associations between MSP2 antibodies and protection; associations with high levels of IgG3 to MSP2 3D7 remained significant (AHR of 0.54, $P = 0.041$), whereas associations with high levels of IgG1 or IgG3 to MSP2 FC27 (AHR of 0.58 [$P = 0.070$] or AHR of 0.54 [$P = 0.065$], respectively) were of borderline significance.

To further understand the contribution of specific antibody responses to protection from symptomatic malaria, we performed a multivariate analysis (adjusted for location) including subclass-specific responses to all four antigens. Only high anti-AMA-1 IgG3 responses (AHR of 0.30 for high versus medium and low levels, $P = 0.001$) and moderate and high anti-MSP1-19 IgG1 responses (AHR of 0.58 for high and medium versus low levels, $P = 0.036$) were predictive of a reduced risk of symptomatic malaria. Adjustment for age did not change the significance or size of these associations (data not shown).

We also examined associations between antibodies and re-infection with a parasitemia of any density (as detected by PCR or light microscopy) or episodes of moderate-density (>500 parasites/μl) or high-density (>5,000 parasites/μl) parasitemia (Fig. 7) (analysis was based on the time to first episode). There was a weak association of borderline statistical significance
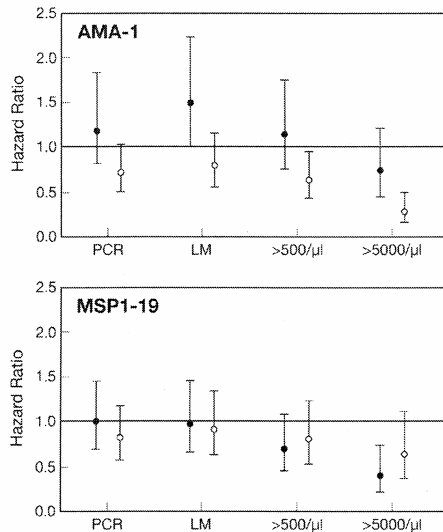
FIG. 7. Association between subclass-specific antibodies against merozoite antigens and *P. falciparum* blood-stage infections of different densities. Values represent HRs ± 95% confidence intervals. Filled circles, IgG1; open circles, IgG3. PCR, *P. falciparum* infection determined by PCR; LM, *P. falciparum* infection determined by light microscopy; >500/μl, *P. falciparum* infection with a density of >500 parasites/μl; >5000/μl, *P. falciparum* infection with a density of >5,000 parasites/μl. The 3D7 allele was used for each antigen.

between high AMA-1 IgG3 levels and time to reinfection, as detected by PCR (HR of 0.72, P = 0.071) (Fig. 7). There were no other total IgG or subclass responses significantly associated with time to reinfection, as determined by PCR or light microscopy (data not shown for MSP2). In contrast, high AMA-1 IgG3 levels were more strongly associated with protection from moderate-density parasitemia (HR of 0.60, P = 0.017) and most strongly associated with protection from high-density parasitemia (HR of 0.24, P < 0.001). High levels of IgG1 to MSP1-19 were significantly associated with protection from only high-density parasitemia (HR of 0.40, P = 0.003) and not moderate-density parasitemia.

DISCUSSION

An important question in understanding human immunity to malaria is whether antibodies to merozoite antigens may contribute to protection against parasitization per se or only act to prevent symptomatic malaria. Our results demonstrate that IgG subclass-specific responses to merozoite antigens are significantly associated with protection from high-density parasitemia and symptomatic malaria but not against parasitization per se. Several previous studies have examined associations between merozoite antibodies and risk of malaria illness, but very few have examined associations with reinfection. To our knowledge, none have examined associations between IgG subclass-specific responses and reinfection, high-density parasitemia, and symptomatic malaria in the same study. To address these important questions in our study, we cleared parasitemia among participants at enrollment with a highly

effective treatment (>90% cure rate), actively screened for reparasitization using sensitive molecular methods, and genotyped infecting parasites to distinguish reinfection from treatment failure. Furthermore, we examined IgG subclass responses in addition to total IgG responses. Although there was a weak association between IgG3 to AMA-1 and reinfection, this was of borderline statistical significance. The association with risk of high-density parasitemia was much stronger and highly significant for IgG3 to AMA-1 and IgG1 to MSP1-19. No other IgG or subclass responses were associated with risk of reinfection, as detected by PCR or light microcopy. Instead, our results suggest that antibodies to merozoite antigens mediate their protective effect by control of blood-stage parasitemia, thereby preventing high-density infections, which has important implications for understanding immunity. High-density parasitemia and overall parasite biomass are linked with the pathogenesis of severe malaria (19), suggesting that an ability to control parasitemia, but not necessarily prevent blood-stage parasitemia, may afford substantial protection from severe and complicated malaria.

The strongest association with protection from symptomatic malaria in multivariate analysis was for IgG3 against AMA-1. Although IgG3 was predictive of protection, IgG1 was only weakly associated with protection, even though it was the dominant subclass and correlated with age and exposure. Furthermore, total IgG to AMA-1 was only weakly associated with protection, emphasizing the value of examining subclasses and not just total IgG in studies of human immunity. The association between protection and IgG3 is unlikely to be explained simply by these antibodies being a marker of an alternate protective immune response. AMA-1 IgG3 was not predictive of the IgG1 or IgG3 response to MSP1-19 or MSP2 and did not indicate an overall propensity of protected individuals to have a greater IgG3 response to malaria antigens; the IgG3 response to AMA-1 and its association with protection appeared specific to AMA-1. These results have important implications for AMA-1 vaccine development, as a recent phase 1 clinical trial demonstrated that the vaccine construct induced predominantly IgG1 (27). Our finding differs from a recent study (31) in which IgG1, but not IgG3, to AMA-1 was associated with protection from symptomatic malaria in an African population. Differences in population genetics, transmission levels, and age groups studied may account for the differences between studies.

IgG1 responses to MSP1-19 were also associated with protection against symptomatic malaria, which remained significant in multivariate analysis. This finding is in agreement with some studies conducted previously with African populations, but not others (5, 11, 15, 25, 31, 39). IgG3 reactivity to MSP1-19 was low overall, which may also explain why it was not associated with a reduced risk of malaria. Consistent with the association with protection, AMA-1 and MSP1-19 are thought to be essential for erythrocyte invasion (10) and have been identified as targets of human invasion-inhibitory antibodies (for example, see references 13 and 21).

Antibodies to MSP2 had a weaker association with protection, and this association did not remain significant in a multivariate analysis of antibody responses. Although there was evidence that levels of allele-specific antibodies were higher if infected with that particular allele, there was not clear evi-

dence of allele-specific protection by antibodies to MSP2 when the genotype of episodes was prospectively related to allele-specific antibodies. MSP2 3D7 IgG3 and MSP2 FC27 IgG1 and IgG3 were all associated with a reduced risk of symptomatic 3D7-type malaria. This may indicate that a component of the protective antibody response is directed against the conserved region of MSP2. It has been shown in previous studies that total IgG and IgG3 to MSP2 are associated with a reduced risk of symptomatic malaria (1, 18, 29, 37, 45). Antibodies to the variable region of MSP2 3D7 are thought to have contributed to the efficacy of the combination B malaria vaccine (18).

In this study, IgG1 and IgG3 levels of reactivity to each antigen increased with age and exposure and were higher among those with active infection. In previous studies, it has been suggested that increasing age (and malaria exposure) is accompanied by an increasing polarization of the subclass response to malaria antigens (45, 48); that is, an individual's ability to produce both IgG1 and IgG3 diminishes and only IgG1 or IgG3 is produced. For example, it has been suggested that a mixed IgG1/IgG3 response to MSP2 in young children evolves into an IgG3-only response as age increases (45, 48). In our cohort, we did not observe polarization of responses; for all antigens, the prevalence and levels of IgG1 and IgG3 increased significantly with age and exposure (measured as antibody reactivity to *P. falciparum* schizont extract). As the age range of our primary cohort was narrow, we studied a second cohort of residents of the same geographical area with a wider age range. Both the dominant and subdominant antibody responses increased with age for all antigens, supporting our previous observation. Reasons for differences between the studies may relate to differences in host population genetics, malaria transmission intensity, and sensitivity of reagents used.

We report here that subclass-specific responses to different antigens are not related among individuals; antigen-specific subclass responses appear to be generated independently. Only in the instance of responses to different AMA-1 variants was there evidence of relatedness. This may be because antibodies are largely directed against conserved epitopes of AMA-1 rather than due to an inherent bias among individuals to produce a particular subclass. The lack of a predisposition to produce a particular subclass response regardless of the antigen further supports the hypothesis that the factors influencing the subclass response are determined mainly by the antigen rather than the host. Prior studies of mice identified a T-cell epitope in MSP2 that appears to promote cytophilic IgG subclass responses (47). Although polymorphisms in AMA-1 were not associated with differences in subclass responses, it is conceivable that the component of the antibody response directed against the conserved epitopes may have affected our ability to detect differences in subclass responses to allele-specific epitopes. Some difference in the subclass responses to MSP2 alleles was observed, and IgG1 and IgG3 responses to the two alleles were not as strongly correlated as other responses and were not significantly related. These observations suggest that responses to each allele may be determined independently and that, in principle, polymorphisms in antigens can influence the profile of IgG subclasses induced.

Our observation that the subclass response to MSP1-19 is a mixed IgG1/IgG3 response, with IgG1 being predominant, and that the response to MSP2 is predominantly IgG3 is generally in agreement with previous studies (8, 11, 15, 25, 29, 31, 37, 42, 43, 46, 48). Studies of African populations report that IgG1 is preferentially induced, with some IgG3 (31, 37); however, one study reported very little or no IgG3 to AMA-1 (48). In our study, although IgG1 was the predominant response to AMA-1, it was also a mixed IgG1/IgG3 response. To confirm our results, we validated our subclass typing reagents against two other commercially available reagents; furthermore, testing plasma from Kenyan children and adults, we detected both IgG1 and IgG3 to AMA-1, with IgG1 being predominant (D. Stanisic and F. McCallum, unpublished data).

In conclusion, these findings have significant implications for understanding and measuring immunity and for the development and evaluation of blood-stage vaccines. They provide strong support for the role of IgG subclass-specific antibodies to merozoite antigens in protection from symptomatic malaria and high-density infections and important insights into how IgG subclass responses are acquired.

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SCIENTIFI PUBLICATIONS

IgG subclass-specific responses against *Plasmodium falciparum* merozoite antigens are associated with control of parasitemia and protection from symptomatic illness.

Stanisic DI, Richards JS, McCallum FJ, Michon P, King CL, Schoepflin S, Gilson PR, Murphy VJ, Anders RF, Mueller I, Beeson JG. Infect Immun. 2009 Jan 12.

Heterogeneous distribution of *Plasmodium falciparum* drug resistance haplotypes in subsets of the host population.

Sonja Schoepflin, Jutta Marfurt, Mary Goroti, Moses Baisor, Ivo Mueller, Ingrid Felger. Malaria J. 2008 May 6; 7:78

The risk of malarial infections and disease in Papua New Guinean children.

Michon P, Cole-Tobian JL, Dabod E, Schoepflin S, Igu J, Susapu M, Tarongka N, Zimmerman PA, Reeder JC, Beeson JG, Schofield L, King CL, Mueller I. Am J Trop Med Hyg. 2007 Jun;76(6):997-1008.

Effect of the malaria vaccine Combination B on merozoite surface antigen 2 diversity.

Flück C, **Schöpflin S**, Smith T, Genton B, Alpers MP, Beck HP, Felger I. Infect Genet Evol. 2007 Jan;7(1):44-51

CONFERENCES AND WORKSHOPS

2008   **Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases**

University of California, Irvine, USA

Oral presentation: "Infection dynamics of *Plasmodium falciparum* and fitness costs of drug resistance"

Award for best presentation by a student

BioMalPar Conference: Heidelberg, Germany

Poster presentation: "Heterogeneous distribution of *Plasmodium falciparum* drug resistance haplotypes in different subsets of the host population"

2007   **PhD student meeting of the Swiss Society of Tropical Medicine and Parasitology (SSTMP)**: Münchenwiler, Switzerland

Oral presentation: "Heterogeneous distribution of *Plasmodium falciparum* drug resistance haplotypes in subsets of the host population"

Joint meeting of the Swiss Society of Tropical Medicine and Parasitology and the French and German Society of Parasitology: Strassbourg, France

Poster presentation: "Mutations conferring drug resistance in *Plasmodium falciparum* are more prominent in new infections compared to chronic infections"

2006   Workshop: **Drug resistance across the tree of life**: Bern, Switzerland

Joint meeting of the Royal Society of Tropical Medicine and Hygiene and the Swiss Society of Tropical Medicine and Parasitology: Basel, Switzerland

1 week **Workshop in Evolutionary Biology** organised by the Zoological Institute of the University of Basel: Guarda, Switzerland

2005   **PhD student meeting of the Swiss Society of Tropical Medicine and Parasitology (SSTMP):** Ascona, Switzerland

Oral presentation: "Infection dynamics and fitness costs of drug resistance in *Plasmodium falciparum* studied in children from Papua New Guinea"

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Languages	German: native language
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